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Blood Culture Technique Based on Centrifugation: Developmental Phase

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A quantitative and flexible blood culture system based on centrifugation of lysed blood over a density layer is described. The effect of relative centrifugal force, centrifugation time, and two different density solutions on the recovery of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albicans* was determined. To demonstrate the versatility of this technique, the reisolation of 23 representative microorganisms from inoculated normal human blood was determined at a fixed centrifugation speed and time. The potential merits of this technique are discussed and compared with those of conventional blood culture methods.

It is well recognized that septicemia represents one of the most serious forms of infection in man. Furthermore, with continued advances in medical techniques, it is now possible to maintain the severely compromised patient for extended periods. Patients with cancer, extensive burns, tissue transplants, or other degenerative diseases are especially prone to septicemia. In addition, the extensive use of immunosuppressive drugs and broad-spectrum antibiotics has not only caused a change in the antibiotic resistance pattern of bacteria, but has also caused an emergence of fungal infections (6).

Although the blood bottle culture technique has been the primary method used for the detection of septicemia, it has several major limitations. In recognition of these restraints, attempts have been made to improve the quality of the technique through changes in media and atmospheric composition (9, 10, 14, 16, 22, 23), inclusion of ^{14}C-labeled substrates (1, 5, 15), and modifications in the procedure, i.e., number of bottles used, number of times of subculture (4), etc.

Recent improvements in the filtration method for blood culturing (8, 11, 17-19, 24) suggest that this system might overcome many of the blood bottle drawbacks. With the filtration system, the antimicrobial factors are removed, the cultural conditions of choice for a given organism are selected, and the time required to obtain a pure isolate is significantly reduced. Furthermore, the quantitative data obtained may prove valuable.

In spite of the potential merit of this approach, even the most recently improved version of the filtration system has a number of possible problems. To use the filtration technique, the corpuscular components of the blood must be chemically solubilized (18). This could be harmful to many pathogenic organisms. Multiple entries are generally required, and the final step requires considerable manipulation in an open atmosphere (18). Hence, the system is susceptible to laboratory contamination and may not be suitable for the recovery of strict anaerobic microorganisms.

Stimulated by the improved blood culturing capabilities of the filtration system, our laboratory has been investigating the potential use of centrifugation for the isolation of microorganisms from a blood specimen. By centrifuging microorganisms on or into a stabilizing density layer, after effective lysis of the erythrocytes, we have developed an alternative method of blood culturing.

The centrifugation method described here potentially has the same advantages as the filtration method. In addition, this method minimizes exposure of the sample to the atmosphere; hence, the system may be more suitable for the recovery of anaerobic microorganisms. Laboratory contamination should also be minimal. Finally, the hypertonic layer provides conditions suitable for the recovery of fragile organisms such as L-forms and *Mycoplasma*.

This report describes the centrifugation system and provides data on the recovery of *Staphylococcus aureus, Pseudomonas aeruginosa, Bacteroides fragilis,* and *Candida albicans* from blood as a function of relative centrifugal

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force (*g*) and time. The percent recovery of 21 microorganisms is also presented for a fixed speed (2,520 × *g*) and time (15 min).

MATERIALS AND METHODS

Preparation of the special centrifuge tube. The centrifuge tube consisted of a Vacutainer no. LS 3200 XF 590 (Becton-Dickinson) containing 1.5 ml of a sucrose-gel or Ficoll (Pharmacia Fine Chemicals)-gel. This special tube, with sucrose as the density material, was prepared as follows. To a tared beaker with a magnetic stirring bar, 50 g of water and 1.5 g of purified pigskin-gelatin (Eastman Organic Chemicals) were added. Upon heating, the gelatin dissolved and 50 g of sucrose was then added. The pH of the solution was adjusted to 6.5 by the addition of 4 N NaOH. Water lost during heating was replaced to yield 100 g, final weight. To each Vacutainer, 1.5 ml of the sucrose-gelatin was added; care was taken not to lose the vacuum within the tube. The tubes were then placed in a rack, stopper down, and autoclaved at 121 C for 15 min. After autoclaving, the tubes were placed at 4 C for at least 4 h. Once formed, the gel was stable up to 26 C, and introduction of the blood sample did not disturb the gel. Once the gel melted, however, the centrifuge tube had to be kept inverted (stopper down) to prevent mixing of the sample and the sucrose or Ficoll density layer.

