JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1977, p. 46-50
Vol. 5, No. 1
Printed in U.S.A.
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Development of a Lysis-Filtration Blood Culture Technique

CHARLES H. ZIERDT,* ROBERT L. KAGAN,[1] AND JAMES D. MACLOWRY

Microbiology Service, Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 10 August 1976

A lysed-blood culture system that quickly lyses patients' blood near neutrality and is relatively noninjurious to more delicate pathogens such as *Haemophilus influenzae* and *Bacteroides fragilis* is reported. The lysing solution includes culture medium, 0.004 M sodium carbonate and bicarbonate, 0.04% Triton X-100, and 0.6% Rhozyme (a mixture of proteases). Most of the pathogens tested multiplied in the lysing solution. The lysed blood normally is immediately filtered. The membrane is transferred to culture broth. The greatest advantage realized from this blood culture technique is separation of pathogens from antibiotics, bactericidal antibodies, complement, opsonins, and phagocytic systems. Another advantage is the concentration of organisms into a small volume of clear medium for faster growth and visualization of growth. It was observed that both gram-negative and -positive organisms were attracted during filtration to the filter material and were not removed from it by backwashing with buffer. Thus, filter membranes with porosities much larger than would nominally be expected to retain bacteria retained all or part of light and heavy *Escherichia coli* and *Staphylococcus aureus* suspensions. Advantage may be taken of this phenomenon to use filters with larger pore sizes and avoid filter clogging by poorly lysed specimens. Poor lysis may result from addition of too much blood to the lysing solution, blood with elevated numbers of erythrocytes or leukocytes, or blood from some people whose blood is naturally more resistant to lysis.

There are many advantages to be gained in blood culture procedures if the pathogenic microorganisms present in blood can be separated from the blood. Some of these are the removal of the pathogens from the presence of antibiotics in blood, opsonins, bactericidal antibodies, and complement and from the phagocytic systems.

Progress has been rapid in blood lysis-membrane filtration culture techniques (4, 5). Earlier techniques (1, 3, 6, 7) that were used without the assistance of an enzyme system, utilizing high pH and relatively harsh lysing agents, perhaps justified the criticism (2, 4) of excessive time requirement and injury to bacteria.

A milder blood lysing system, less damaging to microorganisms than those previously described, has been developed. The requirement for dextran sedimentation is obviated. This system, when carried out as described, results in 100% of blood specimens filtering quickly without clogging of filters.

Certain phenomena were observed of bacterial retention on larger porosity membrane filters during and after filtration. Advantage may be taken of these phenomena in blood lysis-filtration culture systems.

[1] Present address: Nuclear Medicine Department, Clinical Center, National Institutes of Health, Bethesda, MD 20014.

MATERIALS AND METHODS

Lysing solution. The lysing solution contained 0.1% Triton X-100 in 0.01 M $NaHCO_3$-Na_2CO_3 buffer, with 3% of stock Rhozyme 41 solution, added aseptically to the autoclaved Triton buffer solution. (Rhozyme, a complex enzyme mixture from Rhom and Haas, is prepared from *Aspergillus oryzae* culture.) To prepare the stock solution, Rhozyme powder (4%, wt/vol) was dissolved in distilled water and centrifuged to sediment insoluble material. The supernatant was sterilized by filtration through 0.22-μm membrane filters. Stock Rhozyme solution was stored at −20°C.

Lysing procedure. Blood (10%, vol/vol) was received in the laboratory in conventional blood culture bottles with brain heart infusion broth, sodium polyanethol sulfate, *p*-aminobenzoic acid, 3% CO_2 (BBL). A 30-ml portion was transferred into a sterile Nalge disposable filter unit having a porosity of 0.45 μm, and 20 ml of lysing solution was added. The lysing solution was prepared and stored at −20°C in 20-ml volumes.

The addition of lysing solution to the blood-broth mixture resulted in final concentrations of 0.04%

$NaHCO_3$-Na_2CO_3 (0.00 4 M), 0.04% Triton X-100, and 0.6% Rhozyme. Lysing solution was at pH 10.8, until mixed with the blood and broth mixture, when the pH was 7.8.

The filter flask was swirled by hand, and those specimens showing sparkling lysis were vacuum filtered immediately. Those with some turbidity were incubated at 37°C for 30 min and vacuum filtered. Sparkling lysis was not necessary for good filtration. After filtration, the membrane filter was cut around the rim with a sterile scalpel and removed with sterile pointed forceps to the culture bottle.

