Effect of Blood Dilution on Recovery of Organisms from Clinical Blood Cultures in Medium Containing Sodium Polyanethol Sulfonate

JOHN F. SALVENTI,[1] TERRI A. DAVIES,[2] EILEEN L. RANDALL,[3] SUSAN WHITAKER,[4] AND JOHN R. WATERS[5]*

Allentown and Sacred Heart Hospital, Allentown, Pennsylvania 18105,[1] University of Chicago, Chicago, Illinois 60637,[2] Evanston Hospital, Evanston, Illinois 60201,[3] Rhode Island Hospital, Providence, Rhode Island 02902,[4] and Johnston Laboratories, Inc., Cockeysville, Maryland 21030[5]

This clinical study was designed to evaluate the standard laboratory protocol that requires blood specimens be diluted with ≥ 10 volumes of media. Blood was collected from hospitalized patients, and 1 ml was inoculated into each of three vials containing 2.3, 7.3, and 24 ml of BACTEC 6B aerobic medium resulting in dilutions of 1:4, 1:10, and 1:30, respectively. The three test vials were treated identically, and the study was carried out at four hospitals. Of the 2,550 sets of vials inoculated, 174 were positive with clinically significant isolates from 105 patients. There was no difference in the number of positive cultures recovered by 24 h (67%) or 48 h (90%) from any dilution. These percentages agreed with other reports from BACTEC users. The number of positive vials (139, 144, 147, respectively) at each dilution was not significantly different, indicating that all three dilutions showed equal recovery of pathogenic microorganisms. Despite this overall equality, two patients, one on antibiotic therapy, were found to have correlated cultures which failed to grow at the 1:4 dilution. This finding implies that a 1:4 dilution of blood cannot be recommended unequivocably despite the higher overall recovery rate of positive cultures.

It is normal laboratory procedure (1) to dilute blood specimens for culture with 10 or 20 volumes of media. This recommendation is based upon the well-established bactericidal effect of fresh human serum.

However, it has also been shown by Lowrance and Traub (10) that this bactericidal effect can be nullified by the addition of sodium polyanethol sulfonate (SPS) to the medium. Beebe et al. (2) demonstrated that when a mixture of blood and media containing SPS was inoculated with several strains of bacteria, a dilution factor of 1:2 (i.e., equal volumes of blood and culture media) produced bacterial growth at the same rate as a 1:8 dilution. Undiluted blood still supported the growth of the organisms but at a lesser rate. Although the bacteria used in this study were recent clinical isolates, their conclusions apply only to these "mock" blood cultures and cannot be assumed to apply to actual cultures.

Our study was designed to compare the recovery of organisms from actual clinical blood cultures at three different dilutions, namely, 1:4, 1:10, and 1:30. The 1:10 was the control dilution and the 1:4 dilution investigated the effect of adding a substantially larger volume of blood to the same volume of medium. For example, with 30 ml of medium, the control dilution would require 3.3 ml of blood, whereas the 1:4 dilution would require 10 ml of blood; this extra blood would increase the recovery rate of the system, since it is the universal finding from every study of blood culture methodology that, as more blood is cultured, more organisms are recovered (1, 7).

We included the 1:30 dilution because such a large dilution often occurs with blood samples from pediatric patients from whom only small volumes of blood are obtainable. Many pediatricians believe that blood can be overdiluted, leading to nonrecovery of pathogenic bacteria. There appears to be no recent studies on this topic, especially with SPS as part of the culture medium.

Our study was carefully planned so that the three test vials were as alike in preparation and treatment as possible, the only difference being the dilution factor. Thus, any enhancement or inhibition of recovery could be assigned to this factor only.

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MATERIALS AND METHODS

Media and tubes. Blood was collected in a sterile Vacutainer transport tube (no. 4960, Becton, Dickinson & Co., Rutherford, N.J.), which will draw a nominal 8.3 ml of blood into 1.7 ml of saline with 0.35% SPS. In a full tube, each 1 ml of fluid actually contains 0.83 ml of blood.

