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Use of Droplet Plating Method and Cystine-Lactose Electrolyte-Deficient Medium in Routine Quantitative Urine Culturing Procedure

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Droplet plating of 0.01 ml of 10^{-2} dilutions of mixed sonically treated urines onto cystine-lactose electrolyte-deficient agar permits formation of discrete, easily counted colonies within a small circumscribed area without interference by *Proteus* overswarm. Each colony is considered as arising from a single viable cell. The single dilution permits precise reproducible quantitation of urine bacteria population within the range 10^4 to 10^6 cells/ml of sample. Droplet-plated counts were found to be consistently (approximately) double those determined by standard pour plate quantitation. The method requires only inexpensive readily available materials and has been performed routinely in a large-volume clinical laboratory for several years.

Procedures to detect bacteriuria are among the most important functions of a clinical microbiology laboratory and are quite often the most frequently requested. A properly performed urine culture enables the physician to know numbers of organisms present in a sample, the identities of probable pathogens, and their antimicrobial susceptibility patterns. Definitive urine cultures also enable the clinician to monitor the status of patients with indwelling catheters and to learn if a subsequent urinary tract infection was nosocomial. A laboratory method should provide a convenient and reliable means to determine bacteriuria at a significant level and enable technologists to distinguish between gram-positive and gram-negative varieties and have well-isolated colonies free from overswarm.

A flood plate technique was found by Vejlsgaard (5, 6) to have precision and reproducibility to quantitate urine bacteria. Whole or diluted urine was flooded onto the agar surface by pipette, and resulting colonies were counted. Mallmann and Broitman used the droplet plating method to determine milk bacterial counts in which they deposited 0.1 or 0.2 ml of the appropriate dilution (2), and Reed and Reed (3) have described and evaluated the method for general microbiological quantitation.

Cystine-lactose electrolyte-deficient (CLED) medium was designed to halt spreading *Proteus* without inhibiting growth. Its peptone content enables other urinary tract organisms to grow, whereas its dye content permits detection of lactose fermenters by their yellow color (4).

This report describes application of the droplet plating method and CLED agar to determine numbers of urine bacteria. It has been used in the author's laboratory for several years.

MATERIALS AND METHODS

Culture routine. Urines are received in glass screw-capped tubes (20 by 125 mm) which are placed onto a Labquake rocking platform mixer (Labindustries, Inc.) for 5 min to provide gross mixing. Tubes are placed 10 at a time into a low-power sonic oscillation bath (Esterline-Angus sonic cleaner, model EA 12, with a frequency of 40 kHz) for 90 s to break apart bacterial clumps. Greater exposure is unnecessary. However, kill will not occur when urine samples are exposed for up to 900 s, should tubes inadvertently be left in longer.

Potential lethality of exposure by sonic treatment to commonly isolated urine bacteria was investigated by exposing 18- to 24-h Trypticase soy broth cultures in glass screw-capped tubes to the oscillation continuously for 15 min. Base line numbers of viable organisms were determined by droplet plating prior to sonic treatment. Samples were withdrawn at 3-min intervals during treatment and droplet plated onto Trypticase soy agar. Counts per time were determined for *Escherichia coli, Staphylococcus aureus, Streptococcus faecalis, Pseudomonas,* and *Mycobacterium tuberculosis*.

Mixed sonically treated urine is diluted 1:100 by adding 0.1 ml to a 9.9-ml saline blank autoclaved with rubber stopper clamped in to prevent fluid loss. The dilution is mixed thoroughly on a Vortex apparatus, and CLED (3) agar plates are "droplet inoculated" on a level surface using 4 drops (0.01 ml) from a Corning no. 7099 disposable glass pipette (Corning Glass Works, Corning, N.Y.).