Culture procedure. The membrane was transferred to a wide-mouth culture bottle (2 oz [ca. 58 ml], screw-capped, no. B-7565-2; Scientific Products Corp., McGaw Park, Ill.), containing 20 ml of brain heart infusion broth with 0.1% agar and 1.5% of a nutrient supplement mixture. The mixture was composed of equal volumes of IsoVitaleX (BBL, Cockeysville, Md.), Fildes enrichment broth (BBL), and dialyzed yeast extract (BBL). It was added aseptically after the base medium was autoclaved.

The gaskets on the culture bottle caps as supplied were paperbacked and became distorted during autoclaving. These were replaced with disks (diameter, 2 in [ca. 5.1 cm]) cut from silicone rubber (thickness, one-sixteenth inch [ca. 0.16 cm]) glued in place with RTV 108 silicone adhesive (General Electric Co., Schenectady, N.Y.). The paper gaskets were easily removed if the adhesive was softened in boiling water. The caps could then be reused; the seal provided was positive and nontoxic. When used with impedance detection of bacterial growth (2a), the Bakelite caps were drilled and fitted with the electrodes described (see Fig. 1).

Use of different 47-mm-diameter membrane filters having different porosities. Membranes obtained from Nuclepore (Pleasanton, Calif.) were also tested. They are thin (one-tenth as thick as membranes obtained from the Millipore Corp., Bedford, Mass.) polycarbonate membrane filters with perforations made by nuclear particle bombardment, having pore sizes of 0.1, 0.2, 0.4, and 0.6 μm. A cellulose-type membrane filter (Millipore) in pore sizes of 0.45, 0.65, 1.2, 3.0, 5.0, 8.0, and 14 μm was also tested. The filters were tested for speed of filtra-

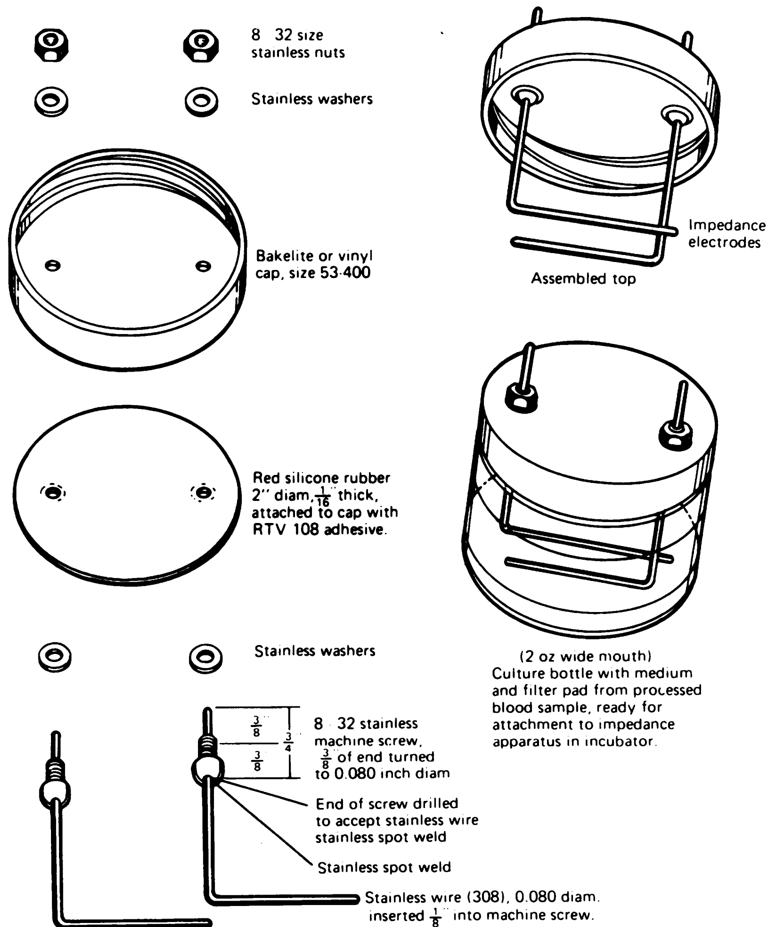

FIG. 1. *Diagram of the parts of a wide-mouth culture bottle.*

tion of lysed blood samples and efficiency of retention of *Escherichia coli* and *Staphylococcus aureus* suspensions.