Three sets of special culture vials were prepared so that when 1 ml of fluid from the collection tube was added to a vial, the dilution of the patient's blood would be exactly 1:4, 1:10, and 1:30, and the final concentration of SPS would be the same in each vial, namely 0.025%. SPS has been reported to inhibit some aerobic bacterial species (5), so keeping the final concentration constant equalized any effect, if present. In addition to these three vials, labeled A, B, and C, respectively, the standard BACTEC 6A aerobic and 7B anaerobic vials (Johnston Laboratories Inc., Cockeysville, Md.) were used. The relevant contents of the test vials are shown in Table 1.

In vial A, the concentration of tryptic soy broth without dextrose (TSB-D) and sodium bicarbonate was 10% higher than in B and C. This was to overcome partially the diluting effect of the blood upon the culture medium. The final concentration of the medium in the three vials A, B, and C was 77, 88, and 96%, respectively, of normal TSB-D, taken as 100%. The other difference between the vials was in the amount of radioactive substrate. This was determined in a preliminary experiment. As the volume of medium is decreased, the cells in the blood generate larger amounts of $^{14}CO_2$ from the metabolism of the ^{14}C-substrates, thus leading to many false positive reactions. Since bacteria are also more efficient in producing $^{14}CO_2$, the quantity of substrates may be reduced with no loss in efficiency. Due to the high specific activity of the radioactive materials, only micromoles are used, whereas much larger quantities, millimoles, of the same, but unlabeled, chemicals are already present in the medium. Thus, these small changes in the radioactive substrates are insignificant and do not affect the composition of the medium with regard to the growth of bacteria.

The three vials A, B, and C were prepared from the same manufacturers' lot numbers of ingredients by the same personnel on three consecutive days with the same equipment and sterilizing cycle.

Test procedure. The procedure was designed so that exactly equal volumes of blood would be put into test vials A, B, and C and that all three vials in one set would be treated in the same way. It is possible that there might be differences in the handling of these sets, especially at different laboratories, but, within a set of vials, differences were minimized.

Blood was drawn into the transport tube and sent to the laboratory. Only tubes that were full, or within 1 cm of being full, were used for the study. Partially empty tubes may have had a high SPS concentration and would have also invalidated the dilution calculations. Using a syringe, we withdrew exactly 3 ml of fluid from a tube and put 1 ml into each of the three test vials. The remainder of the fluid in the tube, nominally 7 ml, was inoculated in the 6A aerobic vial and the 7B anaerobic vial in equal amounts. The three test vials were incubated with agitation and tested periodically on a BACTEC instrument (Johnston Laboratories Inc., Cockeysville, Md.), which measured the amount of $^{14}CO_2$ generated from the substrates by metabolic activity in the test sample. This technique has been shown, for example, by Randall (12), to provide early indication of bacterial presence in blood cultures. The amount of $^{14}CO_2$ is measured in growth index (GI) units, where 100 GI = 0.025 μCi of ^{14}C activity.

The minimum protocol was to test once on day 1 (the day of inoculation), and once on days 2, 3, 5, and 7 and follow with a blind aerobic subculture of all three vials to chocolate agar plates incubated with increased carbon dioxide. All GI values were recorded, as were the values from the two regular culture vials. Data from all positive reading and positive subculture vials and sets was transferred to a standard form for uniform analysis.