By allowing 0.01 ml of diluted urine to flow from the vertical 0.1-ml pipette, a pendant droplet will form but not fall (Fig. 1 and 2). The expulsion of slightly more fluid will cause the droplet to fall, but this should not be allowed to happen. Surface tension will cause the droplet to become spherical, and it will likely roll or bounce prior to breaking on the agar surface. Breaking on contact can create an aerosol hazard plus result in an aggregation of colonies difficult to count. The suspended droplet is applied to the agar medium by allowing the fluid, but not the pipette tip, to touch the surface (Fig. 3 and 4). The fluid spot is allowed to spread naturally on the agar surface, which should be supported on a level working area (Fig. 5 and 6). Its liquid phase will enter the gel matrix by imbibition, depositing bacterial cells on the agar surface (Fig. 7). No glass or wire spreader is used to distribute the deposit, and plates are not moved for 30 min or until all fluid has disappeared. Use of a spreading instrument may pick up organisms from the deposit, thereby disturbing a critical small number. Four deposits are made on each plate. At the end of 18 h discrete round clusters of microcolonies will result, as shown in Fig. 8. The count per milliliter of urine is an average of the total count from four spots (Fig. 9) multiplied by 10^4. The number of colony types is recorded at read out, which takes place under low-power stereomicroscopy by both reflected and transmitted light.

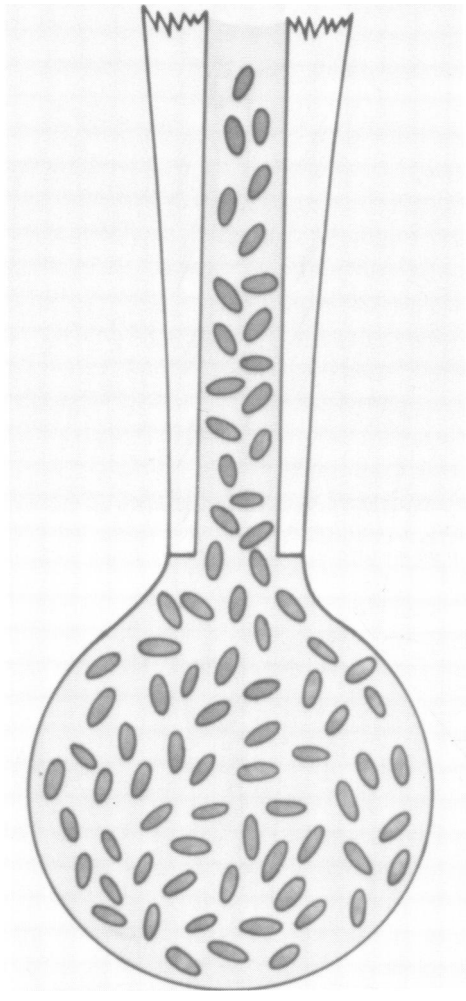

FIG. 1. *Diagram of the formation of a pendant droplet. A 0.1-ml disposable glass pipette marked in 0.01-ml graduations with 0.01 ml of urine diluted 1/100 as a pendant fluid mass containing uniformly dispersed bacteria is shown.*

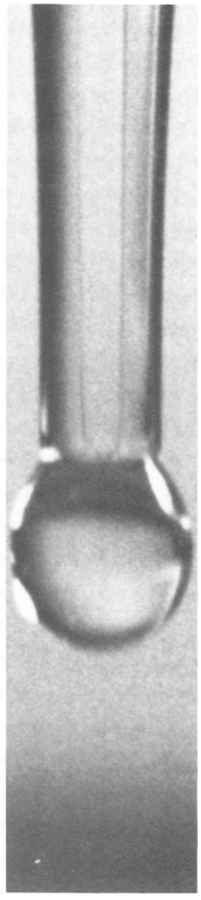

FIG. 2. *Photograph of pendant droplet of diluted urine containing 0.01 ml of fluid.*

Occasionally, large mucoid colonies will merge in a semiconfluent mass, but if the colony count is not excessive ($>10^6$) and distinct arcs of colony periphery are discernible, they are counted (Fig. 10).

Comparison between pour and droplet plate counts. Simultaneous quantitative comparisons were performed between a pour plate (1) method and droplet plating at 10^{-5}, 10^{-6}, and 10^{-7} dilutions on 156 bacteriuric urines. Standard pour plates were made into tryptic soy agar from sonically treated specimens, and colony counts were performed on a standard back-lighted colony counter.

