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A Method for Counting Acid-fast Bacteria [1]

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A reliable, convenient, and accurate method for counting acid-fast bacteria (AFB) is essential for much work with *Mycobacterium leprae*. The procedure used previously in the laboratory ([2, 4]) had two technical difficulties: (a) In order for the drops to spread satisfactorily the microscope slides had to have a uniform wetability of the correct degree; sometimes this requirement was difficult to satisfy and a satisfactory procedure for one batch of slides was sometimes unsatisfactory for the next batch. (b) There was a tendency for bacteria in suspensions devoid of tissue to concentrate in the periphery of the drop during drying. Consequently a new method was developed, and, after the tests described below, it was substituted for the previous method.

MATERIALS AND METHODS

Microscope slides are prepared with three circles, each about 1 cm. in diameter. The slides are prepared either (a) by sandblasting while the area of the circles is protected by three cylinders (of the correct cross-section) held against the slide by a jig, or (b) by the application of colored rings resistant to acids and organic solvents (the rings have been applied by a decal or through a silk-screen process; the slides are obtainable from Mr. Don Lilly, Georgia Institute of Technology, Atlanta, Georgia, or Bellco Glass, Inc., Vineland, New Jersey).[3] We have used circles from 1.00 to 1.13 cm. in diameter (the latter provides circles 1 $cm.^2$ in area), and these have provided the right amount of space for the fluid additions. The size of the circles needs to be measured, especially in the case of those prepared by sandblasting, and the measurement can be made conveniently to an accuracy of 0.01 cm. with a microscope by use of the vernier scale on the mechanical stage.

Micropipettes. The measurement of the small sample to be applied to the slide has been inconvenient in the past. Some of the more accurate measuring devices are cumbersome and allow time for the settling of tissue particles before the sample can be added. Also they may require rigorous cleansing. A convenient and accurate measurement is now possible with the use of disposable capillary micropipettes (Kensington Scientific Corp., Berkeley, California)[3] with a 10 μl. capacity. First 10 μl. of formol-milk (the various solutions are described below) is applied to a circle, and then 10 μl. of the sample to be counted is added with a separate pipette. The sample is mixed immediately before the 10 μl. is taken up. The micropipette is filled by capillarity (complete filling sometimes requires gentle suction), the outside of the pipette is wiped dry (done conveniently with cleansing tissue in a small beaker), the pipette is checked for fullness, the sample is expelled onto the slide, and the pipette is checked for emptiness. Immediately after application the liquids are mixed and spread evenly over the circle with the tip of a platinum wire, which is flamed between samples. Two slides are prepared for each sample. These operations are carried out on a leveling table, where the slides are allowed to remain until dry.

The *fixation* method is the same as that employed previously ([3]). In brief, it consists of formaldehyde vapors for three min-

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[3]Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U. S. Department of Health, Education, and Welfare.

utes, heat (on the lid of a boiling bath) for two minutes, application of gelatin-phenol (added with a pipette; the slide is then drained momentarily), heat for two minutes, formaldehyde vapors three minutes, and heat for two minutes. For *staining* a few slides at a time are flooded with carbolfuchsin and allowed to stand 20 minutes at room temperature. They are then washed with tap water (in a beaker with applied sample away from the stream of water). They are allowed to stand wet with water until the destaining, which is done one slide at a time by gently streaming 1 per cent HCl in 70 per cent ethanol over the slide until the destaining fluid is colorless. After conclusion of destaining the slide is washed in tap water and allowed to stand wet until the counterstaining, which is done with methylene blue for one minute. The slides are then washed in tap water and allowed to drain dry.

Solutions are prepared as follows: (a) For formol-milk, milk (not homogenized) is centrifuged at 3,000 rpm for 15 minutes and the translucent subnatant cream-free milk layer is drawn off with a Pasteur (capillary) pipette. To 10 ml. of this skim milk 1.5 ml. formalin is added, and the volume made up to 100 ml. with distilled water. Storage is at 4°C. (b) Gelatin-phenol is 0.5 per cent gelatin and 0.5 per cent phenol in distilled water. It is stored at room temperature, and before use complete solution of the gelatin is ensured by inspection, and warming if necessary. (c) Carbolfuchsin is prepared by mixing 180 ml. 5 per cent phenol with 20 ml. of a solution in 95 per cent ethanol of 10 per cent basic fuchsin (Matheson, Coleman, and Bell. Catalog No. B300).[3] (d) The methylene blue stain is prepared by dissolving 0.6 gm. methylene blue (Matheson, Coleman, and Bell, Catalog No. 341)[3] in 60 ml. 95 per cent ethanol and adding 140 ml. distilled water.

