Practical Aerobic Membrane Filtration Blood Culture Technique: Development of Procedure

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The advantages of a membrane filter system for blood culturing have been realized for many years. Lysing of the blood prior to filtration is a convenient way to proceed, but previously described lysing procedures result in loss of certain organisms, particularly gram-negative bacilli. Four concentrations of Triton X-100 and sodium carbonate were studied in vitro, and their lysing and antibacterial properties were observed. A solution of 0.08% Na₂CO₃ and 0.005% Triton X-100 was found to have the least antibacterial effect and gave consistently good lysis and filtration times (under 3 min). An 8.3-ml amount of blood added to 190 ml of this concentration of lysing solution, filtered through three 47-mm membrane filters (0.45-μm pore size), led to recovery of 85% or more of various aerobic and facultative organisms in studies of artificially seeded blood.

Despite the availability of excellent antimicrobial drugs, the mortality from bacteremia remains high, especially when shock accompanies sepsis. It is imperative that the identification and susceptibility of the infecting organism be determined as early as possible in the course of bacteremia, since it has been observed that early administration of the appropriate antimicrobial drug greatly improves the chances for survival of the patient.

Our group has been studying different approaches for more efficient blood culturing and has shown several advantages of the membrane filter technique over conventional blood culture procedures for the rapid diagnosis of bacteremia (4, 8, 11). The advantages of the membrane filter procedure include faster growth, ability to remove inhibitory agents (normal bactericidal factors and drugs which have been administered), and growth in the form of discrete colonies on the filter, which facilitates faster identification of the infecting organism. The earlier procedures (2, 10, 12) for membrane filtration have been too cumbersome and time consuming for practical clinical use. The hemolysis of blood cells was tried many years ago by Braun and Kelsh (2) and by Tidwell and Gee (10); however, it was only possible to filter very small quantities of blood with their procedures. An efficient lysing system utilizing 0.8% Na₂CO₃ and 0.05% Triton X-100 was introduced more recently (7). However, two important difficulties with this system were an antibacterial effect of the lysing solution on gram-negative bacilli and certain sensitive gram-positive organisms (3) and significant filtration difficulties related to plugged filters. Unpublished studies in our laboratory of the lysing procedure of Rose and Bradley (7) revealed the same problems described by Farmer and Komorowski (3). Our approach to overcoming these problems involves use of a system employing a larger filter area and the utilization of lower concentrations of sodium carbonate and Triton X-100 (Na₂CO₃-TX-100).

The procedure which we have developed has the advantage of being accurate and yet simple enough for practical use in the clinical laboratory. It minimizes the problems of organism loss and filter plugging.

MATERIALS AND METHODS

Effect of various lysing solutions. Four concentrations of Na₂CO₃-TX-100 were tested: (i) 0.4% Na₂CO₃-0.025% TX-100; (ii) 0.16% Na₂CO₃-0.01% TX-100; (iii) 0.12% Na₂CO₃-0.007% TX-100; and (iv) 0.08% Na₂CO₃-0.005% TX-100. The pH of these solutions was 10.0 to 10.3. Blood (8.3 ml) was collected directly into a Vacutainer tube containing sodium polyanethol sulfonate (SPS, 1.7 ml of 0.35% solution; this yields a final concentration of 0.06%) (BD Inc.) and mixed. The blood was then added via syringe to 190 ml of the appropriate Na₂CO₃-TX-100 (Rohm and Haas) solution. The solution was mixed until good lysis was observed (about 1 min) and then filtered,
using a vacuum of 25 inches (ca. 64 cm) of Hg per min. A 100-ml wash with saline followed the initial filtration in certain studies. The filter first used in the system was a 90-mm membrane filter (0.45-μm pore size; Millipore Corp.), but later studies employed a disposable unit containing three 47-mm filters (0.45-μm pore size; Gelman). This unit (kindly supplied by McGaw Laboratories, Glendale, Calif.) is shown in Fig. 1.

In vitro studies were done with each of the four solutions. Test organisms from human clinical material (Escherichia coli, Streptococcus pneumoniae, and Staphylococcus aureus) were diluted so as to yield approximately 100 to 300 organisms/ml (counts verified in triplicate by pour plates in all experiments). One milliliter of test organism was added to the SPS-containing tube, which already contained 8.3 ml of normal human blood. The contents of this tube were then added to 190 ml of the test lysing solution, mixed for 1 min, and then filtered. The filters were subsequently placed on blood agar plates and incubated aerobically at 35 C. Colonies were counted after 24 h.