Reproducibility. Reproducibility of the method

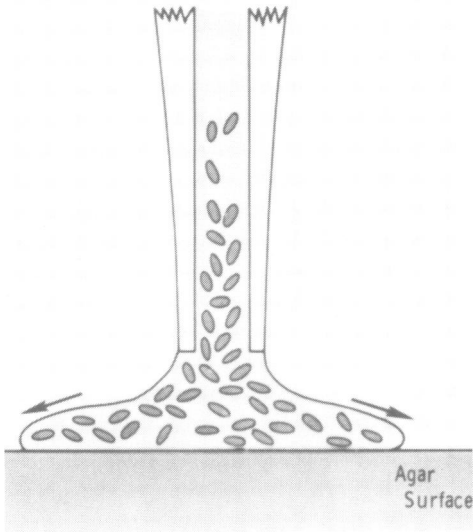

FIG. 3. *Diagram of a droplet touching the surface. Droplet should not be allowed to fall onto agar. May bounce and roll or splatter, causing aerosols.*

was determined by performing 100 sequential plates from the same dilution blank prepared from a bacteriuric urine. The same urine quantitation was performed 50 times using a freshly made dilution blank each time.

Reproducibility between spots on a single plate was expressed as a variability index, determined as the difference between high and low counts on the plate divided by the mean of the four replicates. Perfect reproducibility would equal zero.

RESULTS

In no instances did populations from sonically treated broth culture tubes decline during the 15-min exposure period. Numbers of *S. faecalis*, *Pseudomonas*, and *S. aureus* increased slightly over base line numbers, suggesting breakup of clumps.

Comparison between pour plates and droplet plates resulted in no growth by either method six times, and 54 did not permit direct comparison, either by one or the other being uncountable or by virtue of an extreme count ratio, exceeding either 10× or 0.1×. Mean droplet plate count was compared with mean pour plate count on 96 specimens. A scatter diagram of ratios is shown in Fig. 11. A value above the line (1.0) favors the droplet plating method, and one below favors the pour plate count. Mean droplet plate count was found to exceed mean pour plate count by 1.87×. This would indicate that the droplet plating quantitative method results in formation of approximately 50% more colonies than the standard pour plating technique.

Variability indices between spots on plates are represented in Fig. 12. The range of values for 121 samples was 0.049 to 2.0, with a mean of 0.447. A larger number means a greater variation between numbers of colonies per plate spot.

Mean counts determined by droplet plating

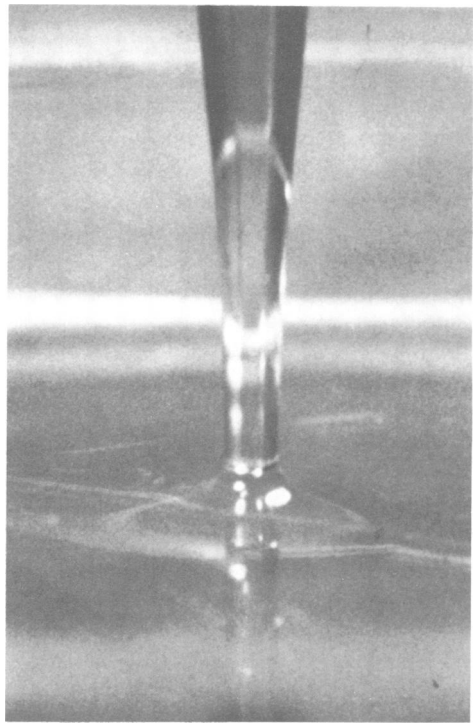

FIG. 4. *Diluted urine droplet being touched off onto agar surface. Pipette does not touch surface.*

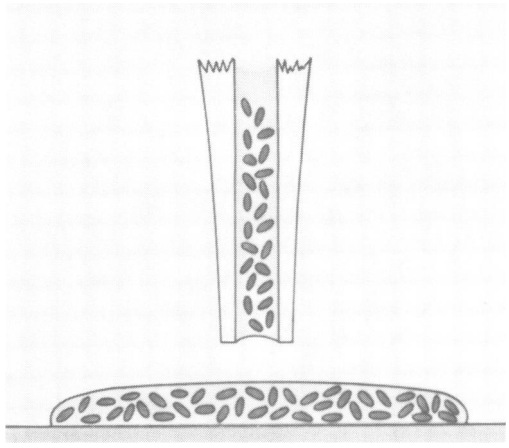

FIG. 5. *Diagram of droplet that is allowed to spread freely on agar and dry 30 min. It is not spread mechanically.*

