THE ENUMERATION OF BACTERIA BY THE MICROSCOPIC METHOD

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The method described by Breed (1911) for estimating the number of bacteria in milk has been widely applied, including the counting of bacteria in other fluids. Despite most careful technic there are two important sources of error: losses of bacteria from the slide during staining and faulty selection of areas on the film for microscopic observations. Since "slide loss" is often influenced by fixatives involved in the suspending fluids themselves and by the complexity of the staining process, it must be admitted that absolute values are seldom attainable. However, reliable, reproducible estimates in successive samples of the same material are more readily assured if appropriate amounts of an effective fixative are used and if the method of sampling smears is improved.

The two sampling methods which appear to have been used most widely are: random movements of the objective over the smears or, possibly, orderly movements along parallel lines so that the sampled areas are arranged in "checkerboard" fashion. These methods of sampling are compatible with the concept of films which are square, "flat" and of haphazard numerical density throughout. This view does not consider the possibility that distribution may be influenced consistently by the surface tension of the suspending fluid (convexity of the drop before drying), migration of fluid and bacteria during drying, or the improbability that slides will be dried in a horizontal position.

The data presented deal with: (a) preparation of smears shaped in accordance with the natural distribution of bacteria

in dried films, (b) factors which influence the adhesion of bacteria in films, and (c) the relation between distribution of bacteria and appropriate methods of sampling when different numbers of fields are to be counted.

METHODS

The materials used in the preparation of smears were mixtures of equal parts of (a) clump-free suspensions of acid-fast bacteria or of staphylococci and (b) a milk fixative of the following formula: Difco dehydrated milk 10 per cent, alum 0.5 per cent and formaldehyde 0.4 per cent. The slides were prepared by careful distribution of 0.01 or 0.02 ml. of these mixtures on 2 areas of each slide. Square areas of 4 sq. cm. and circular areas of 20 mm. diameter were outlined with a diamond point pencil; or the areas were delimited by sealing 20 mm. glass coverslips onto regular glass slides with Dupont metacryalate solution no. RK934. This solution worked well for fixing the coverslips, except for the acid-fast staining procedure.

Unless exceptions are noted, the films were dried on a hot glass plate over a boiling waterbath and then heat-fixed. The acid-fast bacilli were stained by applying Ziehl-Neelsen's carbol fuchsin to the slides on the hot plate for three minutes, decolorizing in 3 per cent HCl-alcohol, and counterstaining with methylene blue. The staphylococci were stained for two minutes with methylene blue and the slides were then rinsed in several changes of distilled water in clean beakers.

The square films were sampled by movements along the diagonals of the films, while the circular areas were sampled along two diameters, at right angles. The number of bacteria seen in each field was recorded on charts designed in accordance with the location of each field observed.

EXPERIMENTAL RESULTS

Distribution on square films

The first series consisted of 36 slides, each carrying two 2 x 2 cm. films, a total of 72; 36 of the films were counted by each of two observers, who made their observations by movements along

the diagonals previously described. The results of these counts are shown in figures 1 and 2. These charts suggest that the organisms are so distributed that the numbers of bacteria per field decrease in proportion to the distance from the center. If one uses the distance of a given field from the center as the radius of a circle, he finds (see fig. 1) that every point on the circumference of that circle contains comparable numbers of bacteria. Thus, the summarized counts in the inner circle

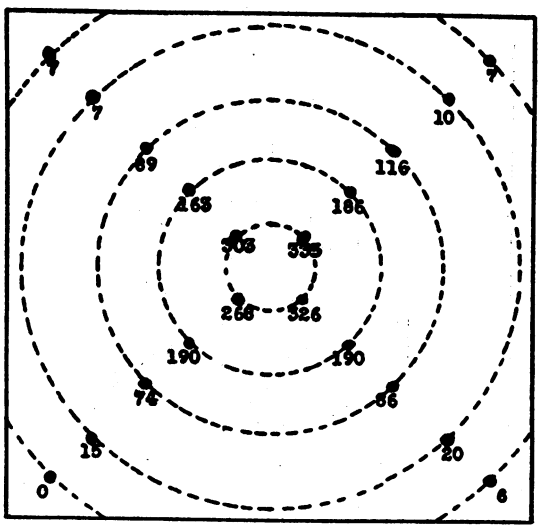

FIG. 1. THE CONCENTRIC DISTRIBUTION OF BACTERIA IN THE SQUARE FILMS COMMONLY EMPLOYED FOR COUNTING BACTERIA BY MICROSCOPIC METHODS

ranged from 268 to 335 bacteria per field, average of 333. In circle 2, the average was 182; in circle 3, average was 91; in circle 4, it was 13. In the outermost circle, whose periphery touched the films only at the corners, the counts averaged five bacteria per field. From these data it is evident that the organisms were arranged in a concentric fashion.

Figure 2 shows a cross section of bacterial distribution in these smears. The values shown were obtained by turning the right-to-left diagonal counterclockwise until it was superimposed on the other diagonal, and by adding the values of the two

diagonals at each distance from the center. These figures illustrate the impropriety of random sampling of square films on which the bacteria tend to be distributed in an orderly, concentric fashion.