Magnification factor. The size of the microscope field must be measured with the microscope, the oculars, and the objective to be used in the counts. With some microscopes the separation of the oculars must be specified. The field is measured with a slide micrometer (e.g., C. Reichert, Catalog No. OBMIP, 2 mm. divided into 200 parts).[3] With these conditions we usually employ a Reichert Zetopan microscope, 12.5x Kompens oculars with 65 mm separation, 100X apochromatic objective; the size of the microscope field is 6.64×10^{-5} cm.^{2} in area.

AFB are counted in fields across the equator of all three circles, either every mm. or every 0.5 mm. The fields are selected by reading the scale of the mechanical stage. Dr. G. R. F. Hilson pointed out that one rotation of the knob controlling lateral movement of this stage corresponded to 5 mm., so that five equally spaced marks on this control could be used for selection of fields, a convenience for those who cannot see the scale of the stage easily. When fields are counted every 0.5 mm., about 20 fields are counted per circle. If desired, fields can also be counted along the vertical diameter.

When 10 μl. undiluted sample (plus the sample of formol-milk) is spread on a circle with diameter-D, and the AFB are counted with the microscope conditions described above, the following formulae are applied:

$$\frac{\text{AFB}}{\text{ml. sample}} = \frac{\text{AFB counted}}{\text{fields counted}} \times \frac{1 \text{ ml.}}{10\ \mu\text{l.}} \times \frac{\pi (D/2)^2 \text{ cm.}^2}{6.64 \times 10^{-5} \text{ cm.}^2} = \frac{\text{AFB counted}}{\text{fields counted}} \times 1.181 \times 10^6 \times D^2.$$

$$\text{When } D = 1.00 \text{ cm.}, \frac{\text{AFB}}{\text{ml.}} = \frac{\text{AFB counted}}{\text{fields counted}} \times 1.181 \times 10^6, \text{ and } \frac{\text{AFB}}{2\text{ ml.}} = \frac{\text{AFB counted}}{\text{fields counted}} \times 2.362 \times 10^6.$$

The microscope conditions should allow clear resolution of the bacillary morphology even at the periphery of the field. Koehler illumination, clean lenses, apochromatic objectives, and compensated oculars are important in achieving sufficient resolution.

Suspensions of *M. leprae* were prepared from infected mouse foot pads or human skin. The method used was the same as that described earlier ([2, 4]) except that the tissue distintegrator was operated with a vibration amplitude of 3 mm. for 1 minute.

RESULTS

Volume delivered by micropipettes. The accuracy of the pipettes is stated to be within 1 per cent. They are calibrated to contain the nominal volume. A series of measurements was carried out in which 10 μl. 0.3 per cent aqueous methylene blue was added (each time with a different pipette) to 5 ml. N/10 NCl. For the delivered volume the methylene blue was placed on a dry glass surface (Petri dish) and the diluent added. For the contained volume the diluent was already in the Petri dish and the methylene blue was washed into it by repeated emptying and filling with the diluent. The concentration of dye was measured with a Beckman DU spectrophotometer (the optical density was about 0.940). The delivered volume was found to average 97.4 per cent (range 96.4-97.8 per cent) of the contained volume. The 2.6 per cent average deficit in delivered volume has been ignored in the calculations.

Counts on erythrocyte suspensions of known concentration. Human erythrocytes were washed in physiologic saline, and exposed overnight to an excess of 10 per cent formalin in BSS (Hanks' balanced salt solution) (the mixture was adjusted to pH 7.00 by pH meter by addition of N/100 NaOH). The cells were then washed in BSS. The resultant suspension was satisfactory in that it was resistant to hemolysis even when diluted in water, and the cells could be readily counted microscopically in unstained, dried smears.