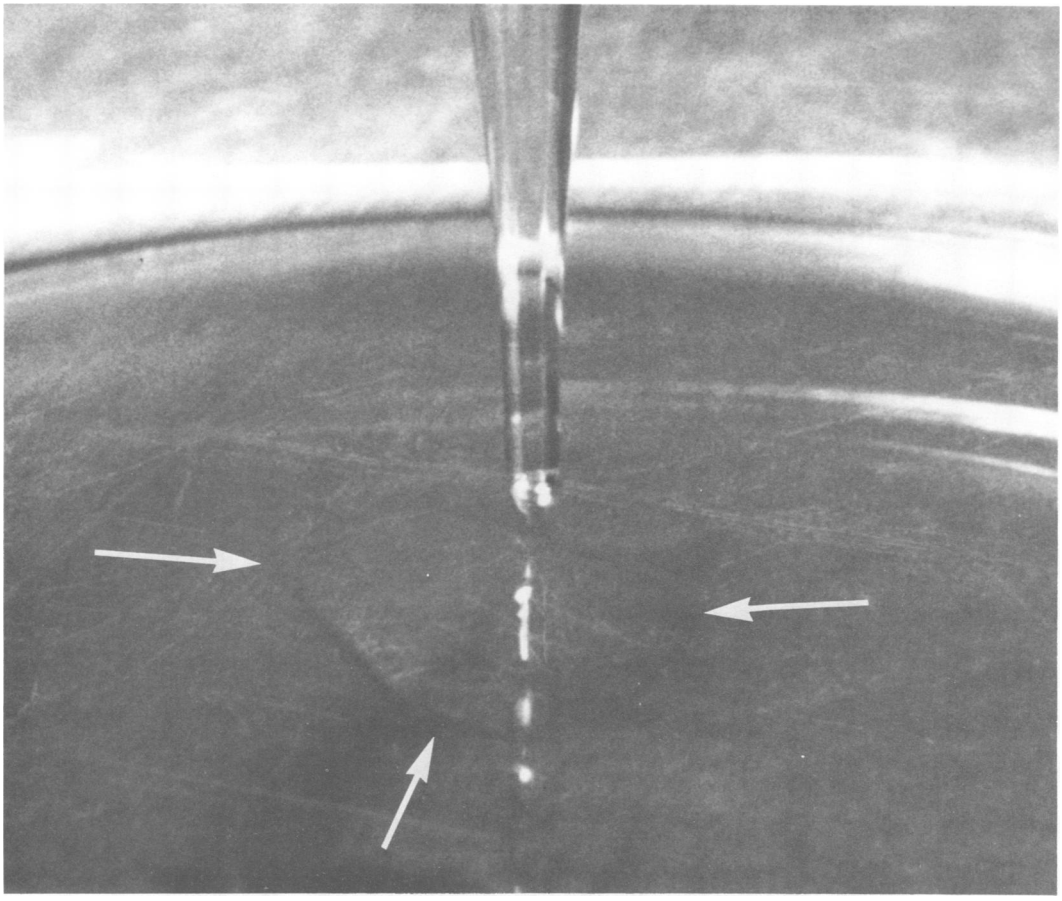

FIG. 6. *Touched-off droplet spreading freely on agar surface.*

onto 100 sequential plates from the same diluted bacteriuric urine on 2 successive days resulted in mean counts remaining fairly constant until replicate 45 (day 1) (Fig. 13) and replicate 75 (day 2) (Fig. 14), respectively. For replicates 1 to 40 on day 1 the average count was 305,500, with a standard deviation of 30,328 and a regression slope of −0.087, and for replicates 1 to 75 on day 2 the average count was 341,400, with a standard deviation of 31,556 and a regression slope of −0.063. Decline in counts attributed to death of cells suspended in the dilution blank indicated that the amount of time the population may be expected to remain constant within a diluted urine is limited. The slightly higher values obtained from the same urine on a second day are attributable to a small psychrophilic population increase during refrigeration.

Quantitation of urine colony counts using 50 sequential different dilution blanks from the same specimen (Fig. 15) resulted in an average

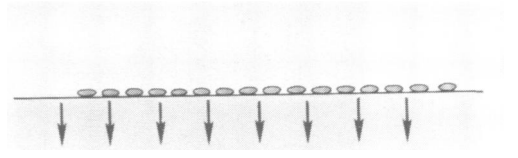

FIG. 7. *Fluid passes agar by imbibition, and separated cells are deposited on the surface as colony-forming units. Average number of colony-forming units multiplied by a factor of 10^4 gives the count.*

count of 283,500, with a standard deviation of 45,300 and a regression slope of + 0.162. The colony count was not found to decline as from the single blank experiment, which indicated the time that organisms were suspended in dilution to be an important factor. These data suggest that the method yields consistent and reliable counts, and that if a urine culture is to be repeated it should be performed on a fresh dilution made from the original specimen. Dilution blanks should not be saved.

