INTERNATIONAL JOURNAL OF LEPROSY

Volume 52, Number 1
Printed in the U.S.A.

Enumeration of Purified Suspensions of *Mycobacterium leprae*[1]

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There are two widely used methods which have been described for enumerating *Mycobacterium leprae* bacilli for research or clinical purposes ([4, 10]). The principal difference between these two methods involves the size of the area over which the *M. leprae* are distributed prior to staining and counting. The "pinhead" method of Hanks, *et al.* ([4]) was developed in an attempt to minimize the often large counting errors due to the uneven and variable distribution of *M. leprae* bacilli over the counting areas. When samples are spread over a large area (≥ 1 cm^2) the number of bacilli per field at the center may exceed those near the perimeter by over ten times ([5] and author's unpublished observations); whereas with smaller areas, this variable is reduced. Hanks, *et al.* pointed out that because of this variation circular bacterial films must be small enough to allow entire film diameters (or, more strictly, the film radius) to be sampled and that any subsequent statistical analysis must be based on nonparametric methods, such as rank analysis ([4]).

Recently it has become necessary at the Armauer Hansen Research Institute (AHRI) in Addis Ababa, Ethiopia, to make accurate estimations of the number of bacilli in purified preparations of viable human *M. leprae*, and a technique has been developed which attempts to meet the following criteria: a) accurate measurement of sample size, both in volume and area of distribution; b) optimal and reproducible distribution, fixation, and staining of the bacillary preparation; and c) accurate estimation of bacillary number and appropriate data analysis.

[1] Received for publication on 1 June 1983; accepted for publication in revised form on 25 July 1983.

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MATERIALS AND METHODS

Bacterial suspensions

M. leprae were purified from the tissue biopsies of untreated multibacillary leprosy patients using a modification of the WHO 79 protocol. Briefly, tissue with a high (≥ 4) Bacterial Index (BI) was homogenized in alkaline saline (pH 8.6), washed once, and incubated with 1 N sodium hydroxide at 37°C for 30 min. Following a second homogenization, the tissue was washed in 0.005 M Hepes buffer (pH 7.4), containing 0.1% Tween 80 and 0.001 M magnesium sulfate, and the pellet incubated at 25°C in the presence of 10 units/ml of deoxyribonuclease I (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) for 1 hr. The pellet after centrifugation was mixed with 30% isotonic Percoll (Pharmacia, Uppsala, Sweden) and centrifuged at 30,000 × *g* in a 23° angle rotor head for 30 min at 4°C. The resulting band of bacilli was removed from the Percoll gradient and washed twice in Hepes Tween 80 buffer. Aliquots of the suspension were kept frozen at −70°C (until required). The *M. leprae* prepared in this way contained little tissue contamination (after staining with soluble blue[12]) and no cultivable bacteria or fungi.

Preparation of standard bacterial smears

The bacterial suspension to be counted was diluted in saline containing 10% fetal calf serum (Flow Laboratories, Irvine, U.K.) following dispersion of any bacterial clumps by vigorous shaking with 10% v/v chloroform. The dispersed suspension was transferred to a slide using a commercially available micropipette (Finnpipette, Helsinki, Finland) which was carefully calibrated to deliver the 5 μl volume within 1.0% and with a reproducibility of better than 0.5%. The films were spread over an area whose

diameter was selected to be exactly 25 microscope fields (approximately 4 mm). This selection of the smear diameter was accomplished by using masks cut from a brass rod of the appropriate diameter. The circular areas were then prepared by spraying polytetrafluoroethylene (PTFE) (fissions, Leicestershire, U.K.) over a suitably masked microscope slide. Provided that the microscope slides are degreased and cleaned before coating with PTFE, the 5 μl drop readily spreads out to the hydrophobic PTFE perimeter of the smear field. Any irregularities can be corrected by spreading the drop with the tip of the micropipette.

Fixation techniques

The effectiveness of three different fixation methods was compared. After air drying, the film was treated in one of the following ways: a) heat fixation over a Bunsen flame; b) each film was covered with 20 μl of 10% formalin and air dried; and c) each film was covered with 20 μl of 95% ethanol and air dried.