Recovery of bacteria from membrane filters. Suspensions of *Staphylococcus epidermidis* containing fewer than 100 colony-forming units (CFU)/ml were first filtered through 0.6- and 0.4-μm pore membranes (Nuclepore). By reversing the flow of 5 ml of buffer through the filters, it was hoped that bacteria grown for culture on different media could be resuspended without the membrane filters.

Toxicity to bacteria. Human blood (10% vol/vol) in brain heart infusion broth was mixed with lysing solution in the proportions described in the lysing procedure. This was divided into 50-ml volumes in milk dilution bottles. Approximately 10^6 CFU of the test organisms taken from log-phase growth was added. The bottles were held in the 37°C water bath and sampled for colony counts at 30 min, 1 h, and 2 h. The species tested are seen in Table 1.

RESULTS

Examination at 1,250× magnification by Nomarski interference optics of wet-mounted Nuclepore membrane filters through which lysed blood had been filtered disclosed erythrocyte stroma and disrupted, condensed, apparently dead leukocytes.

It is seen in Table 2 that *E. coli* and *S. aureus* are retained on Millipore and Nuclepore membrane filters of pore sizes that, on a strictly mechanical basis, would pass at least part of the bacterial suspensions tested. This retention is most evident at low bacterial numbers of about 500 to 1,000 CFU/ml. At higher bacterial numbers of 10^8 to 10^9 CFU/ml, increasing percentages of *E. coli* passed through filters: 0.0% through the 0.65-μm filter, 0.000003% through the 0.8-μm filter, 0.0003% through the 1.2-μm filter, 0.20% through the 3.0-μm filter, 8.5% through the 5.0-μm filter, 27.4% through the 8.0-μm filter, and 100% through the 10.0-μm filter. For *S. aureus* the values were: 0.65 μm, 0%; 0.8 μm, 0.000008%; 1.2 μm, 0.00006%; 3.0 μm, 0.009%; 5.0 μm, 0.01%; 8.0 μm, 0.03%; 10.0 μm, 18.0%; and 14.0 μm, 21.0%.

At low bacterial numbers, 6.2×10^2 CFU for *E. coli* and 7.3×10^2 CFU for *S. aureus*, no bacteria were grown from the *filtrates* until membranes having pore sizes of 5.0 μm (*E. coli*) and 8.0 μm (*S. aureus*) were used.

Blood samples were lysed sparkling clear with most samples as the lysing solution was added. These were filtered immediately by vacuum through 0.45-μm Millipore or 0.6-μm Nuclepore membrane filters. Those showing opacity resulting from unlysed erythrocytes were incubated at 37°C for 30 min and filtered. A few did not lyse sparklng clear in this time, but *filtration* was still 100% within 2 min.

Attempts were made to recover, by backwashing, small numbers (480 CFU) of *S. epidermidis* that had been deposited by filtration onto 0.4- and 0.6-μm Nuclepore membrane filters and 0.45-μm Millipore membrane filters. These attempts were unsuccessful (Table 3). After backwash with 5 ml of M-100 phosphate buffer (pH 6.8), recovery of CFU was 11 (2.3%) for the 0.6-μm Nuclepore membrane filter, 60 (12.5%) for the 0.4-μm Nuclepore membrane filter, and 24 (5.0%) for the 0.45-μm Millipore membrane filter.

The effect of the lysing system on 29 representative bacterial species is seen in Table 1.

Most of the bacterial pathogens tested in the lytic system either held approximately the same numbers over the 2-h test period or multiplied. Unexpectedly, the *Haemophilus* species increased in numbers over 2 h.

TABLE 1. *Tolerance to blood lysing solution of representative bacteria*

Organism	CFU/ml			
	0 min	30 min	60 min	120 min
Staphylococcus aureus	170	800	1,560	2,260
S. epidermidis	210	230	480	1,200
Streptococcus group A	540	620	660	880
S. pneumoniae	117	89	67	43
Streptococcus group D	550	1,080	1,140	1,460
S. viridans	770	305	67	71
Listeria monocytogenes	362	440	292	400
Bacillus subtilis	660	626	520	298
Corynebacterium sp. (*diphtheroids*)	181	140	115	153
Escherichia coli	540	440	1,290	8,200
Klebsiella pneumoniae	490	690	1,190	8,000
Proteus vulgaris	550	520	400	450
Pseudomonas aeruginosa	550	790	1,010	2,120
Haemophilus influenzae	16	50	55	164
H. aphrophilus	250	248	221	276
H. haemolyticus	53	65	115	643
H. parainfluenzae	121	144	250	440
H. vaginalis	180	188	167	143
Neisseria meningitidis	330	23	18	11
N. gonorrhoeae	300	42	14	11
N. lactamica	210	93	62	30
Branhamella catarrhalis	472	105	52	11
Candida albicans	109	90	50	137
Eikenella corrodens	380	100	38	23
Veillonella	370	160	148	88
Peptococcus sp.	89	95	71	60
Fusobacterium nucleatum	476	284	160	5
Bacteroides fragilis	4,000	2,500	2,250	1,800
Clostridium perfringens	2,000	1,000	600	200