The preparation of the Ficoll-gel centrifuge tube varied slightly from the preparation of the sucrose-gel tube. First, 20 g of a 7.5% (wt/wt) aqueous solution of gelatin was prepared and filtered hot through a 0.22-μm membrane filter (Millipore Corp.). Next, 80 g of a 25% (wt/wt) aqueous Ficoll solution, pH 4, was passed through the same filter apparatus. The Ficoll and gelatin were mixed, and a 1.5-ml aliquot was aseptically transferred to each sterile Vacutainer.

Media. With the exception of complete medium, L-medium, rich broth, and rich agar, dehydrated media available from Difco or BBL were used (see footnote *a* of Table 3). The L-medium was prepared by the method of Maltman (13). The formulations of the remaining three media are as follows. Complete medium consisted of (per liter of deionized water): glucose, 10 g; neopeptone (Difco), 10 g; yeast extract (Difco), 10 g; and agar (Difco), 15 g. The pH was adjusted to 6.5 with 4 N NaOH. Rich broth consisted of (per liter of deionized water): glucose, 10 g; nutrient broth (Difco), 10 g; and Casamino Acids (Difco), 10 g. The pH was adjusted to 7.0 with 4 N NaOH. Rich agar consisted of (per liter of deionized water): glucose, 10 g; nutrient broth (Difco), 10 g; Casamino Acids (Difco), 10 g; and agar (Difco), 20 g. The pH was adjusted to 7.0 with 4 N NaOH.

Effect of relative centrifugal force and time on recovery. Clinical isolates of *Escherichia coli, S. aureus, P. aeruginosa,* and *C. albicans* were maintained by weekly transfers on rich agar. For a given experiment, a single colony of one of the test organisms was transferred to rich broth and incubated 18 to 24 h at 35 C.

Human donor blood citrate phosphate dextrose (21 days old) obtained from the Wadley Blood Bank, Dallas, Tex., was used in this study. Blood (8.3 ml) was introduced into a Vacutainer no. 4960 containing 1.7 ml of 0.35% sodium polyanethol sulfonate (SPS). Into each Vacutainer, 0.3 ml of a sterile 12% (wt/vol) SOLRYTH (Medical Research Inc. of Dallas) solution was added. This product can be autoclaved at 121 C for 15 min and is stable for at least 6 months at room temperature. SOLRYTH was found to be an effective erythrocyte-lysing agent. Based on the technique of tube dilution, it was neither bacteriostatic nor bactericidal at 50 times the dilution used against all of the organisms employed in this study (Smith and Dorn, unpublished data). These preceding two steps were performed to parallel the sample preparation used in a concurrent clinical study (7). Lysed blood (8 ml) was removed from the Vacutainer and inoculated (range: 50 to 2,500 colony-forming units) with the test organism. The inoculum for each experiment was determined by the pour plate technique. At this point, the sample was processed as a clinical specimen (see Fig. 1).

After introduction of the sample (Fig. 1, step 1), the sucrose- or Ficoll-gel was melted by immersion of the tube in a 45 C water bath for 5 min (Fig. 1, step 2). The tube was placed, stopper down, in the HB-4 rotor of a RC-2 Sorvall centrifuge. A Corning adapter (no. 4881) with the bottom 1 cm removed was used to center the tube in the bucket.