Staining technique

The suitability of three staining methods was compared: a) standard Ziehl-Neelsen (ZN); b) fixed slides were stained in carbolfuchsin (Neo-fuchsin, Merck, Darmstadt, Germany) with a measured absorption maxima of 544 nm for 7 min with heating (until steam was produced), then rinsed in tap water and decolorized in either 20% sulfuric acid or 1% hydrochloric acid, and counterstained with methylene blue ([6]), and c) the methenamine silver method ([2]). The fixed films were oxidized for 1 hr with 5% chromic acid. Following a brief wash in 1% aqueous sodium bisulfite and rinsing in double-distilled water, the smears were stained in the dark for 60 min at 50°C in a freshly prepared silver nitrate and methenamine mixture (2% v/v methenamine, 0.2% w/v silver nitrate, and 0.1% borax w/v in double-distilled water). The slides were then rinsed three times in double-distilled water and toned with 0.1% gold chloride for 3 min. The unreduced silver was removed by washing in 2% sodium thiosulfate for 2 min, followed by rinsing in distilled water. The smears were counterstained with methylene blue.

Smear assessment

Ten replicate smears from a standard *M. leprae* suspension were made for each of the techniques that were assessed. All slides were read by one observer with a Leitz Orthoplan microscope under ×100 oil immersion objective (NPL) using Köhler illumination.

RESULTS AND DISCUSSION

Preparation of bacterial smears

Several methods are available for the preparation of counting areas, however it is of obvious importance to reduce the field size so that accurate sampling can be performed. The pinhead method of Hanks, *et al.* ([4]) provides an accurate and reproducible solution. However, the technique requires some skill in preparing "perfect" circles and has the disadvantage that with purified *M. leprae* preparations accurate location of the field's edge is often difficult. In addition, and perhaps more importantly, there are relatively large differences in the volume transferred with this technique (mean standard deviation of 7.4% cited by Hanks[3]). For these reasons, it was decided to increase the volume so that commercially available micropipettes with high reproducibility (<0.5%) could be used.

In order to overcome the difficulty of locating the edges of the field, it was decided that the bacterial suspension would be spread over a defined area as in the technique described by Shepard, *et al.* ([11]). However, because of the differences in distribution across a large area and the impracticality of sampling an entire radius, circles with a much smaller area were chosen. The diameter of the sampling circle was chosen so that it was exactly equal to a whole number of high power fields, in this case, 25 (Fig. 1a). The slides were prepared by spraying suitably masked areas with PTFE spray. This technique is rapid and, with care, well-defined sample areas can be produced. In practice the masks can be made either by machining a piece of metal rod to the required diameter or (as in this case) by trial and error with available materials. An alternative is to insert an appropriate size mask into the microscope eyepiece, in which case commercially available 4 mm diameter multiwell test slides (Flow Laboratories) can then be used. (These multiwell test slides

THE TABLE. *The effect of different fixation and staining techniques on bacillary counts of replicate samples.*[a]

Stain	Fixation technique		
	Heat	Alcohol	Formalin
Standard ZN	42.2 ± 6.6	4.8 ± 1.7	4.2 ± 2.0
ZN after periodate	82.3 ± 12.6	9.8 ± 3.5	8.4 ± 2.4
Methenamine silver	88.3 ± 5.7	11.6 ± 4.2	9.8 ± 3.0

[a] Mean of 10 counts (one count = 25 fields as described in the text). Number of bacilli per ml × 10^6 ± standard error calculated using stratified analysis.

cannot, however, be used with Ziehl-Neelsen staining solutions.) Using these methods, the first set of criteria regarding greater accuracy of suspension volume and area of distribution combined with the ability to sample the entire film radius were fulfilled.

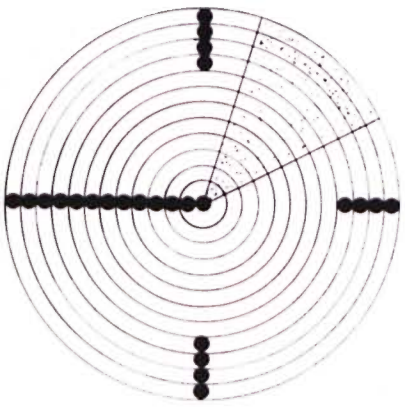
Fixation and staining of slides

The results of using different fixation and staining techniques are shown in The Table. It is apparent that heat fixation provided better retention of *M. leprae* than either formalin or alcohol. In a comparison of formalin fumes and heat fixation, Ridley and Ridley (9) also found that heat fixation gave a considerably higher BI in slit skin smears. More bacilli were visualized with methenamine silver staining and with Ziehl-Neelsen staining after prior oxidation with periodic acid than with the standard Ziehl-Neelsen method. Consistently fewer acid-fast bacilli were seen following decolorization with 20% sulfuric acid than with 1% hydrochloric acid. This difference was less marked in smears which had been oxidized with periodic acid (data not shown). Initial preparations using formal-milk and formalin vapor followed by gelatin phenol (11) were disappointing, since lower numbers of bacilli were often found. Since this method was relatively complex, it was not investigated further.