**Effect of prolonged exposure of organisms to least harmful lysing solution.** To establish the maximal length of time organisms could remain viable in the lysing solution, a killing curve experiment was performed. E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and S. aureus were tested. Relatively small numbers of each test organism were added directly to 190 ml of the lysing solution. Three 1-ml pour plates were made from each test setup at the following time intervals: 0 (control), 5, 10, 15, 30, and 60 min. One milliliter of the solution was added to 19 ml of melted cooled Brucella agar for the pour plates. These were incubated aerobically at 35 C and counted after 24 h.

**Need for wash after filtration.** In our earlier filtration procedures (utilizing smaller volumes of fluid during filtration), it was found necessary to wash the filter because residual antibiotics and normal serum inhibitory factors remained on the filter (4, 12).

Figure 2A from earlier studies with our previous technique shows a filter, improperly washed, with residual antibiotic. There is inhibition of the indicator organism by antibiotic in the filter strip in the center of the petri dish. Figure 2B shows a similar setup with proper washing. The necessity of a wash for the currently described procedure (using 190 ml of the 0.08% Na2CO3-0.005% TX-100 lysing solution) was therefore tested. Four antibiotics were used in concentrations higher than or equal to those normally attained in serum during antimicrobial therapy. The test organisms and antibiotics were: E. coli and 20 μg of chloramphenicol per ml, group A Streptococcus and 2 μg of penicillin G per ml, Enterobacter aerogenes and 20 μg of chloramphenicol per ml, E. aerogenes and 30 μg of tetracycline per ml, and P. aeruginosa and 100 μg of polymyxin B per ml. The bacterium (to be used as an indicator of residual antimicrobial activity) was diluted to 10⁸ organisms/ml in broth and then spread evenly over a blood agar plate with a cotton swab. The corresponding antibiotic, in the appropriate concentration, was added to two Vacutainer-SPS tubes containing 8.3 ml of normal human blood. The tubes were mixed, added to the lysing solution, mixed well for 1 min, and then filtered. One filtration in each pair was followed by

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**Fig. 1.** Filtration unit (McGaw) with three membrane filter holders. The lysed blood specimen is divided evenly between the three filters.

**Fig. 2.** Need for wash after filtration with old membrane filter technique. (A) Unwashed filter has adsorbed antibiotic from blood specimen and inhibits test organism on plate; (B) washed filter; antibiotic has been washed away so there is no longer inhibition of test organism.
filtration of 100 ml of normal saline; the other was not. The filters were cut in half and placed on the blood agar plates which had been seeded with sensitive indicator strains and incubated aerobically at 35 C. Control broths containing 10^6 organisms/ml and the corresponding antibiotics in the same concentrations were incubated at the same time as the filters.

RESULTS

Effect of various lysing solutions. Ten to 20 filtrations were run with each of the four concentrations of Na_2CO_3-TX-100, and the filtration time was noted. The average filtration time with the 0.4% Na_2CO_3-TX-100 was 41 s; with the 0.16% Na_2CO_3-0.01% TX-100, 61 s; with the 0.12% Na_2CO_3-0.007% TX-100, 42 s; and with the 0.08% Na_2CO_3-0.005% TX-100, 42 s. The slowest filtration time was always under 3 min in each group.

The results of the in vitro experiments with the four concentrations of Na_2CO_3-TX-100 are given in Table 1. The gram-positive cocci (S. pneumoniae and S. aureus) were affected little, if at all, by even the strongest lysing solution tested. E. coli, however, sustained significant killing in the higher concentrations of lysing solution and showed only a small loss, if any, with the lowest concentration. One study employing E. aerogenes was done and, although only a minor bacterial loss was observed even at the highest concentration, colonial morphology was somewhat distorted at this concentration.

Effect of prolonged exposure of the organism to the least harmful lysing solution. Figures 3 and 4 show the results from the killing curve experiments. The mean number of colonies per 1-ml pour plate is plotted against the time the organisms were exposed to the lysing solution. It was found that a small loss sometimes occurred between 10 and 15 min, but that not until 30 to 60 min was there an appreciable loss of organisms (and then only with two of the four test organisms, E. coli and K. pneumoniae).

Need for wash after filtration. A zone of inhibition around the filter and no growth under the filter would be observed if residual antibiotic remained on the filter. Failure of growth in the broth control would verify susceptibility of the indicator strain to the corresponding drug. Table 2 shows the results of the experiment. In all cases there was no growth in the broth control containing the test organism plus the antibiotic, and full growth was observed under and around all filters. Therefore, with the present procedure, washing is unnecessary.