DISCUSSION

Recovery of filtered bacteria by backwashing from the filter pad was so low that this was

TABLE 2. *Membrane filter retention of E. coli and S. aureus*

Filter	Filtration time (s)	% of 50 ml filtered	Membrane filter retention of: *E. coli* 6.2×10^8 CFU: CFU in filtrate	% of total	*E. coli* 6.2×10^2 CFU: CFU in filtrate	% of total	*S. aureus* 1.0×10^9 CFU: CFU in filtrate	% of total	*S. aureus* 7.3×10^2 CFU: CFU in filtrate	% of total
Nuclepore (μm)										
0.1	120[a]	5	0		0		0		0	
0.2	120	30	0		0		0		0	
0.4	23	100	0		0		0		0	
0.6	10	100	0		0		0		0	
Millipore (μm)										
0.20	120	85	0		0		0		0	
0.45	12	100	0		0		0		0	
0.65	21	100	0		0		0		0	
0.8	6	100	2.0×10^2	0.000003	0		8.0×10^1	0.000008	0	
1.2	<6	100	1.8×10^3	0.0003	0		6.3×10^2	0.00006	0	
3.0	<6	100	1.2×10^6	0.2	0		9.2×10^4	0.009	0	
5.0	<6	100	5.3×10^7	8.5	3.0×10^1	4.8	1.1×10^5	0.01	0	
8.0	<6	100	1.7×10^8	27.4	3.0×10^2	48.4	2.5×10^5	0.03	8.9×10^1	12.2
10.0	<6	100	6.4×10^8	103.2	7.1×10^2	114.5	1.8×10^8	18.0	6.8×10^2	93.2
14.0	6	100	5.1×10^8	82.3	6.9×10^2	111.3	2.1×10^8	21.0	7.5×10^2	102.7

[a] Arbitrary time limit.

TABLE 3. *Retention of S. epidermidis on membrane filters despite backwash procedure*

Trial	No. of CFU in 50 ml of lysed blood	Filter membrane	Colonies on membrane after filtration	CFU in 5 ml of backwash buffer
1	480	Nuclepore, 0.6 μm	TNTC[a]	11 (2.3%)
2	480	Nuclepore, 0.4 μm	TNTC	60 (12.5%)
3	480	Millipore, 0.45 μm	TNTC	24 (5.0%)
4	480	Nuclepore, 0.6 μm	TNTC	Not backwashed

[a] TNTC, Too numerous to count.

abandoned as a means of recovering washed pathogens. It was necessary to continue culture using the filter pad itself, either on or in media.

Backwashing experiments indicate that pore diameter only partially explains the phenomenon. Since, at the most, only 12.5% of *S. epidermidis* CFU was recovered by backwashing Nuclepore filters, there must be an electrostatic charge attraction between bacteria and filter. This applies to both cellulose (Millipore)- or polycarbonate (Nuclepore)-type filters. This phenomenon applies to gram-positive as well as gram-negative bacteria. For a blood sample of higher erythrocyte or leukocyte content, i.e., resistances to lysis (and then to filtration), it might be justified to use a membrane filter of larger pore size, with assurance that bacteria present would remain adherent to the membrane. This possibility should be further explored, using a wide selection of test organisms.

The cellulose-type filter (Millipore; Gelman Instrument Co., Ann Arbor, Mich.; Amicon Corp., Lexington, Mass.) is 10-fold thicker than the polycarbonate-type filter (Nuclepore) and has pores that do not pass straight through the filter but twist and interlace through the pad. Because of this, it might be expected that a proportion of the pores would be much smaller than the maximum pore size designation. This would explain partial retention of bacteria in those filters having pore sizes up to 8.0 μm in diameter. Polycarbonate filters are very thin and nonfibrous, and the pores are "punched" out by particle bombardment. Unfortunately, the largest pore size available for testing was 0.6 μm. In the tests conducted, no bacterial cells passed through Nuclepore filters. It might be expected that a few bacteria would pass through the 0.6-μm pores, but this was not observed.

