

## Detection of Bacteria in Blood by Centrifugation and Filtration

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Culture of blood is the most frequent means of diagnosing bacteremia. However, conventional blood culturing methods are slow in isolating bacteria. We developed a method for isolation of bacteria by centrifugation and filtration. Fresh human whole blood was inoculated with facultatively anaerobic and aerobic microorganisms (3 to 172 microorganisms per 5 ml). Seeded blood was then mixed with Ficoll-Hypaque (density,  $1.149 \pm 0.002$  g/ml) and centrifuged ( $386 \times g$ ) for 30 min at ambient temperature. The entire gradient (plasma, leukocytes, and Ficoll-Hypaque) was removed and filtered through a 0.22- $\mu$ m membrane filter (Millipore). The filters were then placed on chocolate agar plates and incubated at 35°C in a humidified atmosphere containing 5% CO<sub>2</sub>. For each bacterium tested, approximately 35 to 100% of the viable microorganisms were recovered when compared with control cultures (pour plates of seeded blood). All bacteria produced isolated colonies on filters after overnight incubation (18 h). This procedure may prove to be a more rapid method for isolating bacteria from clinical blood samples than the blood culture bottle technique.

Septicemia is potentially life threatening. Approximately 200,000 cases of septicemia occur annually in the United States with a mortality rate of 40 to 50% (24). Rapid detection and isolation of the infecting microorganism(s) are paramount for the administration of effective antimicrobial therapy and survival of the patient.

Recent advances toward rapid detection of bacteremia have included the BACTEC system (Johnston Laboratories, Inc., Towson, Md.), the Oxoid Signal (Oxoid, U.S.A., Inc., Columbia, Md.) blood bottle, the Roche Septi-Chek system (Roche Diagnostics, Division of Hoffmann-La Roche Inc., Nutley, N.J.), and the Isolator (E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.). With the exception of the Isolator, these methods require culturing of blood in liquid medium. Several hours or days may be required for detection and isolation of bacteria. Although the BACTEC system, Oxoid Signal, Roche Septi-Chek, and Isolator provide enhanced detection of bacteria, some drawbacks have been documented such as contamination and different recovery rates for members of the family *Enterobacteriaceae*, staphylococci, yeasts, streptococci, and anaerobes (5-8, 14, 16, 18, 20, 25).

The use of membrane filter procedures for isolating microorganisms from blood has long been established (1, 21) but the procedure has been considered slow, cumbersome, and impractical for clinical use (20). Sullivan et al. (21, 22) encountered problems with filter clogging and subsequent decreased recovery of bacteria when lysing solution was used. Herlich et al. (6) and Lamberg et al. (11) described a centrifugation-filtration system that uses Ficoll-Hypaque. Their studies showed that centrifugation and filtration of blood constituted a sensitive method for recovery of microorganisms. However, whole blood had to be gently layered on top of a Ficoll-Hypaque gradient so as not to disrupt the blood-gradient interface.

We have developed a more practical procedure which allows mixing of blood with Ficoll-Hypaque. Whole blood samples, each containing one of a variety of aerobic and

facultatively anaerobic microorganisms, were mixed with the gradient, centrifuged, and filtered. We recovered 35 to 100% of viable microorganisms from seeded blood compared with the control inoculum.

### MATERIALS AND METHODS

**Microorganisms.** The microorganisms prepared for use as stock cultures were *Escherichia coli*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Streptococcus salivarius*, *Enterococcus faecalis*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus pneumoniae*, *Streptococcus sanguis*, and *Staphylococcus aureus*. All cultures were identified at the Wisconsin State Laboratory of Hygiene, the reference laboratory for the state of Wisconsin, by referred methods (12). Microorganisms were chosen to represent those frequently isolated from bacteremic individuals or having fastidious growth requirements.

Stock cultures were prepared by inoculating Todd-Hewitt broth (streptococci), Schaedler broth with 5% Fildes (*H. influenzae* and *N. meningitidis*), or dextrose broth (other bacteria) with one to five colonies of a recent clinical isolate. The suspensions were incubated at 35°C until slightly turbid. Each culture was diluted serially 10-fold in its respective broth with 10% rabbit serum, and 1.0-ml aliquots were dispensed into vials (catalog no 72.694.006; Sarstead, Princeton, N.J.) The vials were sealed and stored in liquid nitrogen until used.