Classification. The standard forms from the four hospitals were collected and reviewed for any anomalies. For an isolate to be called significant and counted, it must, at least, have grown on subculture to solid media from one of the three vials A, B, and C. It was not necessary that it be detected radiometrically or that it be seen on a direct Gram stain from the vial. Isolates were classed as contaminants and not counted if they were identified as *Staphylococcus epidermidis*, *Bacillus*, *Micrococcus*, *Corynebacterium*, or *Propionibacterium*, unless at least two of the four vials (the three test vials plus the 6A aerobic vial) yielded the same organism, and, in addition, the same organism was recovered from another blood culture set drawn the same day. This definition might result in a significant isolate being classed as a contaminant, but it is completely objective and does not require subjective interpretation of the patients' condition or history. Such misclassifications are likely to be rare because standard practice at each laboratory is to draw multiple blood cultures.

RESULTS

Each significant isolate was assigned to a group identified by the number and type of vials that were positive. For example, if the A and C vials were positive and the B vial showed no growth, this isolate would be classed as "A+C" and counted once only in that specific group and no other. Table 2 gives the number of isolates in each group. Note that a positive vial was only

TABLE 1. *Contents of the three test vials*

Vial	Vol. of medium (ml)	Tryptic soy broth without dextrose (%)	Sodium bicarbonate (%)	SPS (%)	^{14}C-substrates (μCi)
A	2.3	3.025	0.041	0.01	0.5
B	7.3	2.75	0.0375	0.02	1.0
C	24	2.75	0.0375	0.024	2.0

TABLE 2. *Number of isolates in each group*

Assigned group[a]	No. of isolates in each group	Summary	
A	8	Total A bottles positive	= 139
B	13	$[\sum (A) + (A+B) + (A+C) + (A+B+C)]$	
C	12	Total B bottles positive	= 144
A+B	5	$[\sum (B) + (A+B) + (B+C) + (A+B+C)]$	
A+C	9	Total C bottles positive	= 147
B+C	9	$[\sum(C) + (A+C) + (B+C) + (A+B+C)]$	
A+B+C	117	Bottles A or B or C positive	= 173

[a] Each significant isolate was assigned to only one group defined by the letter code of the vials that were positive.

counted once even if two different bacterial strains were isolated from it.

Table 3 summarizes the number of positive test bottles and the number of positive patients. Gram-negative bacilli comprised 53% of the single isolates, gram-positive cocci comprised 30%, and yeasts comprised 10%. About 7% of the positive bottles contained two or more organisms. Altogether, there were 173 positive sets out of the 2,550 inoculated (6.8%).

The three vials A, B, and C are matched samples with similar recovery rates. Application of Cochran's Q-test (3) to evaluate the hypothesis that their recovery rates are equal, using the experimental results from Table 2, resulted in $Q = 1.75$. Therefore, there is no significant inhibition or enhancement of recovery as a function of dilution over the range of 1:4 to 1:30 (two degrees of freedom, $P = 0.4$).

Were dilution to have an effect, it would be expected to be most pronounced when comparing the A and C vials because these have the largest spread in dilution. From the table, there were 13 instances when A was positive and C was negative and 21 instances with the opposite situation. With McNemar's test (11), there is no statistically significant difference ($\chi^2 = 1.44$; $P = 0.23$) between recovery rates for A and C (ignoring B).

However, if there really is a small inhibitory effect in A when compared to C, how large would this have to be to have been detected by this study? This can be estimated by using Cohen's tables (4). If there was a 20% inhibition in A, there would be an over 99% chance of detecting it. There would be a 95% chance of detecting a 14% inhibition. However, there would be only a 70% chance of detecting a 10% difference with the present number of positive cultures. It is estimated that it would take almost double the number of cultures to have a 90% chance of detecting such a 10% inhibition (at the 5% level of significance, $P = 0.05$). Even an 80% chance of detection would still take half again as many samples as contained in this study.