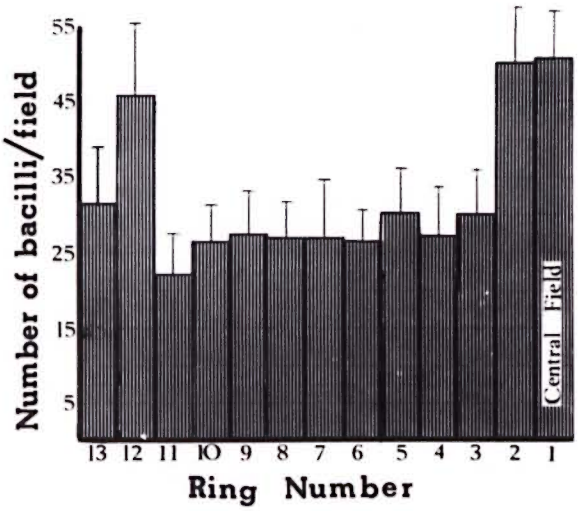
The requirements for fixation and staining of the *M. leprae* smears were that fixation should ensure that a reproducible maximum number of bacilli be retained on the slide. Subsequent staining should visualize a high and consistent number, such that either the entire population or a constant proportion of the bacilli are being sampled in samples from a variety of patients, both treated and untreated. Previously Reich (8) has shown that unlike the situation for Gram-stained organisms where microscopic counts were, as might be expected, consistently higher than estimates for viable organisms, microscopic counts for cultivable mycobacteria stained with Ziehl-Neelsen could be up to 100 times lower than the actual viable counts. In an evaluation of the effect of periodate oxidation, Levy, *et al.* (7) showed that although this treatment resulted in a higher (36%) bacterial count in samples taken from treated patients, it resulted in a lower (16–33%) bacterial count in samples from mouse foot pad cultures. It has also been shown (9) that the number of bacilli seen in smears stained with Ziehl-Neelsen was dependent on the staining time, staining temperature, and the differentiating agent. Taken together, these reports suggest that neither the standard Ziehl-Neelsen staining technique nor prior periodate oxidation results in either the visualization of the whole population or a constant proportion of the bacilli fixed onto the slide. This suggestion is supported by the data presented here because although periodate treatment increased the number of bacilli visualized, still more were seen using the methenamine silver technique.

Acid-fast staining is a cytoplasmic feature, and the cell wall or cell membrane are not stained but probably act as selected barriers to retain the dye in the presence of an acid decolorizer (8). Previous investigations have shown that the acid-fast proportion of a mycobacterial population may vary depending on age, viability, source, etc., and it is therefore unlikely that counting techniques based on this staining procedure will be reliable. The methenamine silver staining technique visualizes more bacteria than the other two methods investigated, and it is likely that a technique which visualizes the most mycobacteria, even when they are partly degraded, would be more appropriate for bacillary enumeration. However, further investigations would be required to ascertain whether a constant proportion of samples taken from different smears are, in fact, being visualized by the methenamine silver technique.

a)



b)



c)

Multiplication Factor	96	88	80	72	64	56	48	40	32	24	16	8	1
Calculated Sampling Rate (%)	21	23	17	9	6	5	5	5	2	3	2	1	1
Actual Sampling Rate (%)	16	16	16	16	4	4	4	4	4	4	4	4	4

FIG. 1. a) Diagrammatic representation of a circular bacterial field showing areas of stratification and location of samples taken. Note that the width of each strata is equal to the diameter of the central circle which, in turn, is the area of the field of view. Inset in the top right hand quadrant shows the approximate distribution of bacilli. b) Bar graph showing distribution of bacilli across the radius of a circular bacterial field. The bars represent the mean values ± 1 S.E. in each strata from ten replicate smears stained with methenamine silver. c) The area of each succeeding ring is defined by $8a(i - 1)$ where i is the ring number and a is the area of the central ring. This formula gives the series of multiplication values shown in the table. The total number of bacilli in each ring is therefore obtained by multiplying the mean number of bacilli per sample by the appropriate factor. The calculated sampling rate was determined from the formula $N_i \cdot S_i$ where S_i is the standard deviation of the sampling units in the ith stratum. The actual sampling rate is that obtained by taking the samples shown in (a). By taking the additional samples suggested in the text the rate becomes: 22; 22; 15; 15; 4; 4; 4; 4; 4; 4; 1; 1; 1.