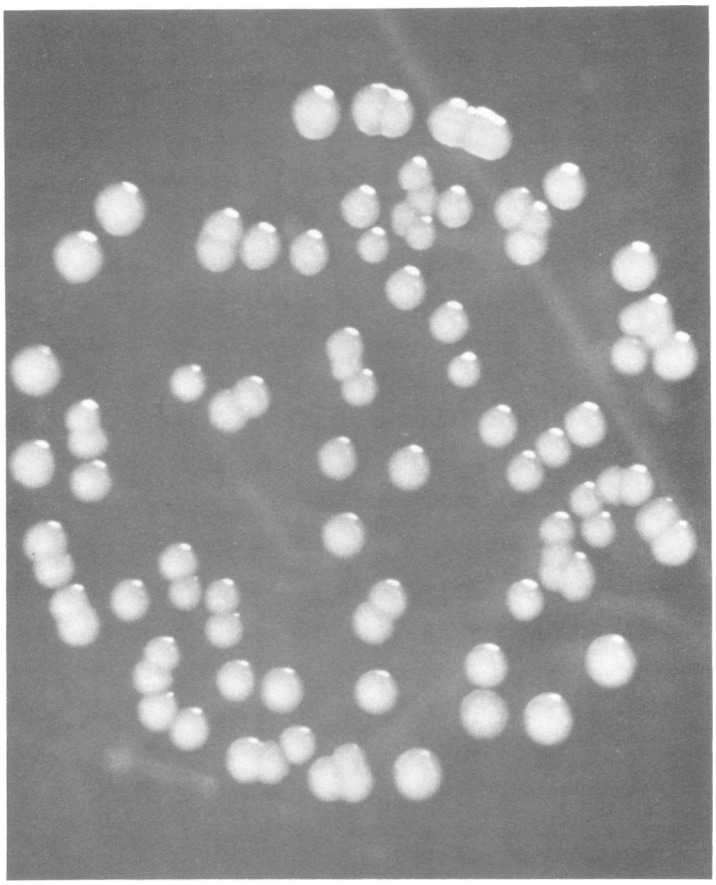

FIG. 8. *Colonies formed within droplet-inoculated area. Diameter of spot is approximately 7 to 9 mm.*

