Automated Count and Size Evaluation of Colonies of Bacteria Grown in a Zonal Concentration Gradient of Antimicrobial Agent

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The quantitative study (counting and size and surface evaluation of bacterial colonies) of the activity of an antimicrobial agent against a microbial population growing in solid medium can be performed by an electronic image analyzer. The Zeiss Micro-Videomat allowed the detection of even slight antimicrobial effects, which would be difficult to detect by colony counting alone and would escape the manual procedures of observation. The potential of the new method of investigation was illustrated by the examination of *Staphylococcus aureus* inhibition zones produced by disks of penicillin G and sulfadiazine.

Advances in opto-electronic image analyzers have opened up new horizons in the quantitative study of bacterial multiplication. In preliminary studies (E. Yourassowsky and E. Schoutens, Symposium on Rapid Methods and Automation in Microbiology, Stockholm, 1973, Abstr. B73, p. 49), it was shown that the Zeiss Micro-Videomat was capable of counting colonies of bacteria grown in agar with great accuracy and precision when cultures were developed in triple layers.

In the present work, the image analysis technique was applied to the study of a more complex phenomenon: enumeration and size evaluation of colonies of bacteria grown in a concentration gradient of antimicrobial agents that diffused into the surrounding medium from paper disks applied to the surface of the medium. Two antimicrobials were selected as examples: (i) penicillin G, which generally produces zones with clearly defined margins, and (ii) sulfadiazine, which produces zones having diffuse edges and containing some partially resistant cells.

**MATERIALS AND METHODS**

**Equipment.** The equipment used during this study is shown in Fig. 1. A Tessovar Zeiss Zoom System lens (A) allows a continuous magnification from 0.4 to 12.8×. (Further electronic amplification determines a final magnification of 8 to 256× on the television screen). Its depth of field (5.2 mm maximum) varies as a function of magnification. The plates are illuminated by a diffuse light source (B) to ensure homogeneous light distribution over the observation field. A motorized stage (C), movable along X- and Y-axes (speed adjustable) allows observation of Petri dishes in sizes up to 150 mm. With this accessory, plates can be examined at an optimal magnification chosen independently of the size of the zones to be studied. A Siemens Plumbicon scanning head (D) detects and records differences in optical density between a bacterial colony and the agar background. The Zeiss Micro-Videomat image analyzer (E) applies linear analysis to examine and describe the microscopic images. A simple maneuver allows one to pass simultaneously from the normal image of a particle on the video to its automatic enumeration or to its surface evaluation (2). Analogue output of results (F) is possible; the actual equipment is coupled to a galvanometer recorder (G).

**Preparation of samples.** To give the colonies a homogeneous character and to ensure their even distribution into the medium over the whole surface area of the Petri dish, the cultures were developed in triple layers (bottom layer, inoculated layer, and top layer). The total thickness of the three layers did not exceed 6 mm. All tests were performed in quintuplicate. Trypticase soy agar (BBL) inoculated with *Staphylococcus aureus* (ATCC 9801) was poured into Petri dishes 150 mm in diameter. Inoculum consisted of ± 10⁴ microorganisms per plate. A paper disk containing either 1 µ of penicillin G or 1,000 µg of sulfadiazine was placed on the surface of the top layer. Inhibition zones were studied after overnight incubation at 37°C.

**Size evaluation.** Size evaluation of colonies is based on the principle illustrated in Fig. 2. All particles present in the observation field are counted globally, as indicated by the illuminated dot on the video superimposed over each colony which has been counted (Fig. 2A). The image of each colony on the monitor can be electronically duplicated, and one of them can be laterally displaced over a variable length. As long as one of the two images of the same particle...
partially covers the other (Fig. 2B), the enumeration does not vary. If the distance between the two images of the same colony extends to a point where they no longer touch, the particle is no longer counted (Fig. 2C). The true length of displacement can be determined by scanning a micrometer object. A distribution curve of colonies in function of their size, based on the successive elimination of elements ranging from the smallest to the largest, can be easily established with the equipment described above.

**Enumeration of colonies.** The enumeration of colonies of bacteria grown in a concentration gradient of antimicrobial agent is done according to the technique illustrated in Fig. 3. At zero time, the measuring field is tangent to the paper disk. By means of the motorized stage, the inhibition zone will regularly move through the measuring field. The resulting curve, obtained on the galvanometer recorder, corresponds to the formula:

\[ N(L) = L^{L+\Delta} C(X) dX \]

where \( N \) is the number of colonies, \( L \) is the distance from the disk on \( X \) axis, \( \Delta \) is the width of the measuring field, and \( C \) is the density of colonies.

When the inhibition zone is clearly defined, the curve is linear. Its slope depends on the width of the measuring field and on the maximal number of colonies enumerated; its intersection with the \( X \)-axis corresponds with great accuracy to the edge of the zone.

If the colony-size discrimination procedure is used, this method allows either the elimination or the study of the phenomenon of “fringe,” observed from time to time in an inhibition zone, resulting from a concentration gradient of antimicrobial drug. Surface evaluation or planimetry may be read immediately in analogue values. It is a relative measurement indicating, in parts per thousand of the total measuring area, the surface of the colonies in the observation field. If necessary, these relative measurements can be directly transformed (after the instrument has been properly calibrated) into absolute measurement and thus results can be shown in square millimeter or square micrometer units.

**RESULTS**

Numerous small colonies were observed within the inhibition zone of sulfadiazine. A distribution curve of these colonies in function of their size was established in an area tangent to the disk and compared to that obtained in a similar control area situated far from the inhibition zone (Fig. 4). Nearly all colonies grown in the zone were small in size as only 7% of them, contrasting with 90% in the control zone (outside of the apparent zone of inhibition), had a diameter larger than 0.1 mm.