DISCUSSION

The need for better techniques for the diagnosis of bacteremia has been appreciated for many years. The conventional broths used today grow organisms relatively slowly, and additional time is required for subculture before even preliminary identification can be determined.

The potential of the membrane filter system in preventing the rapid killing of bacteria by the normal bactericidal factors in serum (5) and in avoiding inhibition or killing by residual antimicrobial compounds which cannot be inactivated has been recognized for some time. There are also certain other desirable features of the

<table>
<thead>
<tr>
<th>Conc of lysing solution</th>
<th>Avg recovery (%) (range)</th>
<th>Gross colonial morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli (6 strains)</td>
<td>S. pneumoniae (3 strains)</td>
</tr>
<tr>
<td>0.4% Na_2CO_3-0.025% TX-100</td>
<td>20.6 (40-9)</td>
<td>100 (100)</td>
</tr>
<tr>
<td>0.16% Na_2CO_3-0.01% TX-100</td>
<td>50.6 (75-33)</td>
<td>—*</td>
</tr>
<tr>
<td>0.12% Na_2CO_3-0.007% TX-100</td>
<td>59 (75-50)</td>
<td>98 (100-95)</td>
</tr>
<tr>
<td>0.08% Na_2CO_3-0.005% TX-100</td>
<td>86.4 (100-60)</td>
<td>100 (100)</td>
</tr>
</tbody>
</table>

* No studies done at this concentration.
membrane filter technique, such as rapid growth of organisms as colonies with typical morphology. Earlier filter systems did demonstrate the ability of the technique to provide early detection and identification of organisms causing bacteremia (12); however, the problems of a cumbersome system and inability to filter an adequate volume of blood limited the practical application of this technique. A later system (7) permitted use of somewhat larger volumes of blood, but filter plugging was still a problem and there was significant loss of certain organisms caused by the solution used to lyse the blood in this system.

**Table 2.** Studies concerning need for wash after filtration procedure

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Antibiotic (µg/ml)</th>
<th>Normal saline wash (100 ml) Zone of inhibition around filter</th>
<th>No wash Zone of inhibition around filter</th>
<th>Control broth with antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>Chloramphenicol (20)</td>
<td>None +</td>
<td>None +</td>
<td>No growth</td>
</tr>
<tr>
<td>Group A <em>Streptococcus</em></td>
<td>Penicillin G (2)</td>
<td>None +</td>
<td>None +</td>
<td>No growth</td>
</tr>
<tr>
<td><em>E. aerogenes</em></td>
<td>Chloramphenicol (20)</td>
<td>None +</td>
<td>None +</td>
<td>No growth</td>
</tr>
<tr>
<td><em>E. aerogenes</em></td>
<td>Tetracycline (30)</td>
<td>None +</td>
<td>None +</td>
<td>No growth</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>Polymyxin B (100)</td>
<td>None +</td>
<td>None +</td>
<td>No growth</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of least harmful lysing solution (0.08% Na₂CO₃-0.005% TX-100) versus *P. aeruginosa, E. coli, and S. aureus* as a function of time. There is no antibacterial effect for at least 15 min.

Fig. 4. Effect of least harmful lysing solution versus Klebsiella pneumoniae as a function of time. There is no antibacterial effect for the first 15 min.
Using a smaller concentration of Na₂CO₃ and TX-100 and a larger filter area, we have usually been able to recover 85 to 100% of the organisms used in artificial bacteremias. Since there may be very few organisms per milliliter of blood in clinical bacteremia, particularly when patients are on antimicrobial therapy (4), the more blood which is cultured the better the chance of detecting a low level bacteremia. In our present system, using the commercially available Vacutainer tube with SPS (BD, Inc.), 8.3 ml of blood can be filtered rapidly. The entire procedure for processing the specimen from the time of blood drawing ordinarily takes only 2 to 5 min. The SPS in the tube is an anticoagulant; it promptly neutralizes the normal bactericidal power of blood, interferes with phagocytosis, and inactivates certain antibiotics (1, 4, 11). Sodium amylosulfate shows the same types of activities as SPS and may offer some advantages (6).

With our system, filtrations taking longer than 10 to 15 min might lead to loss of organisms. However, such filtration problems are extremely rare. It should be pointed out that the late loss of gram-negative bacteria in lysing solutions may be related simply to the pH itself of the lysing solution, rather than to the specific ingredients.