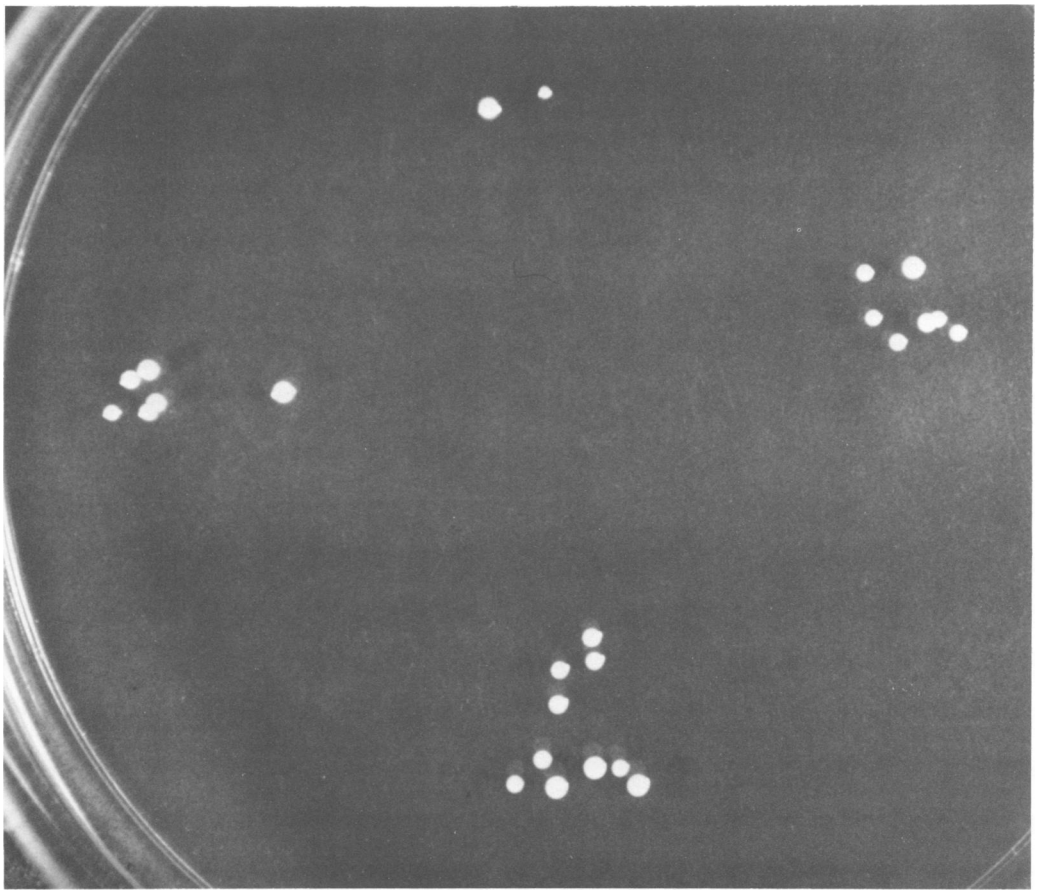

FIG. 9. *Droplet-inoculated CLED plate after 18 h, showing four discrete colony clusters.*