For counting, the fixed erythrocytes were diluted in 1 per cent bovine albumin to a concentration that was suitable for use in a hemocytometer and by the present slide method. Because of rapid settling care had to be taken to stir the suspension immediately before the sample was removed. The concentration of erythrocytes determined by hemocytometer was 2.59×10^7 per ml. (based on counts of 510, 514, 513, 484, 532, and 553 in six separate fillings of the hemocytometer). The concentration determined by the slide method was 2.61×10^7 per ml. (based on counts of 2.67×10^7, 2.55×10^7, 2.68×10^7, and 2.53×10^7 on four separate slides with counts made on fields every 0.5 mm. with an average of 21 cells per field). Thus the count by the slide method agreed very well with the count by hemocytometer.

Repeatability of counts of *M. leprae*. Counts made along the horizontal equator were compared with those made along the vertical equator of a series of 27 slides that had been prepared for routine use in the laboratory. These represented suspensions of 20 mouse foot pad tissues, six of human biopsy tissues, and one nasal washing. They were selected on the basis of high concentrations of AFB, and in all but one the estimated concentrations were greater than 1×10^6 AFB/ml. Fields were counted every 0.5 mm. in both dimensions of all three circles. The ratio of AFB/field in the horizontal counts to that in the vertical counts averaged 1.11, and the median was 1.04; the range was 0.45 to 2.41, with two-thirds of the ratios falling between 0.75 and 1.35. A few large clumps of tissue and bacilli were responsible for all instances where there were large differences between horizontal and vertical counts. Our method of tissue dispersion does not result in complete homogenization.

Comparison of counts obtained by old ([2, 4]) and new method. For a time all bacillary counts in the laboratory were carried out by both methods. For 23 tissue suspensions the ratio of the counts by the new method to that by the old averaged 1.04 and ranged from 0.28 to 1.94. The concentration of AFB/ml. ranged from 5.0×10^6 to 2.0×10^4, as calculated by the new method. The recorded results showed that discrepancies between the results by the two methods were the greatest when a clump of tissue containing many bacilli was encountered by one method only.

Concentration of bacilli in lepromin. Counts by the new method of AFB in several lepromins are given in Table 1. The dispersion of bacilli in these materials was much more satisfactory than in materials prepared for inoculation of mice and the variation in counts between fields was much less. Most of these products had been used extensively in skin tests and their skin reactivities were known to be acceptable. Presumably some were identical with the

TABLE 1. *Concentration of AFB determined by the new method in several lepromins of established potency.*

Preparer, date	AFB/ml.
1. Mabalay, 1959	9.4×10^6
2. Mabalay, 1960	3.1×10^7
3. Mabalay, Sept. 25, 1962	7.6×10^6
4. Mabalay, May 12, 1964	2.5×10^7
5. Wade, 1956	2.5×10^7
6. Wade, 1958	1.2×10^7

([1]). The bacillary concentrations (given in lepromins studied by Lechat and Hanks the lower part of their Table 2) were stated by them to be in the vicinity of 1.8×10^8 bacilli/ml., or approximately ten times what we have found. We have previously counted the AFB in the Mabalay 1960 lepromin ([5]) by our old method, and found a value of 4.2×10^7 AFB/ml., in essential agreement with the present value.

DISCUSSION

The method described here has been in use for two and half years and no technical difficulties have been encountered. Since the pipettes were shown to deliver the stated volume onto a glass surface, and the method shown to give the same concentration of erythrocytes as was given by an independent and accepted method, the possibility of systematic errors appears to be eliminated. Such systematic errors might have arisen from errors in the pipettes, in the size of the circles, in the size of the microscope fields, or in the calculations. The volume delivered by the capillary pipettes is not influenced significantly by surface tension of the liquid to be measured, as it is when wire loops are used. Also errors due to settling of tissue particles can be avoided.