TABLE 3. *Significant isolates from positive test vials*

Organism	Cultures	Patients
Enterobacteriaceae	68	45
Pseudomonads	23	11
Staphylococci	31	19
Streptococci	21	13
Yeast	17	6
Haemophilus	1	1
Mixed cultures	12	10

Table 2 shows that B+C = 9, i.e., no growth in A but growth in B and C, A+C = 9 and A+B = 5. All 14 sets in which the B or C vials were negative were single cultures from separate patients. This was not true of the nine sets with A negative. There were four single cultures from four patients, two consecutive cultures from patient MM from which *Pseudomonas aeruginosa* was recovered, and three consecutive cultures from patient AL from which non-enterococcal group D streptococci were recovered. It is very unlikely that such correlated results occurred by chance.

To evaluate the rapidity with which positive test bottles were detected, the number of each type of vial that became positive within 24 and 48 h after receipt by the laboratory was determined. By 24 h, 67% of all vials (63% of A vials, 67% of B vials, 70% of C vials) were positive; by 48 h, 90% of all vials (90% of A vials, 91% of B vials, 89% of C vials) were positive.

On the assumption, justified above, that the three test vials had essentially equal efficiency for recovery of organisms, we can determine the effect of increased blood volume on recovery. For 1 ml of inoculum, only a single vial of the three need be positive; there was an average of 143 such cases. For 2 ml, one vial of any pair of vials must be positive; there was an average of 162 cases. For 3 ml, there were 173 cases. When plotted on either semi-log or log-log scales, these figures yield a straight line. For the log-log plot (Fig. 1), this relation is: log (number positives) = 2.155 + 0.174 log (milliliters of blood).
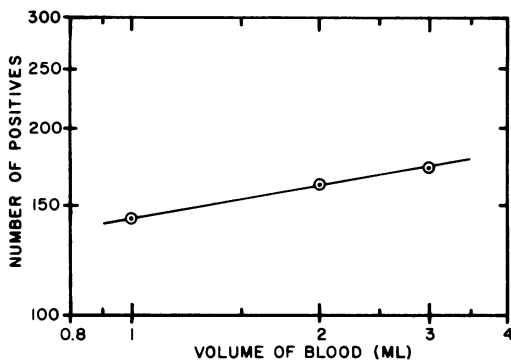
FIG. 1. *Number of positive vials as a function of volume of blood cultured.*

On the assumption that this relation can be extrapolated, we can estimate the percentage increase in positive cultures as the volume of blood cultured is increased: from 5 to 10 ml, 13% increase; 10 to 15 ml, 7.5% increase; 15 to 20 ml, 5% increase; 20 to 25 ml, 3.7% increase; or from 10 to 20 ml, 13% increase; 20 to 30 ml, 7% increase; 30 to 40 ml, 5% increase.

DISCUSSION

Since the advent and widespread use of SPS, there have been no well-controlled clinical studies to investigate the continued need for diluting blood 1:10 to 1:20 (vol/vol) with media when culturing. This present study was designed to address this question.

The study was carefully controlled to ensure that there were no differences in medium, additives, or laboratory handling. Under these conditions, there was no statistically significant difference in the recovery rate of clinically significant organisms when the blood sample was diluted 1:4, 1:10, or 1:30 by the culture medium. There would have been a 95% probability of detecting a 14% difference between the recovery ability of the 1:4 and 1:30 dilutions and a 70% probability of finding a 10% difference. For blood cultures performed with the BACTEC system this implies that: (i) there will be no inhibitory effect if up to 10 ml of blood is inoculated into a vial that contains 30 ml of medium; (ii) there will be no inhibitory effect if as little as 1 ml of blood is inoculated into the vial. Because pediatric blood cultures were not a part of this study, no conclusions can be drawn as to the relevancy of the above data to this particular group of patients.

Although the overall results show no effect of blood dilution on recovery, some effect becomes apparent when the results are scrutinized. In particular, there was lack of growth at 1:4 dilution for five cultures from two patients, MM and

AL, whereas no such effect was seen at 1:10 or 1:30 dilutions.