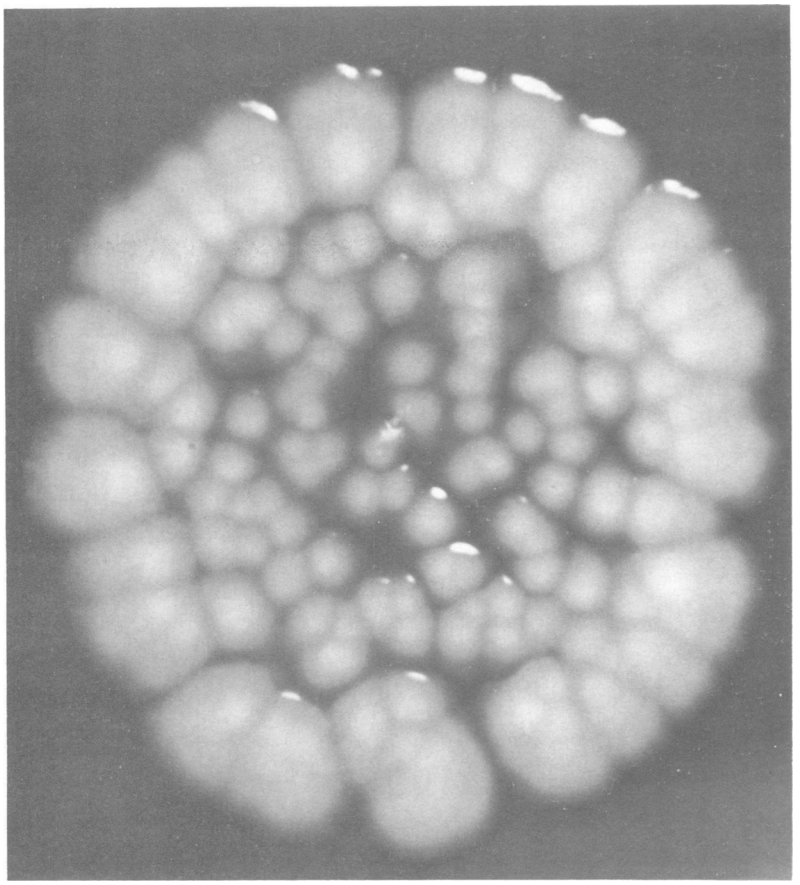

FIG. 10. *Crowded, yet discernible, mucoid colonies.*

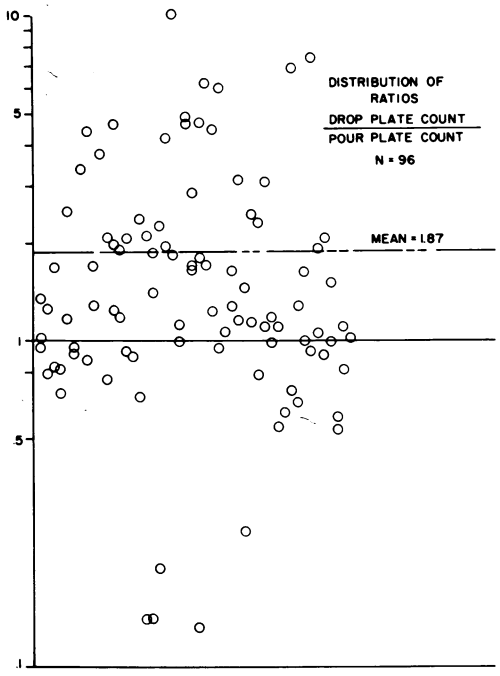

FIG. 11. *Scatter diagram of ratios of mean droplet plate count and mean pour plate count of 96 specimens.*

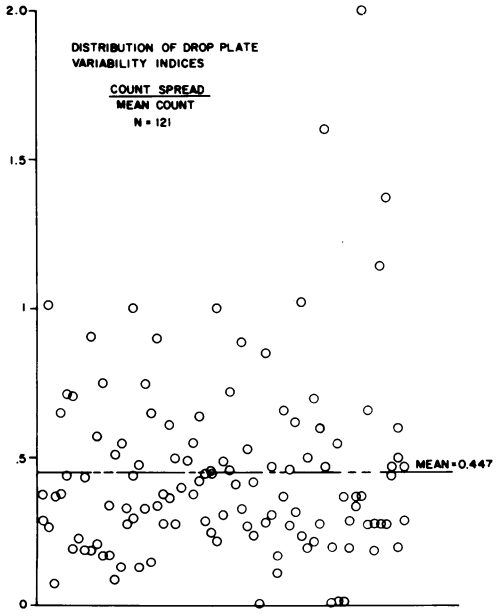

FIG. 12. *Variability indices between spots on plates.*

DISCUSSION

The droplet inoculation method permits the formation of discrete, well-distributed colonies within the inoculated area. If dispersal of cells within the urine is complete and its dilution is properly performed, resulting colonies may be considered as arising from single viable cells. Failure to achieve complete dispersal will result in a reduced number of colony-forming units per spot, and a falsely low count may result (Fig. 16). The findings from this investigation that droplet-plated urines produce consistent counts approximately double those obtained by pour plating seems to cast reasonable question upon the viewpoint of Bailey and Scott (1) that the pour plate technique "remains the most accurate procedure yet devised for measuring the degree of bacteriuria." The comparison indicates that organisms detectable by the droplet plate method are not revealed by the pour plate technique. This may have far-reaching significance, since many of the rapid

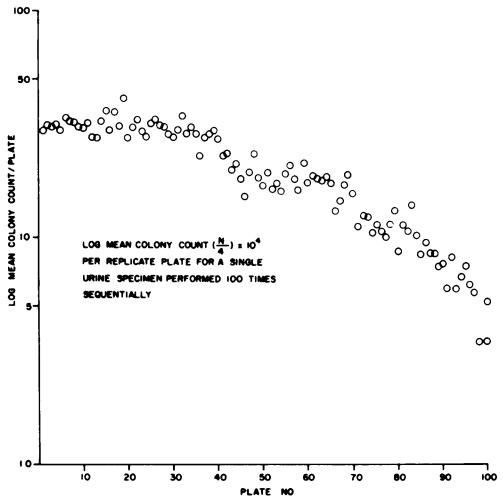

FIG. 13. *Determination of mean counts by droplet plating onto 100 sequential plates from the same diluted bacteriuric urine on 2 successive days, resulting in fairly constant mean counts until replicate 45 (day 1).*

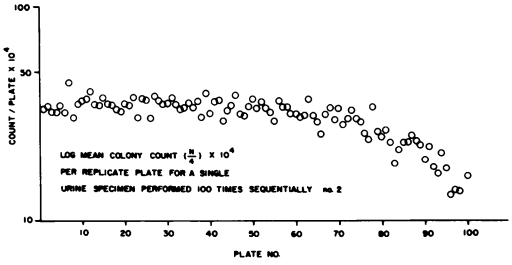

FIG. 14. *Determination of mean counts by droplet plating onto 100 sequential plates from the same diluted bacteriuric urine on 2 successive days, resulting in fairly constant mean counts until replicate 75 (day 2).*