After centrifugation at ambient temperature, under the desired conditions (see Tables 1–3), the microorganisms in the density layer were quantitated. The supernatant above the density layer was removed with a 1.5-inch (ca. 3.8-cm), 21-gauge hypodermic needle and a 10-ml syringe (Fig. 1, step 4). The remaining contents of the tube, 0.5 ml of supernatant and 1.5 ml of the density layer, were mixed vigorously and withdrawn into a 3-ml syringe. The volume was measured, and aliquots (0.2 ml) of the mixture were placed on each of two rich agar plates. The samples were spread with a glass rod. Samples containing *E. coli, S. aureus* or *P. aeruginosa* were incubated for 24 h at 35 C, and the colonies were then counted. Samples of *C. albicans* were incubated for 48 h at 35 C before the colonies were counted.

Recovery of 23 microorganisms by the centrifugation technique. Blood sample preparation and inoculum range were the same as in the preceding study; however, the maintenance media, broth media, and diluents varied with the organism tested (Table 3). After introduction of the seeded lysed blood into the centrifuge tube, the gel was melted and the tube was centrifuged for 15 min at 2,520 × *g*. Two methods were used to remove the sucrose density layer for quantitation. Method A consisted of first withdrawing 7.5 ml of supernatant, followed by mixing and plating of the density layer. In method B, 1.5 ml of the density layer and 0.5 ml of supernatant were drawn into a syringe and mixed vigorously, and then the density material was plated. For each method, aliquots (0.2 ml) were plated on the maintenance media for the test organism and incubated under the appropriate atmospheric conditions.

RESULTS

Use of the special centrifuge tube. A diagram of the manipulations required to process a