**Quantitation of inocula.** Frozen vials containing suspensions of each microorganism were thawed and used to inoculate the above respective medium. The cultures were incubated and serially 10-fold diluted. Fresh human whole blood was seeded with selected dilutions to obtain a final concentration of 3 to 172 microorganisms per 5 ml of blood. This inoculum was chosen to simulate a low-grade bacteremia and to determine the sensitivity of the procedure. The actual number of microorganisms in the seeded blood was determined by mixing the seeded blood with molten antibiotic medium no. 11 (Difco Laboratories, Detroit, Mich.) maintained at 45 to 50°C. Four plates (100-mm diameter)

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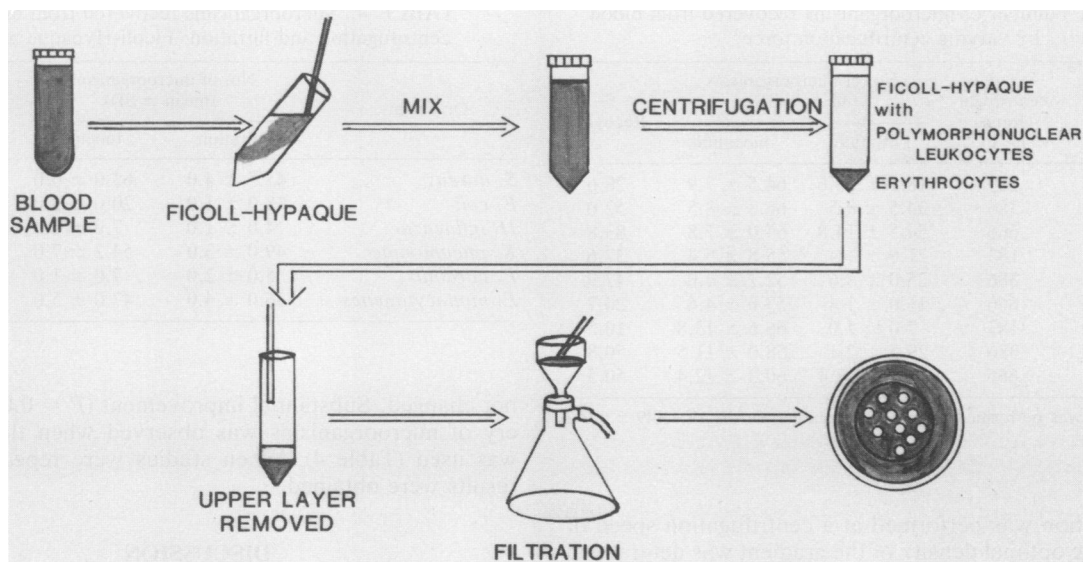


FIG. 1. Ficoll-Hypaque centrifugation and filtration procedure.

received a mixture of 2.5 ml of seeded blood and 10 ml of antibiotic medium no. 11. After the seeded blood and agar mixture solidified, the number of CFU was determined after 24 to 48 h of incubation at 35°C. The bacterial colonies were readily counted from the thin mixture of seeded blood and agar.

**Preparation of whole blood.** Fresh human whole blood (60 ml) was obtained from healthy volunteers who had not received antibiotics during the preceding 2 weeks. Blood was collected in 10-ml Vacutainer tubes containing 0.35% sodium polyanetholesulfonate (Becton Dickinson and Co., Rutherford, N.J.). The blood of a single donor was then seeded with bacteria. Immediately after seeding of the whole blood with bacteria, pour plates were poured (to determine inoculum counts) and the centrifugation-filtration procedure was initiated.

**Centrifugation and filtration procedure.** The centrifugation and filtration procedure is illustrated in Fig. 1. Initially, various densities ( $1.114 \pm 0.002$ ,  $1.149 \pm 0.002$ , and  $1.174 \pm 0.002$  g/ml) of Ficoll-Hypaque were prepared. Briefly, the Ficoll-Hypaque gradient density of  $1.149 \pm 0.002$  g/ml was prepared by dissolving 45 g of Ficoll 400 (molecular weight, 400,000; polymer of sucrose; Sigma Chemical Co., St. Louis, Mo.) and 2.5 g of NaCl in 500 ml of distilled water. The final density was achieved by adding 188 ml of Hypaque-M (diatrizoate meglumine; Winthrop Laboratories, New York, N.Y.) to the solution of Ficoll. The mixture was sterilized by filtration (Nalgene, 0.22- $\mu$ m pore size; Nalge Co., Div. of Sybron Corp., Rochester, N.Y.) and maintained at 4°C until used.