Since the natural distribution of the bacteria is concentric and not in accordance with the square shape of the film, one is in a quandary to know whether it is correct to count only 16 fields (omitting the four corner fields) or to obtain an estimate by dividing the total number of bacteria by 20 fields.

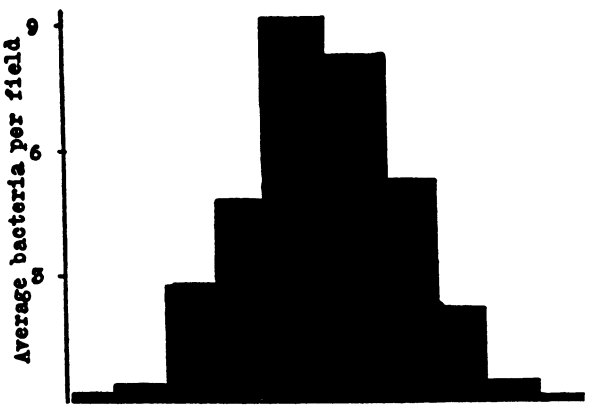

FIG. 2. A CROSS-SECTION OF THE DISTRIBUTION OF BACTERIA IN SQUARE FILMS, OBTAINED BY AVERAGING THE COUNTS ALONG TWO DIAGONALS OF 72 FILMS

Distribution on circular films

To avoid the difficulties encountered on square films, smears were next prepared on round coverslips (22 mm. diameter). The two diameters (vertical and horizontal) were sampled by observations at distances of 1 mm. Since the first of the 22 observations on each diameter was made at the extreme margin of the coverslip, the last observation was located about 1 mm. from the opposite edge. To summarize the figures and overcome this defect in sampling, the vertical diameter was rotated counterclockwise until it coincided with the horizontal. Thus the right hand field (on the edge) of the horizontal diameter was matched with the bottom field of the vertical diameter (1 mm. from the edge). Such counts will be referred to as "pooled diameters."

Figure 3 shows the results of counts in an individual circular film, in order to illustrate the data obtained in each diameter and the results of pooling the two diameters. The dotted lines show the bacterial distribution along single diameters; the solid line depicts the results of pooling. Each case demonstrates the tendency of counts in single diameters to reach a peak near the center of the film. When these two diameters are pooled (solid line), the symmetry of distribution is improved. Furthermore, if the pooled diameters from two films are combined, as in counting for the purpose of enumerating bacteria, the picture of the distribution begins to approach the ideal.

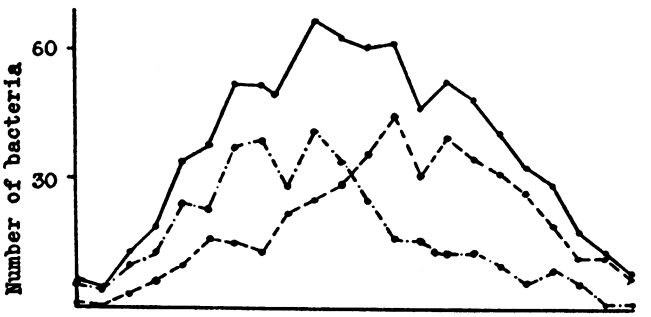

FIG. 3. THE DISTRIBUTION OF BACTERIA IN A CIRCULAR FILM
Showing the counts along the vertical (-·-·-) and the horizontal (- - - -) diameter and the effect of pooling the two diameters.

A true picture of the distribution of bacteria in these round films is illustrated in figure 4, showing the results of summarizing the 64 diameters of 32 films. The values for this figure are based on counts of 1408 microscopic fields.

Modifying factors

Three methods of drying the films were tested. After the suspension was smeared on round coverslips, separate groups of films were allowed to dry in the refrigerator, at room temperature, and on a hot glass plate over a boiling water-bath. Drying in the refrigerator required about 24 hours; at room temperature, some 15 minutes; and on the hot plate two or three minutes. The smears which were dried rapidly (room temperature or on

hot plate) showed comparable and satisfactory microscopic distribution of material and symmetrical distribution of bacteria. However, the films dried in the refrigerator demonstrated gross and unpredictable variations in the distribution of material at different points on the films, due mainly to the slow drying which allowed otherwise insignificant sources of error to magnify their effects disproportionately.

Errors in distribution of material due to such variations from the horizontal as are encountered by films drying under routine

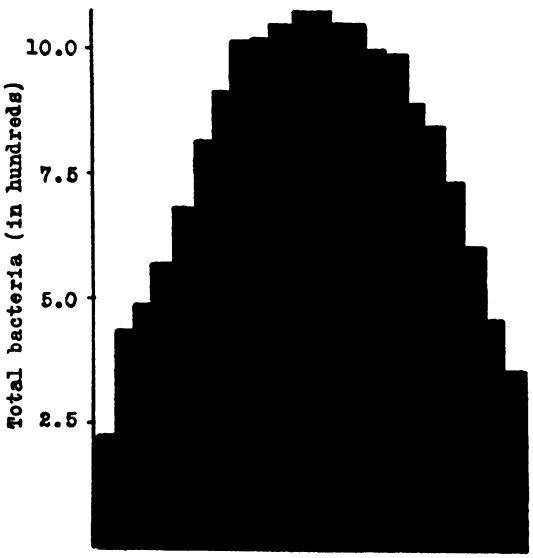

FIG. 4. THE DISTRIBUTION OF BACTERIA IN CIRCULAR FILMS, BASED ON THE TOTAL BACTERIA IN 32 FILMS

laboratory conditions were corrected to a large extent by the counting method used.