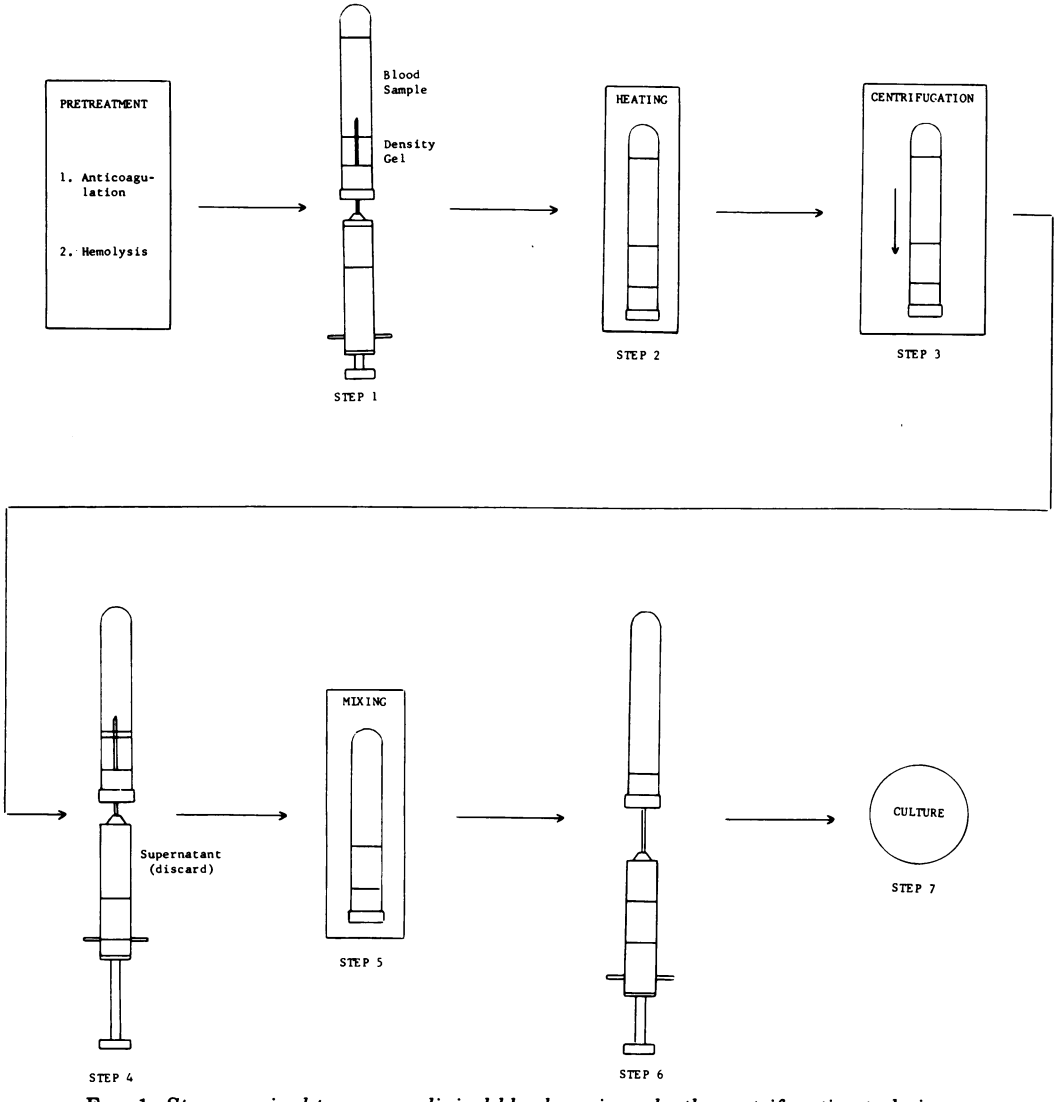

FIG. 1. *Steps required to process clinical blood specimen by the centrifugation technique.*

clinical blood specimen is presented in Fig. 1. The pretreatment step involves the addition of SPS and SOLRYTH. The latter compound causes hemolysis and prevents packing of the erythrocytes at the blood-density layer interface during centrifugation. The anticoagulant SPS was chosen because of its ability to neutralize antimicrobial factors present in blood (2, 20, 21).

A variety of solutions were evaluated for their ability to support whole blood during centrifugation at 5,820 × *g*. In addition to 50% (wt/wt) sucrose and 20% (wt/wt) Ficoll, 20% (wt/wt) solutions of the dextran T's (2000, 500, 110, 70 and 10, Pharmacia Fine Chemicals), dextran sulfate (Pharmacia Fine Chemicals), and diethylaminoethyl-dextran (Pharmacia Fine Chemicals) supported whole blood. However, 50% Hypaque (Winthrop), 40% polyvinylpyrollidone (40,000 molecular weight, MCB Manufacturing Chemists), and 40% polyethylene glycol (6,000 and 30,000 molecular weight, Fisher Scientific) would not support whole blood at 5,820 × *g*. Solutions of sucrose and Ficoll showed the most promise as density materials and were studied further.

Gelatin was added to stabilize the density layers. Incorporation of at least 1.5% (wt/wt) gelatin was found to prevent mixing of the sample and the density layer prior to centrifugation. Heating the tube at 45 C for 5 min converted the gel into a liquid.

Effect of relative centrifugal force and time on recovery. The recovery of four pathogenic microorganisms from blood by centrifugation was studied at six different centrifugal forces and three different times: 10, 20, and 30 min. When Ficoll was used as the density material, the recovery for each organism increased to a maximum as a function of g and time (Table 1). However, with extended times of centrifugation, e.g., 30 min, or at high centrifugal force, e.g., 4,000 to 6,000 $\times$ g, some decrease from optimal recovery was observed. This effect was most pronounced with *S. aureus* and *C. albicans*. With Ficoll as the density material, the best conditions found for recovery of all four microorganisms were 1,465 $\times$ g and 20 min.