Sterile, double-ended, rubber-stoppered, glass tubes (16-mm diameter; 110 to 120 mm long) were loaded with 8.0 or 10 ml of sterile Ficoll-Hypaque. Aliquots, 5 ml, of seeded blood were added to each tube containing Ficoll-Hypaque. The tubes were inverted five times to mix thoroughly the Ficoll-Hypaque with the seeded blood. The tubes were then centrifuged (IEC Centra-8; International Equipment Co., Div. of Damon, Needham Heights, Mass.) at various speeds at room temperature for 30 min. A single organism was evaluated in each run. Triplicate tubes were prepared for each microorganism.

Centrifugation of the blood-gradient mixture resulted in erythrocytes being pelleted at the bottom of the tubes. Mononuclear and polymorphonuclear cells were distributed predominately in the upper portion of the gradient. After centrifugation the entire gradient, except erythrocytes, was removed and filtered through 0.22- $\mu$ m-pore-size filters (diameter, 47 mm; Millipore Corp., Bedford, Mass.) under negative pressure with a single-place sterility test manifold (Millipore) attached to a vacuum pump (Gast Mfg. Corp., Benton Harbor, Mich.).

The filters were removed from the filtration apparatus, placed with the filtrate side up on chocolate agar plates, and incubated at 35°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Colonies of bacteria were detected within 18 h. The number of CFU on the filters was compared with the inoculum counts. Percent recovery was calculated by dividing filter counts by inoculum counts and multiplying by 100.

**Analysis of data.** Statistical analysis was performed by analysis of variance. The alpha level was set at 0.05 before the experiments were started.

## RESULTS

**Density gradient, centrifugation, and filtration.** An initial evaluation was performed to determine the effects of Ficoll-Hypaque density on recovery of microorganisms (Table 1).

TABLE 1. Number of microorganisms recovered from blood by centrifugation and filtration with three densities of Ficoll-Hypaque<sup>a</sup>

| Organism             | No. of microorganisms (mean $\pm$ SD) recovered with given Ficoll-Hypaque density (g/ml) |                |                | Inoculum (no. of microorganisms) |
|----------------------|--|----------------|----------------|----------------------------------|
|                      | 1.114  | 1.149          | 1.174          |                                  |
| <i>E. coli</i>       | 12.0 $\pm$ 1.0   | 12.0 $\pm$ 1.0 | 13.0 $\pm$ 1.0 | 16.0 $\pm$ 3.0                   |
| <i>S. aureus</i>     | 8.0 $\pm$ 1.0  | 17.0 $\pm$ 2.0 | 19.0 $\pm$ 3.0 | 29.0 $\pm$ 5.0                   |
| <i>H. influenzae</i> | 3.0 $\pm$ 1.0  | 4.0 $\pm$ 1.0  | 3.0 $\pm$ 2.0  | 7.0 $\pm$ 1.0                    |

<sup>a</sup> Procedure was performed at a centrifugation speed of  $386 \times g$ .

TABLE 2. Number of microorganisms recovered from blood by varying centrifugation force<sup>a</sup>

| Organism             | Relative centrifugal force ( $\times g$ ) | No. of microorganisms (mean $\pm$ SD) |                 | % Recovery |
|----------------------|---|---------------------------------------|-----------------|------------|
|                      |   | Filtration                            | Inoculum        |            |
| <i>E. coli</i>       | 183                                       | 18.5 $\pm$ 10.6                       | 64.5 $\pm$ 7.9  | 28.6       |
|                      | 386                                       | 34.5 $\pm$ 8.5                        | 66.3 $\pm$ 8.5  | 52.0       |
|                      | 666                                       | 54.5 $\pm$ 14.8                       | 65.0 $\pm$ 7.8  | 83.8       |
| <i>S. aureus</i>     | 183                                       | 21.0 $\pm$ 1.4                        | 55.8 $\pm$ 5.4  | 37.6       |
|                      | 386                                       | 25.0 $\pm$ 5.6                        | 52.2 $\pm$ 4.0  | 47.9       |
|                      | 666                                       | 11.0 $\pm$ 1.4                        | 53.0 $\pm$ 4.6  | 20.7       |
| <i>H. influenzae</i> | 183                                       | 7.0 $\pm$ 7.0                         | 66.6 $\pm$ 13.8 | 10.5       |
|                      | 386                                       | 29.5 $\pm$ 2.1                        | 58.0 $\pm$ 11.5 | 50.8       |
|                      | 666                                       | 30.5 $\pm$ 13.4                       | 60.3 $\pm$ 12.4 | 50.5       |

<sup>a</sup> Procedure was performed at a Ficoll-Hypaque density of 1.149  $\pm$  0.002 g/ml.