The accuracy of the method is affected by the presence of aggregations of bacilli in pieces of tissue or in globi. If large particles containing bacilli are present they may affect the counts in several ways. If the material is not stirred immediately before filling the pipette, the sample in the pipette may not be representative of the total material. If the sample placed on the slide is not immediately stirred and spread, the larger particles may sediment onto a restricted area of the circle, usually near the center. (It may be shown that such concentrations of material, even if symmetrically placed, act to raise the calculated AFB/ml.) There is also a random sampling error due to clumps, which may be overcome by counting more fields. In the materials used for inoculation of mice, tissue disruption was kept to a minimum in order to prevent damage to bacilli, and large clumps of bacilli were sometimes encountered. In the lepromins, however, tissue and bacillary aggregates were much less common, and there was better agreement between counts for each circle.

SUMMARY

A method for counting acid-fast bacilli is described. Disposable micropipettes are used to deliver 10 μl. of the suspension to be counted onto circular areas marked on microscope slides. Fixation and staining are carried out as previously described.

The accuracy of the micropipettes for delivery was found satisfactory. Counts of suspensions of erythrocytes made by the method described agreed well with those made by hemocytometer. The repeatability of counts of *Mycobacterium leprae* in suspensions prepared in routine practice in the laboratory was affected by the infrequent encounter of tissue clumps containing many bacilli. The ratio between duplicate counts made on the same slide varied from 0.45 to 2.41 with two-thirds of the ratios falling between 0.75 and 1.35.

In lepromins the bacilli are much more evenly suspended. Counts were made on several preparations of known skin-test potency. The counts fell in the range 0.8 to 3.1×10^7 AFB/ml.

The counting method has proved satisfactory in two and a half years of routine laboratory use.

RESUMEN

Se describe un método para contar bacilos ácido-resistentes. Micropipetas descartables se usan para llevar 10 μl. de suspensión con objeto de ser contado en áreas circulares marcadas en placas de microscopio. La fijación y teñido se

llevan a cabo como se describe previamente.

La exactitud de las micropipetas para medir se encontró satisfactoria. El recuento de suspenciones de erythrocitos hecho por el método descrito concuerda bien con aquellos hechos mediante el hemocitómetro. La repetición de recuentos de *Mycobacterium leprae* en suspensiones preparadas en el trabajo regular en el laboratorio se vió afectada por el hecho infrecuente de grupos de tepidos que contenían muchos bacilos. La relación entre recuentos repetidos dos veces en una misma placa variaron de 0.45 a 2.41 con dos tercios de la razón que correspondieron entre 0.75 y 1.35.

Los bacilos están mucho mas homogeneamente distribuídos en las leprominas. Se hicieron recuentos en varias preparaciones de potencia conocida en pruebas en la piel. Los recuentos estuvieron en un rango 0.8 a 3.1 $\times$ 10^7 AFB/ml.

El método de recuento se demostró satisfactorio en dos y medio años de trabajo rutinario de laboratorio.

RÉSUMÉ

On décrit ici une méthode pour compter les bacilles acido-résistants. Des micro-pipettes à jeter ensuite (disposable micropipettes) sont employées pour délivrer 10 μl. de suspension, quantité qui est ensuite dénombrée sur des aires circulaires marquées sur des lames microscopiques. La fixation et la coloration sont pratiquées comme il a été décrit antérieurement.

L'exactitude des micropipettes à délivrer cette quantité a été trouvée satisfaisante. Des numérations d'érythrocytes en suspension faites par la méthode décrite ont été en accord avec celles effectuées au moyen de l'hémocytomètre. La reproduction des valeurs obtenues à la suite de numérations successives de *Mycobacterium leprae* dans des suspensions préparées de manière routinière au laboratoire a été affectée par l'apparition peu fréquente d'amas tissulaires contenant beaucoup de bacilles. Le rapport entre des numérations effectuées deux fois sur la même lame a varié de 0.45 à 2.41; dans les deux tiers des cas, ce rapport se situait entre 0.75 et 1.35.

Dans les lépromines, les bacilles sont suspendus de manière beaucoup plus uniforme. Les numérations ont été pratiquées sur plusieurs préparations dotées d'une activité connue pour l'épreuve cutanée. Les numérations se sont situées dans une gamme allant de 0.8 à 3.1 $\times$ 10^7 AFB/ml.

Cette méthode de numération s'est révélée satisfaisante au cours de deux ans et demi d'utilisation continuelle au laboratorie.

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