Optimal conditions for the recovery of the four test organisms changed when sucrose was used as the density material (Table 2). The same centrifugal forces were used as in the preceding experiment. However, the time of centrifugation was 10 or 20 min. One of the organisms, *E. coli*, failed to show 100% recovery under these conditions, and the time of centrifugation in this one instance was extended to 30 min. In general, there was an increase in the recovery of a given organism with an increase in centrifugal force or time. With sucrose as the density material, the best conditions found for optimum recovery of all four microorganisms

TABLE 1. *Recovery of four pathogenic organisms from blood by centrifugation—Ficoll density layer*

Organism	Time (min)	Recovery[a]											
		164[b]		650		1465		2520		4080		5860	
		$\bar{X}$ ± SD	No.	$\bar{X}$ ± SD	No.	$\bar{X}$ ± SD	No.	$\bar{X}$ ± SD	No.	$\bar{X}$ ± SD	No.	$\bar{X}$ ± SD	No.
C. albicans	10	74 ± 35	14	104 ± 39	14	99 ± 22	14	67 ± 32	14	63 ± 34	14	71 ± 49	12
	20	92 ± 19	13	78 ± 47	13	86 ± 28	11	80 ± 38	10	88 ± 53	11	67 ± 33	11
	30	64 ± 24	12	72 ± 24	12	67 ± 20	12	55 ± 18	12	26 ± 20	12	29 ± 25	10
S. aureus	10	69 ± 22	13	92 ± 44	13	107 ± 46	12	88 ± 26	13	82 ± 49	13	65 ± 36	13
	20	72 ± 30	16	93 ± 44	16	96 ± 35	15	85 ± 34	16	64 ± 39	16	49 ± 22	16
	30	64 ± 30	14	86 ± 18	14	77 ± 27	14	64 ± 29	14	52 ± 36	14	39 ± 37	14
P. aeruginosa	10	37 ± 20	16	31 ± 16	14	63 ± 23	16	46 ± 34	15	63 ± 25	16	59 ± 21	15
	20	45 ± 16	13	75 ± 18	13	85 ± 30	13	72 ± 29	10	67 ± 21	13	73 ± 21	12
	30	43 ± 19	15	75 ± 18	15	80 ± 29	15	89 ± 30	14	59 ± 22	14	69 ± 30	15
E. coli	10	19 ± 13	26	36 ± 19	26	57 ± 32	25	57 ± 24	25	54 ± 24	23	52 ± 26	24
	20	36 ± 11	17	72 ± 20	16	85 ± 17	17	78 ± 13	17	65 ± 15	16	56 ± 20	14
	30	39 ± 20	13	76 ± 22	12	82 ± 29	14	79 ± 20	13	107 ± 59	13	87 ± 32	12

[a] Percent recovery given as mean ($\bar{X}$), standard deviation (SD), and number of experiments (No.).
[b] RCF, Relative centrifugal force.

TABLE 2. *Recovery of four pathogenic organisms from blood by centrifugation—sucrose density layer*

Organism	Time (min)	Recovery[a]											
		164[b]		650		1465		2520		4080		5860	
		$\bar{X}$ ± SD	No.	$\bar{X}$ ± SD	No.	$\bar{X}$ ± SD	No.	$\bar{X}$ ± SD	No.	$\bar{X}$ ± SD	No.	$\bar{X}$ ± SD	No.
C. albicans	10	51 ± 37	11	69 ± 26	11	83 ± 30	11	95 ± 13	11	76 ± 13	11	99 ± 46	11
	20	60 ± 37	10	91 ± 50	10	105 ± 24	10	98 ± 37	10	132 ± 33	10	108 ± 26	10
S. aureus	10	58 ± 49	15	90 ± 52	12	87 ± 40	14	105 ± 52	15	115 ± 55	15	114 ± 47	15
	20	66 ± 54	16	88 ± 61	15	92 ± 47	15	101 ± 55	15	105 ± 61	15	97 ± 53	13
P. aeruginosa	10	30 ± 19	12	45 ± 18	12	53 ± 21	12	72 ± 27	12	83 ± 22	12	94 ± 31	12
	20	48 ± 33	11	67 ± 28	11	72 ± 28	11	103 ± 56	11	99 ± 48	11	101 ± 50	11
E. coli	10	16 ± 17	13	28 ± 13	14	37 ± 16	15	48 ± 21	15	64 ± 20	15	65 ± 17	15
	20	28 ± 16	13	55 ± 20	12	62 ± 31	13	61 ± 32	13	73 ± 20	11	71 ± 26	13
	30	46 ± 24	13	65 ± 26	12	83 ± 21	12	91 ± 38	13	104 ± 31	12	89 ± 37	13