This evaluation was performed at a centrifugation speed of 386  $\times g$ . The optimal density of the gradient was determined to be 1.149  $\pm$  0.002 g/ml. Although slightly improved recovery was obtained with *E. coli* and *S. aureus* at a density of 1.174  $\pm$  0.002 g/ml compared with inoculum, unpelleted erythrocytes tended to clog the filters. Therefore, subsequent studies were performed with Ficoll-Hypaque at a density of 1.149  $\pm$  0.002 g/ml.

An additional study was performed to determine the effects of centrifugation speed on recovery of microorganisms (Table 2). This evaluation was performed with the Ficoll-Hypaque density at 1.149  $\pm$  0.002 g/ml. A centrifugation force of 386  $\times g$  produced optimal recovery of bacteria. At a lesser relative centrifugal force (183  $\times g$ ), recoveries of *E. coli* and *H. influenzae* were substantially reduced. At a greater relative centrifugal force (666  $\times g$ ), recovery of *S. aureus* was compromised.

**Recovery of microorganisms from whole blood.** In general, fewer microorganisms were recovered by the filters compared with the original inoculum (pour plate of seeded blood) (Table 3). The amount recovered by filtration ranged from 35 to 107%. All microorganisms, however, were detected on the filters within 18 h after filtration. Occasionally the inoculum (pour plates) required 24 to 48 h for determination of the number of CFU. A subsequent study with additional Ficoll-Hypaque (10 ml) improved recovery of bacteria on filters. The centrifugation speed and gradient density were

TABLE 3. Microorganisms recovered from blood by centrifugation and filtration: Ficoll-Hypaque at 8 ml

| Organism               | No. of microorganisms (mean $\pm$ SD) |                  | % Recovery |
|------------------------|---------------------------------------|------------------|------------|
|                        | Filtration                            | Inoculum         |            |
| <i>S. aureus</i>       | 15.3 $\pm$ 8.7                        | 37.5 $\pm$ 4.9   | 40.8       |
| <i>E. faecalis</i>     | 28.0 $\pm$ 4.5                        | 32.0 $\pm$ 8.4   | 87.5       |
| <i>S. mitis</i>        | 22.6 $\pm$ 6.6                        | 65.0 $\pm$ 8.4   | 34.7       |
| <i>S. mutans</i>       | 141.3 $\pm$ 10.0                      | 172.6 $\pm$ 20.5 | 81.8       |
| <i>S. pneumoniae</i>   | 52.3 $\pm$ 6.3                        | 56.0 $\pm$ 1.4   | 93.3       |
| <i>S. salivarius</i>   | 12.3 $\pm$ 8.0                        | 15.5 $\pm$ 2.1   | 79.3       |
| <i>S. sanguis</i>      | 40.0 $\pm$ 6.5                        | 49.0 $\pm$ 4.2   | 81.6       |
| <i>E. coli</i>         | 38.6 $\pm$ 3.5                        | 79.0 $\pm$ 9.8   | 48.8       |
| <i>H. influenzae</i>   | 19.0 $\pm$ 6.2                        | 46.3 $\pm$ 16.6  | 41.0       |
| <i>K. pneumoniae</i>   | 35.3 $\pm$ 5.1                        | 59.5 $\pm$ 7.7   | 59.3       |
| <i>N. meningitidis</i> | 93.0 $\pm$ 7.8                        | 131.0 $\pm$ 13.4 | 70.9       |
| <i>P. aeruginosa</i>   | 171.3 $\pm$ 12.0                      | 159.5 $\pm$ 9.2  | 107.3      |