The effect of varying the amounts of fixative was investigated. In the presence of constant numbers of bacteria, increasing the amount of fixative to 0.02 ml. lowered the counts, probably because of the density of the films. This effect occurred especially in the presence of small numbers of bacteria. When 0.005 and 0.01 ml. of fixative per film were used, no significant difference was noted. Smaller amounts were not tried.

The number of fields and bacteria which need to be sampled

The number of fields which must be observed depends on two factors: the counting of sufficient bacteria to nullify errors due to small numbers, and the observation of enough fields to sample the distribution of bacteria. A series of 32 staphylococcus films containing differing average numbers of bacteria per field was counted by observing 44 fields in each film. To learn the effect of observing smaller numbers of fields, the results obtainable in 22 fields were compiled by the selection of alternate fields, and in but 12 fields per film by recording the results from every fourth field of the original counts. In both cases the results on each diameter included the first field at the edge of the film. Note that the 12 fields were selected because they permitted sampling completely across each diameter at equidistant points. The average bacterial counts per field were obtained for each sampling method in the usual way. As expected, the counts from the 12-field sampling were more variable than those based on 22 or 44 fields. Curiously, however, they were significantly lower than those obtained in 22 or 44 fields. Inquiry into this abnormal result revealed but one factor which might account for the low estimates in the 12-field samples, this being the relative number of fields observed near the periphery of the films. In the 44-field samples, 4 marginal fields had been counted; in the 22-field, 2 had been included; but in the 12-field samples, two (rather than one) of these marginal fields had been selected. A disproportionate number of fields known to give low counts had been included. This explanation of the low estimates was tested by excluding one field (the first marginal field) on the edge of one diameter. By thus establishing the proper ratio of marginal to total fields, the abnormally low counts were corrected. This showed the importance of maintaining a constant ratio of marginal and total fields, and of examining the effect produced when the sampling method is changed to observe a smaller or larger number of fields than usual.

The 32 films were next classified according to average number of bacteria per film, as determined by the results in the 44 fields. The relative numbers of bacteria which would have been esti-

mated had the results been based on 22 or on 11 of these 44 fields were calculated. Since the results were compiled from identical sites in the films, the influence of variables other than the number of fields was minimized. By regarding the number of bacteria from 44 fields in each slide as 100 per cent, it was possible to determine the percentage by which 22 and 11 field samples failed to agree with these values. Estimates departing more than 10 per cent from the 44-field estimates were regarded as inaccurate and were designated "discrepancies." Among the 10 films giving average counts of 21 to 40 bacteria per field there were no discrepancies when 22 or 11 fields were considered instead of 44. Among 10 slides containing from six to 20 bacteria per field, one discrepancy was encountered by considering only 22 fields and three occurred in the 11-field estimates. One to five bacteria per field produced two discrepancies in the 22-field estimates and four discrepancies when only 11 fields were considered.

From these limited data it is impossible to state the frequency with which 10 per cent errors should be expected in practical work. It must be remembered that these figures were obtained by considering the same 44 fields on each film. They do not provide for differences arising from variations in amount and distribution of material placed on duplicate films. However, the results suggest that certain minimum standards must be met in order to obtain reliable agreement of estimates under ideal conditions. If it is assumed that the counting of bacteria in duplicate films will reduce by one-half the number of fields to be examined in each film, at least 22 fields should be counted in a film containing an average of six to 20 bacteria per field. In the presence of 20 or more bacteria per field, 11 fields per film should be acceptable.

Although it should be possible to devise methods of sampling films by observing fewer than 11 fields, it appears that there are few situations in which this limitation of sampling points would be desirable, because of the excessive numbers of bacteria required per field to produce reliable estimates.

The relation between the average number of bacteria per field and the number of fields to be examined has been discussed by

Wilson (1935) in connection with microscopic counts in milk. Because of the desirability of adequately sampling the distribution of bacteria in films, even to obtain the estimates permitted in grading milk, a reciprocal relation between the bacterial numbers and the fields to be counted (see Wilson) is perhaps inapplicable below a minimal number of fields.

SUMMARY AND CONCLUSIONS

It has been shown that the natural distribution of bacteria in films prepared for microscopic counting is not taken into consideration when square films are used or random observations are made. Preparation of circular films and microscopic sampling along two diameters at right angles takes into account the concentric distribution of bacteria and minimizes the effect of abnormal distribution.

The production of reliable counts necessitated the use of a suitable fixative; the amount required on a given slide area has been mentioned.

An attempt was made to learn the minimal number of fields permissible to sample in order to obtain estimates substantiated by counting a larger number of fields. The relation between the average number of bacteria per field and the number of fields is discussed.

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