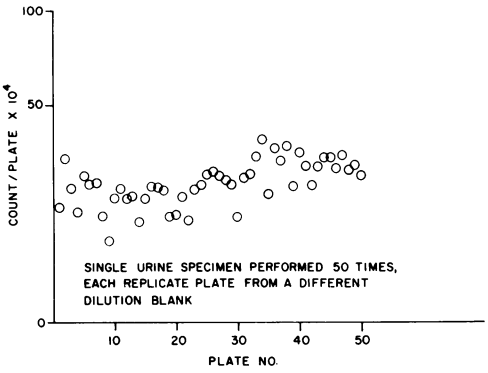

FIG. 15. *Quantitation of urine colony counts using 50 sequential different dilution blanks from the same specimen.*

screening methods, such as dip-slides, etc., are calibrated to the pour plate count as a standard. However, the difference between attainable counts by the two methods may not assume practical significance until the variation exceeds one order of magnitude.

The 10^{-4} dilution permits formation of countable colonies from urines having populations between 10^{-4} and 10^{-6} organisms/ml. Only one dilution is needed to cover the range extending one order of magnitude on either side of numerically significant bacteriuria. In routine practice no more than 50 colonies would be counted from a single spot. Such urines would be reported as "colony count exceeds 500,000 colony-forming units/ml."

The method has distinct advantage when coupled with use of CLED medium. However, minor disadvantages are seen. Certain streptococci and lactobacilli appear at 18 h as tiny transparent colonies. These enlarge upon prolonged incubation but may be missed without low-power stereomicroscopy to read plates initially.

The urine culture procedure in our laboratory also includes streaking 0.1 ml of undiluted urine on MacConkey agar, blood, and colistin-nalidixic acid blood agar plates. These latter plates are not inoculated by the droplet method because in heavily populated urines isolated colonies may not result. Colistin-nalidixic acid inhibits gram-negative organisms and allows gram positives to grow. However, certain strains of *S. aureus* produce stunted colonies and sometimes occur in reduced numbers on colistin-nalidixic acid. *Pseudomonas* strains will often grow on colistin-nalidixic acid.

In conclusion, the droplet plate method to estimate bacteriuria is reliable, reproducible, and able to detect microbial populations between 10^4 and 10^6 organisms with a single dilution step. Precision of the method is dependent upon thorough dispersal of cells within the dilution fluid and a careful pipetting technique. Its successful application to urine culturing is enhanced by the use of CLED agar that inhibits swarming of *Proteus* without toxicity or detriment to other species. The method yields counts greater than are attainable by pour plating and has the additional advantage that an average of four counts per specimen is possible instead of a single one. Numbers obtained represent viable colony-forming units. Equipment and materials to perform the technique are readily available and inexpensive.

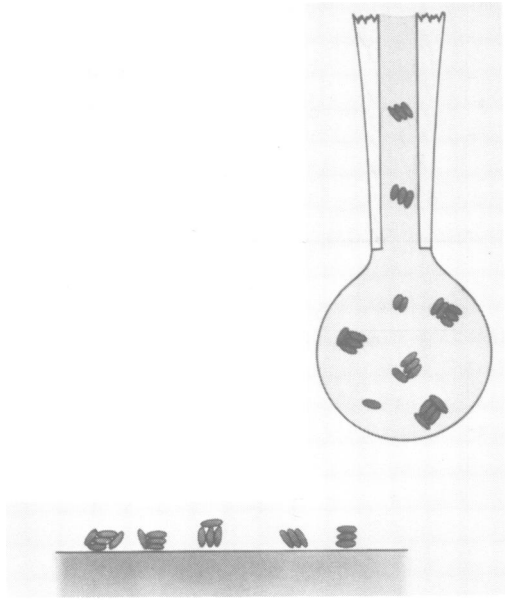

FIG. 16. *Uneven dispersion of cells by failure to break up clumps can result in falsely low numbers of colony-forming units, hence, low apparent count.*

ACKNOWLEDGMENTS

I am indebted to Barbara Ross (retired) and Jonathon Geiger (Harper Hospital, Detroit) for technological efforts during standardization and evaluation of the method.

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