TABLE 4. Microorganisms recovered from blood by centrifugation and filtration: Ficoll-Hypaque at 10 ml

| Organism                | No. of microorganisms (mean $\pm$ SD) |                | % Recovery |
|-------------------------|---------------------------------------|----------------|------------|
|                         | Filtration                            | Inoculum       |            |
| <i>S. aureus</i>        | 45.5 $\pm$ 4.0                        | 63.0 $\pm$ 6.0 | 72.0       |
| <i>E. coli</i>          | 18.0 $\pm$ 1.0                        | 20.0 $\pm$ 2.0 | 90.0       |
| <i>H. influenzae</i>    | 4.0 $\pm$ 1.0                         | 7.0 $\pm$ 1.0  | 57.1       |
| <i>K. pneumoniae</i>    | 49.0 $\pm$ 3.0                        | 54.2 $\pm$ 7.0 | 90.4       |
| <i>P. mirabilis</i>     | 5.0 $\pm$ 2.0                         | 7.0 $\pm$ 1.0  | 71.4       |
| <i>L. monocytogenes</i> | 36.0 $\pm$ 4.0                        | 47.0 $\pm$ 5.0 | 76.0       |

not changed. Substantial improvement ( $P = 0.01$ ) in recovery of microorganisms was observed when this approach was used (Table 4). When studies were repeated, similar results were obtained.

## DISCUSSION

Our data show that the Ficoll-Hypaque gradient and filtration system is sensitive for the detection of simulated low-grade bacteremia. Microorganisms were detected on filters within 18 h after filtration. To simulate low-grade bacteremia, blood was seeded with a small number of microorganisms. The inocula (mean  $\pm$  standard deviation) ranged from 3.0  $\pm$  2.0 to 172.6  $\pm$  20.5 microorganisms per 5 ml.

Overall, fewer microorganisms were recovered on the filters than were present in the inoculum (Table 3). When longer tubes and additional Ficoll-Hypaque were used, substantially improved recovery of *S. aureus*, *E. coli*, *H. influenzae*, and *K. pneumoniae* was obtained (Table 4). Some loss of microorganisms was expected due to retention of small quantities of seeded blood in the pipette used to transfer blood to the gradient. In addition, the tubes retained a small portion of the gradient containing seeded blood. It is also possible that bacteria adhered to the sides of the glass tubes and were missed by filtration. The increased recovery of *P. aeruginosa* (107.3%) may be due to multiplication in the blood during the time required to complete the centrifugation-filtration procedure. Similar results were obtained with repeated trials.

Previously developed blood filtration systems were not used in the clinical setting because they were considered technically slow and cumbersome (20). Also, problems with clogging of the filters due to blood components and decreased recovery of gram-negative microorganisms with the use of blood lysing reagents have been reported (21, 22). Recent work by Lamberg et al. (11) and Herlich et al. (6) with a Ficoll-Hypaque density gradient has overcome those problems. The disadvantage of their procedure was that blood had to be layered on top of the gradient.

We have developed a more practical procedure in which seeded whole blood was mixed with the Ficoll-Hypaque, centrifuged, and filtered. Isolated bacteria were detected within 18 h. Like the work by Lamberg et al. (11) and Herlich et al. (6), our system did not require lysing agents (4, 21, 26), multiple filters (10), dilutions (10), sophisticated equipment (13, 20, 21), or excessive centrifugation speeds (3). Clogging of filters with erythrocytes and leukocytes did not occur. Filtration of the entire gradient (plasma, leukocytes, and Ficoll-Hypaque) took <1 min and no contamination was observed.

While there are no commercial filtration systems currently available, filtration does offer potential advantages. These include faster isolation of bacteremic agents, collection of leukocytes that may harbor phagocytized bacteria, no subculturing, removal of inhibitory agents (antibiotics) from blood, detection and faster identification of the causative organism, and detection of mixed infections or contaminants (1, 2, 20, 21). Antibiotic susceptibility tests can also be accelerated due to growth of distinct isolated colonies that do not need further purification. In addition, efficacy of treatment can be determined by quantitation of bacteria on the filters.

To better aid in the rapid detection of bacteremia, several detection systems have been developed, including the BACTEC system (radiometric and nonradiometric), the Oxoid Signal bottle, and the Roche Septi-Chek (14, 18, 20, 25). While detection times of the BACTEC system and the Oxoid Signal may be comparable (9, 25), both still require subculture for isolation of bacteria. Although the Roche Septi-Chek system has a subculture attachment, repeated inoculations may be required for isolation of bacteria. Our system does not require subculturing attempts (7, 9, 12, 15). The Isolator uses direct plating and may isolate bacteria faster. Additional studies are needed to determine whether our centrifugation-filtration system compares with the Roche Septi-Chek and the Isolator methods.

The current effort is an extension of previous work (6, 11) which showed that centrifugation-filtration detected bacteremia more rapidly than conventional blood culture. We have improved the system by removing the requirement for layering blood over Ficoll-Hypaque. The present system allows for mixing of blood with gradient, making the procedure more practical for routine clinical laboratory use.

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