With the large volume of lysing solution used it proved unnecessary to wash the filter to remove residual inhibitory substances.

Two clinical blood culture studies have been done using this system. In one study, 300 patients undergoing genitourinary tract manipulation were studied for transient bacteremias (8). Various blood culture systems were compared. The membrane filter system did not do as well as a prereduced osmotically stabilized broth in terms of total number of recoveries, but positive cultures were detected fastest in nine cultures by this procedure. The other study, reported in the accompanying paper (9), demonstrated the advantages of the improved membrane filter system; it far surpassed the other systems, both in total recovery of organisms and in speed of detection of the bacteremia.

We were working primarily with uninfected people in the first clinical blood culture study and had no problems with the filtration process. However, in the second study we noted filters plugging on occasion. The blood of patients with high leukocyte counts gelled in the lysing solution, thereby causing the filter to plug before all of the solution could be filtered. A commercial streptokinase-streptodornase (SK-SD) compound (Varidase, Lederle Laboratories) added to the lysing solution-blood mixture resulted in breaking up of the gelled mass and permitted filtration without problem. Further details on this problem and its solution are presented in the accompanying paper (9).

A membrane filter setup comprised of three 47-mm filters (0.45-μm pore size) and a lysing solution of 0.08% Na₂CO₃-0.005% TX-100 plus SK-SD constitutes an excellent system for the diagnosis of bacteremia due to aerobic and facultative organisms. Since this system has not yet been tested with anaerobes, it is suggested that an appropriate broth for anaerobes be included in the system. There are a few important points that must not be overlooked if the system is to function properly.

(i) The blood which has been anticoagulated with SPS must not be refrigerated and must be filtered before 6 h to avoid coagulation. One of the major advantages of the system is to greatly expedite detection of bacteremia. Since speed in diagnosis and therapy is a major factor in improving the patient’s prognosis, specimens should always be processed immediately whenever possible.

(ii) The lysing solution must be at room temperature before use; below 16 °C it does not function properly.

(iii) Only 3 ml of SK-SD (of a 1:20 dilution in water) should be added to the 190 ml of lysing solution (add immediately after the blood); 9 ml or more of SK-SD is bactericidal.

APPENDIX

The complete procedure for the membrane filter system is as follows.

An 8.3-ml amount of blood is collected directly into a Vacutainer tube (BD, Inc.) containing 1.7 ml of a 0.35% SPS solution and mixed immediately. The Vacutainer tube stopper is decontaminated by vigorous scrubbing and a 2-min exposure to 70% alcohol. The blood is then injected into 190 ml of 0.08% Na₂CO₃-0.005% TX-100 (Rohm and Haas) solution (Fig. 5) after first removing the protective metal cap (similar to that on a standard bottle of solution for intravenous administration). If the patient is known to have an elevated leukocyte count, addition of 3 ml of a 1:20 dilution of SK-SD in water to the lysing solution is necessary. This solution is mixed for 1 min. The rubber diaphragm over the stopper of the lysing solution bottle is removed. The protective cap is removed from the spike at the bottom of the filter unit. The spike is then pushed through the central depression in the stopper of the bottle of lysing solution. A vacuum hose is attached to the port on top of the filter unit and a vacuum of 25 inches (ca. 64 cm) of Hg is applied. The unit contains three 47-mm filters (0.45-μm pore size) (Fig. 6). A 90-mm filter (0.45-μm pore size) may also be used. When the
solution is completely filtered, the vacuum is released slowly to prevent the filters from puffing. The filters are aseptically taken from the unit (Fig. 7) and rolled onto the solid media to insure proper contact without air pockets (Fig. 8). If a 90-mm filter is used, the filter can be cut, aseptically, into thirds. The media we recommend are: a chocolate blood agar plate under 10% CO₂, a blood agar plate under 10% CO₂, and an eosin-methylene blue or MacConkey plate aerobically. Incubation is at 35 to 37 C.

The filters are observed periodically; positive cultures can be recognized as early as 9 to 12 h in certain bacteremias. Most positive cultures will be evident on the filters within 1 to 4 days, but filters are not discarded until at least 14 days of incubation. Colonies on filters are observed carefully for unique morphological features. Hemolysis beneath the filter is observed from the under surface of the petri dish or by gently lifting the filter to observe the agar underneath. Colonies are then used for various rapid biochemical and other identification procedures and are subcultured for conventional identification and susceptibility studies.

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LITERATURE CITED