Nevertheless, the demonstration of an inhibition zone, based on colony counting alone, could only be done if enumeration was performed after elimination of all colonies less than 0.1 mm (most discriminating value between inhibi-
No colony was observed within the inhibition zone of penicillin G, which was clearly defined. The distance between the disk and outer edge of the zone was 5.7 mm, as indicated by extrapolation of the linear part of the enumeration curve to the X-axis. The upper portions of the curves (Fig. 6) were slightly concave, probably because of an inner fringe of growth stimulation by increases in nutrient availability (5).

No antimicrobial effect could be detected beyond the zone: enumeration and planimetric curves were similar and had the same position on the abscissa.

Global surface evaluation of colonies (displayed in parts per thousand of the total measuring area) was also a suitable parameter for studying the effects of the concentration gradient, more especially as it was independent of the overlap phenomenon. Compared with the enumeration curve, the top of the planimetric curve shifted to the right, reflecting an antimicrobial effect of sulfadiazine detectable beyond the apparent zone of inhibition.

Global count: an illuminated dot is superimposed over each colony which has been counted. (B) Images of all colonies are electronically duplicated, and one the duplicates is laterally displaced over a variable length (—). (C) When the two images of the same colony no longer touch, the particle is no longer counted.

Fig. 2. Principle of size evaluation of colonies. (A) Global count: an illuminated dot is superimposed over each colony which has been counted. (B) Images of all colonies are electronically duplicated, and one the duplicates is laterally displaced over a variable length (—). (C) When the two images of the same colony no longer touch, the particle is no longer counted.

Fig. 3. Enumeration of colonies of bacteria grown in a zonal concentration gradient, using the motorized stage. Upper diagram: the measuring field travels from disk (hatched area) to zone of normal growth (dotted area). Lower diagram: curve recorded on the galvanometer. C, number of colonies; Δ, width of measuring field; X, distance from disk.

Fig. 4. Distribution (cumulative form) of colonies grown in two similar observation fields: one tangent to the disk containing sulfadiazine, the other in a zone of normal growth. On the ordinate: cumulative percentage of colonies with diameters equal to or higher than the values plotted on the abscissa.
FIG. 5. Enumeration and surface evaluation of colonies grown in a zonal concentration gradient of sulfadiazine. Ordinate: left scale, number of colonies; right scale, surface evaluation; 0/00 indicates relative measurements in parts per thousand of total measuring area. Abscissa: distance from disk.

DISCUSSION

Benefits provided by automatic bacteria colony counters have been mentioned by diverse authors (3, 4, 6-8). Among the instruments used, the computer-controlled flying-spot scanner described by Glaser (3) seems to be the best performer at the present time, as it is capable of counting, sizing, and identifying large numbers of colonies of bacteria grown on nutrient agar on the basis of their optical characteristics. It is, however, a non-commercialized prototype.

Several colony-counting instruments are commercially available, the majority of which, based on television, apply linear analysis to count particles. The simplest models enumerate the colonies (Aminco Petri-Scan, NBS BioTran, Artek colony counter). In addition to counting, more sophisticated instruments (Imanco Quantimet 720 P, Zeiss Micro-Videomat) provide other parameters (sizing, surface evaluation) which allow more rigorous experimental investigation.

The Zeiss instrument (approximate cost: $28,000), including the Micro-Videomat and the Tessovar lens, has a good performance: counting, area determination, and size evaluation of particles as small as 0.01 mm in diameter can be carried out, and distribution curves of colonies in relation to their size can be easily established. Differences exist between automated and manual counts which are related to touching and overlapping of colonies and directly proportional to the number of colonies per plate: the machine is unable to resolve clusters of colonies into their component numbers. This bias has been accurately defined by Malligo (4), who concluded that it was stable and correctable with a single overall equation. Increased precision for automated counts can be gained by growing cultures in triple layers. This technique reduces formation of clusters of colonies that characterize surface inocula, and subsurface colonies are small (but perfectly visible) with almost identical dimensions, allowing colony densities of $10^4$ to $10^5$ per plate to be accurately counted (E. Yourassowsky and E. Schoutens, Symposium on Rapid methods and Automation in Microbiology, Stockholm, 1973, Abstr. B73, p. 49). Nevertheless, correlation with the Bauer and Kirby procedure, for example, in which the spread surface layer technique is used, needs

FIG. 6. Enumeration and surface evaluation of colonies grown in a zonal concentration gradient of penicillin G. Ordinate: left scale, number of colonies; right scale, surface evaluation; 0/00 indicates relative measurements in parts per thousand of total measuring area. Abscissa: distance from disk.
further investigation.

In the present work, the performance of the Zeiss instrument has been applied to the quantitative study of the activity of a zonal concentration gradient of either penicillin G or sulfadiazine on *S. aureus*. A gradient of penicillin G determines a very clear-cut inhibition zone without any antibacterial effect detected beyond the outer edge of the zone. In contrast, an inhibition zone around a disk containing sulfadiazine can only be demonstrated, by colony counting, if enumeration is done after elimination of all colonies with diameters less than a critical value previously determined. Moreover, as demonstrated by the surface evaluation of colonies grown within and outside the apparent zone of inhibition, slight antibacterial effects are detected beyond the zone, which would escape manual procedures of observation.

The present study is only an example of a new technical approach in microbiological research. If the time spent in preparing and handling the plates is not taken into account, 30 s are required to analyze and record a zonal concentration gradient, the technique being suitable for studying any diffusable substance enhancing or inhibiting the growth of a bacterial population. Analogue output of the results allows their graphic recording. Connection, on-line or off-line, to a computer could yet increase the overall efficiency of the equipment.

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LITERATURE CITED