An advantage of the lysis-filtration system is the clarity of the medium. With no confusing turbidity from blood cells, a positive culture is detected easier and earlier by visual inspection. A cause of incomplete blood lysis and filter clogging, and therefore the most compelling

reason for the use of filters of 0.6-, 0.65, or 0.8-μm porosity, is the bedside addition by the physician of greater than 10% blood to the broth medium. The lysing system is designed to work at blood-to-broth levels of 10% or less. When the proportion of blood is above 10%, lysis may be less complete. Other causes of incomplete blood lysis are: (i) abnormally high erythrocyte counts; (ii) abnormally high leukocyte counts; and (iii) blood from certain patients is invariably difficult to lyse. The reasons are not understood why some individuals have blood that is difficult to lyse in the absence of high erythrocyte or leukocyte counts.

The remarkably low toxicity of the lysing system described is probably due to two factors. The first factor is the presence of adequate quantities of protein, peptone, amino acids, and other organic molecules known to protect organisms from injury. The second factor is the moderate pH, about 7.5, at which lysis is conducted. This is far lower than other systems, using sodium carbonate alone, rather than sodium carbonate-sodium bicarbonate buffer.

Loss of the more fastidious bacteria in the lysing solution after 30 min in no case was great enough to raise doubt of the usefulness of the technique. A further mitigating factor was the consistent reduction of these same species while standing as stock suspensions in brain heart infusion broth or Trypticase soy broth at room temperature. This reduction for some species approached that which occurred in the lysing solution. Also, lysing time for most processed specimens was kept well within 30 min, and most were filtered immediately after mixing with lysing solution.

Increased numbers of *Haemophilus* during the 2-h period in the lysing solution is evidence of the gentleness of the lysing procedure and indicates, moreover, that the combined action of Triton and Rhozyme proteases on the blood sample produces substances that promote growth of *Haemophilus*.

The technique has recently been simplified for blood cultures from the leukemia service of the hospital. Bottles (screw capped) of blood culture medium with the final concentration of lysing reagents are kept at 4°C on the wards. Blood is transferred at bedside to this bottle and brought to the laboratory, where the mixture is poured into a disposable filter unit and filtered. The filter membrane is then transferred to the 2-oz blood culture bottle. At that point it is incubated, either attached to the impedance detection machine or separated for visual growth detection. The time required is, thus, little more than that required for a conventional culture. Overall cost is greater than the conventional culture only by the cost of a disposable filter unit. Potential for increased contamination over the conventional system is restricted to the filtration and filter retrieval step. This has been of an acceptable level.

ACKNOWLEDGMENTS

The technical assistance of Harry (Mark) Strauss and Deborah Rubin is gratefully acknowledged.

LITERATURE CITED

1. **Braun, W., and J. Kelsh.** 1954. Improved method for cultivation of Brucella from the blood. Proc. Soc. Exp. Biol. Med. 85:154–155.
2. **Farmer, S. G., and R. A. Komorowski.** 1972. Evaluation of the Sterifil lysis-filtration blood culture system. Appl. Microbiol. 23:500–504.
2a. **Kagan, R. L., W. H. Schuette, C. H. Zierdt, and J. D. MacLowry.** 1977. Rapid automated diagnosis of bacteremia by impedence detection. J. Clin. Microbiol. 5:51–57.
3. **Rose, R. E., and W. J. Bradley.** 1969. Using the membrane filter in clinical microbiology. Med. Lab. 3:22–23, 29, 43.
4. **Sullivan, N. M., V. L. Sutter, and S. M. Finegold.** 1975. Practical aerobic membrane filtration blood culture technique: development of procedure. J. Clin. Microbiol. 1:30–36.
5. **Sullivan, N. M., V. L. Sutter, and S. M. Finegold.** 1975. Practical aerobic membrane filtration blood culture technique: clinical blood culture trial. J. Clin. Microbiol. 1:37–43.
6. **Tidwell, W. L., and L. L. Gee.** 1955. Use of membrane filter in blood cultures. Proc. Soc. Exp. Biol. Med. 88:561–563.
7. **Winn, W. R., M. L. White, W. T. Carter, A. B. Miller, and S. M. Finegold.** 1966. Rapid diagnosis of bacteremia with quantitative differential-membrane filtration culture. J. Am. Med. Assoc. 197:539–548.