[a] Percent recovery given as mean ($\bar{X}$), standard deviation (SD), and number of experiments (No.).
[b] RCF, Relative centrifugal force.

were 4,080 × *g* and 30 min. If one is willing to sacrifice some recovery of *E. coli*, 2,520 × *g* for 10 to 20 min will provide satisfactory conditions.

These studies indicate that either a Ficoll-gelatin mixture or a sucrose-gelatin mixture can be used as the density material. The Ficoll-gelatin mixture, however, can only be sterilized by filtration, whereas the sucrose-gelatin mixture can be autoclaved. Since absolute sterility is required for blood culture analysis, the sucrose-gelatin mixture was selected as the density solution of choice.

Recovery of 23 microorganisms at 2,520 × g for 15 min. To verify the potential clinical use of centrifugation in the analysis of blood cultures, 23 representative microorganisms were selected for analysis. Since few table top centrifuges are capable of achieving 4,000 × *g* with a swinging-bucket rotor, the conditions of 2,520 × *g* and 15 min were chosen (Table 3). The majority of organisms listed in Table 3 show a recovery of 50% or greater. The recovery of three organisms, *B. fragilis*, *S. pneumoniae*, and *Listeria monocytogenes*, was below 50%. A higher centrifugal force or a longer time of centrifugation may be required for these organisms. Also, these organisms may be more sensitive than other organisms to SOLRYTH or to the bactericidal factors found in normal human blood. Studies on the effect of centrifugal force and time on the isolation of these three organisms is currently in progress.

The data in Table 3 also emphasize the great flexibility of this technique since anaerobes, aerobes, and fungi were easily recovered. Even fastidious organisms such as *Mycoplasma* and L-forms were isolated by this process. To our knowledge, no reliable method for quantitation of these two organisms has been developed.

Two methods were used for the removal of the gradient for quantitation (Table 3). Method A consisted of the removal of the lysed blood above the density layer, leaving the density material in the centrifuge tube. The density layer containing the organisms was mixed, removed, and quantitated. In method B, the density layer was removed directly from the centrifuge tube, leaving the lysed blood behind. The density material was mixed in the syringe and quantitated for microorganisms.

Method B has two important advantages over method A. The number of entries into the system is reduced from six to five. In addition, the patient's blood remains in the centrifuge tube, and pathogenic viral agents in the sample, e.g., hepatitis, are contained. Since there is no significant difference in the recovery by method A or method B, B would be the method of choice for the processing of clinical specimens.

DISCUSSION

It is apparent that centrifugation of microorganisms into a high-density solution has potential as an alternative to conventional blood culture techniques. The method is quantitative and very flexible with regard to the choice of growth conditions. Sedimentation of organisms into a hypertonic solution should protect microbial species with defective cell walls (12). The density solution containing the microorganisms is plated directly onto conventional media. This technique should shorten the time to detection and to isolation of pure strains. Furthermore, since single, characteristic colonies are observed, identification is simplified, and the possibility of overgrowth by a second organism is minimized. The theoretical dilution of antimicrobial factors achieved with this system is approximately 1:400. Finally, the data suggest that virtually every clinically significant bacterium or fungus could be isolated. This includes strict anaerobes like *B. fragilis* and organisms without complete cell walls, e.g., staphylococcal L-forms and *Mycoplasma*.