Patient MM, a 25-year old male heroin user, presented with a diagnosis of acute bacterial endocarditis. Three blood cultures were drawn the next day and were positive for *P. aeruginosa* from the routine aerobic vial (6A) and all three study vials, A, B, and C. The patient was started on gentamicin and carbenicillin; the gentamicin was replaced by tobramycin on day 5. Four days later, two more sets of blood cultures were drawn and *P. aeruginosa* was recovered on the following day from the routine aerobic vial and only study vials B and C. No growth occurred in either of the A vials. Thus, he was on an intensive course of appropriate antimicrobial therapy at the time the blood was drawn for culture. The SPS in the culture medium would tend to inactivate the aminoglycoside antibiotic (8) but has no reported effect with the penicillin class of antibiotics. It appears that the failure to recover the organism at the 1:4 dilution was probably due to the presence of the carbenicillin because the pretreatment cultures demonstrated the ability of the test vial to grow this organism. A similar effect was reported by L. B. Reller, D. A. Lichtenstein, S. Mirrett, and W.-L. L. Wang (Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, C177, p. 306), who found that cultures from 12 patients receiving high-dosage anti-staphylococcal drugs were positive at 1:10 dilution but negative at 1:5 dilution, although all had been positive at both dilutions before treatment.

Patient AL, a 61-year old male, was transferred from another hospital with a diagnosis of bacterial endocarditis and severe iron-deficiency anemia. Three cultures were drawn on admission and were all positive in the routine aerobic (6A) and anaerobic (7B) culture vials and study vials B and C, but not A, the 1:4 dilution. The organism isolated was a non-enterococcal group D streptococcus. Hospital records do not indicate that any antibiotics were being administered at the time the blood was drawn for culture. The patient's chart shows a 3- to 4-week history of fever of unknown origin and a subacute bacterial endocarditis possibly of greater than 6 weeks' duration. During this subacute phase of the patient's disease, significant antibody levels could have developed in the patient's serum (9), requiring greater dilution of the blood before growth could occur. However, because this was the only isolate of this type of organism in the study, it is possible that it may be an unusual characteristic of this strain requiring the greater dilution.

The rapidity with which positive cultures were detected was the same for all three dilutions.

Detection times agreed with those reported by other users of this radiometric technique (12). These results are in contrast to those of Reller et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, C177, p. 306). Using a conventional culture system with blind subculture and Gram stains from Supplemented Peptone Broth with SPS (B-D, Rutherford, N.J.), they found that significantly faster detection was obtained at the 1:5 dilution than at 1:10. Such a difference in detection time must be due to the nature of the systems employed because it was not apparent in our study, probably due to the greater overall rapidity for the detection of positive cultures with the BACTEC system.

As the volume of blood being cultured is increased, the recovery rate for positive cultures also increases. This is a universal finding of all such studies (7). Since there is pressure to minimize the amount of blood drawn from the patient for diagnostic purposes (6), one should select an optimum volume for culture. For our system, this optimum appears to be 20 to 30 ml because there is a 13% increase in recovery from 10 to 20 ml, a 7% increase from 20 to 30 ml, but only a 5% increase from 30 to 40 ml. This optimum may vary for other culture systems and must be determined empirically.

We have shown that for the BACTEC culture medium used in this study, the dilution of the blood sample had no statistical effect on recovery; this may or may not hold for other media. For a single blood culture in which 3.3 ml of blood is inoculated into each of a pair of bottles containing 30 ml of medium, there will be a 21% increase in positive samples if the blood volume is increased to 10 ml to give a 1:4 dilution rather than 1:10. This is clearly valuable. Nevertheless, there were two sets of cultures in our study that showed growth at 1:30 and 1:10, but not at 1:4.

One patient was being treated with antibiotics, but one was not. Thus, we can not unequivocally recommend the 1:4 dilution. A dilution factor of ca. 1:7 (5 ml into 30 ml of medium) might be preferable because it would yield more positive cultures, due to the increased blood volume, and yet be closer to the 1:10 dilution at which no inhibitory effects were found.

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