With sucrose as the density material, this study indicates that at a low centrifugal force and short time interval one does not recover 100% of the organisms in a blood sample. However, reconstruction experiments with seeded donor blood indicate that recovery does improve if a greater relative centrifugal force or a longer centrifugation time is employed. Unfortunately, swinging-bucket centrifuges capable of providing 4,000 to 6,000 × *g* are not commonly found in a routine clinical microbiology laboratory. However, with moderate centrifugal forces (2,520 × *g*) and a centrifugation time of 15 min, an average recovery of 69% was observed from a study of 21 representative pathogenic microorganisms. Preliminary experiments indicate that, when an angle rotor is substituted for a swinging-bucket rotor, a substantial reduction in recovery is observed. This loss in recovery is probably due to the sedimentation of the organisms along the side of the centrifuge tube.

To ascertain the possible merits of the centrifugation method as an alternative to conventional blood culture techniques, the following protocol is suggested for clinical evaluation. Blood is taken by venipuncture and aseptically placed in a Vacutainer containing 0.35% SPS (no. 4960). The specimen is transported to the laboratory where 0.3 ml of a 12% solution of SOLRYTH is introduced to lyse the erythro-

TABLE 3. *Percent recovery of various pathogens by centrifugation—2,520 × g, 15 min*

Organism[a]	Method A[b]			Method B[c]		
	No. of Samples	$\bar{X}$[d]	SD[e]	No. of Samples	$\bar{X}$	SD
Aspergillus nidulans (spores)	12	61.9	19.0	12	54.2	20.1
Candida albicans	24	76.6	24.1	22	70.7	32.4
Cryptococcus neoformans	24	80.6	31.3	23	91.6	37.0
Torulopsis glabrata	23	76.2	23.2	23	73.7	39.6
Citrobacter freundii	22	62.3	30.0	24	66.4	22.4
Enterobacter cloacae ATCC 23355	22	76.9	23.9	21	85.8	29.2
Escherichia coli	24	61.8	22.7	24	55.1	20.4
Klebsiella pneumoniae ATCC 23357	22	66.1	14.1	24	75.0	30.5
Proteus vulgaris ATCC 6380	24	71.2	25.2	23	63.5	18.0
Salmonella choleraesuis ATCC 10708	24	49.8	14.0	21	57.1	12.6
Pseudomonas aeruginosa	22	56.2	19.7	22	54.9	25.4
Listeria monocytogenes		NT[f]		24	35.5	8.6
Staphylococcus aureus	20	95.7	37.4	20	106.5	39.0
Staphylococcal L-forms		[f]			NT	
Streptococcus pyogenes	12	128.7	45.1	12	137.1	60.9
Streptococcus pneumoniae	24	25.5	10.4	24	28.4	13.9
Haemophilus influenzae ATCC 8149	10	53.2	18.8	10	51.8	17.7
Neisseria meningitidis ATCC 13077		NT		24	55.0	31.9
Brucella suis ATCC 4312		NT		24	61.3	20.0
Bacteroides fragilis ATCC 25285	24	52.6	50.3	24	38.3	19.5
Clostridium sphenoides	22	119.4	36.5	22	121.1	47.6
Propionibacterium shermanii	24	78.7	13.4	24	72.6	16.4
Mycoplasma hominis ATCC 23114		[f]			NT	

[a] For the appropriate culture techniques, see reference 3.
[b] Method A: Supernatant withdrawn prior to removal of density layer.
[c] Method B: density layer directly removed.
[d] Mean.
[e] Standard deviation.
[f] Recovered but not quantitated.

cytes. The lysed blood sample is removed and placed into the special sucrose-gel centrifuge tube (see Materials and Methods). The centrifuge tube is placed, stopper down, in a 45 C water bath for 5 min. After centrifugation, the sucrose layer (1.5 ml) plus a small amount of the supernatant (0.5 ml) is removed with a syringe and needle. The contents of the syringe are mixed, and aliquots (0.2 ml) are placed on the following media and incubated as specified: two blood agar plates, one aerobic at 35 C, one anaerobic at 35 C; one chocolate agar plate under 10% CO_2 at 35 C; two Sabouraud dextrose agar plates, one at 35 C and the other at 25 C.

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