Distribution of bacilli

Figure 1b shows the distribution of the number of bacilli per field across the radius of the circular fields used in this technique. The distribution is consistently bimodal with approximately twice the number of bacilli in the two central and the two outer rings than in the rest of the area. In addition, it should be noted that these two areas of higher bacterial concentration are relatively well defined with immediately adjacent fields having lower counts, although in individual films clumps may disturb this distribution. In particular, this bimodal distribution is often much more pronounced with smears obtained from low-density suspensions of *M. leprae*. If the *M. leprae* were distributed randomly within the circular fields, the sampling distribution should correspond to a Poisson curve. However, the number of acid-fast bacilli per field did not correspond to Poisson distribution since in all of the 50 fields analyzed large χ^2 values were obtained, all of which exceeded the critical value (at the 5% level).

Statistical consideration

It is apparent that because of the unpredictable but invariably bimodal distribution of bacilli across circular bacterial films, an estimation of bacillary concentration based on a mean value, obtained by aver-

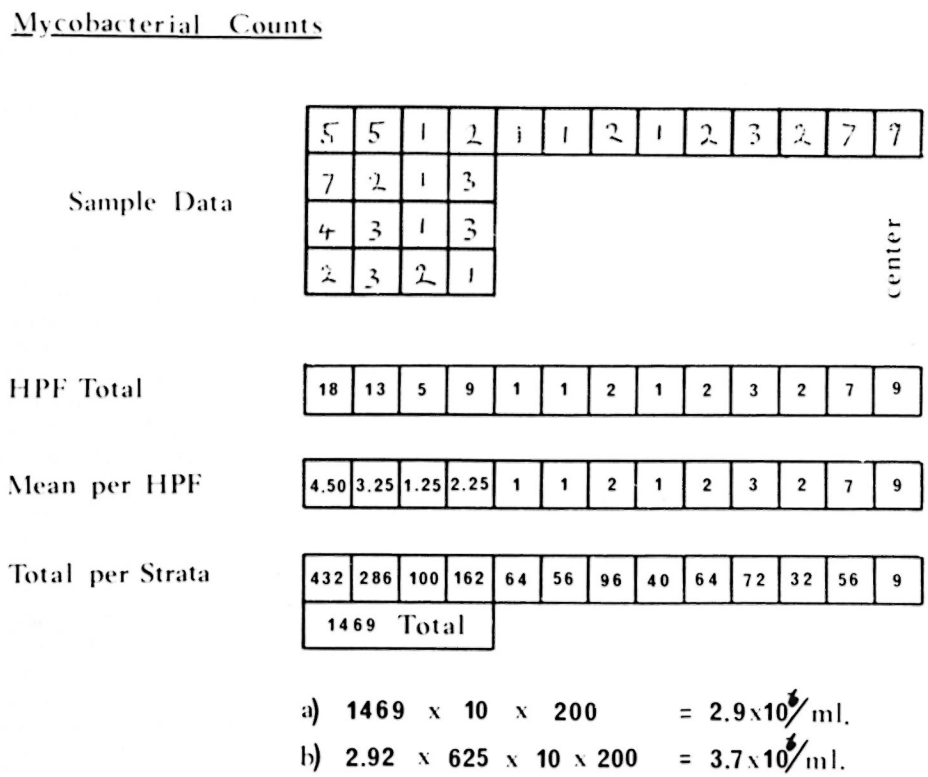


FIG. 2. A set of sample data obtained by the stratified sampling technique. The total number of bacilli per strata are obtained by multiplying the mean number per high power field (HPF) for each strata by the appropriate multiplication factor in Figure 1. The number of bacilli/ml are then calculated by summation of the individual strata followed by multiplication with the dilution factor and the volume factor [line (a)]. Line (b) shows the result obtained using the conventional technique of multiplying the overall average number of bacilli per HPF (73 bacilli in 25 fields or 2.92 bacilli per field) by the area (it can be shown that the area of the circle is 625 times the area of one field since the circle has a diameter of 25 fields), dilution, and volume factors.

aging samples taken from areas differing in bacterial density, leads to a biased estimate. Random sampling is intuitively fair and free from distortion; its weakness, however, is that it does not use any relevant information or judgment that we have about the nature of the population.

One method that accommodates this advantage is stratified sampling techniques ([1]). True random sampling of a bacterial film is, in practice, difficult and time-consuming to achieve. However, stratified sampling techniques are easily applied to the bacterial counts obtained from counting entire radii of circular bacterial films. In addition, the application of a stratified sampling technique allows the optimum determination of the sample sizes in each strata (Fig. 1c). Such analysis clearly shows that the sampling rate should be much higher (>70%) in the first four strata. In practice, therefore, one entire radius is sampled in each film together with three additional samples in each of the outer four rings (a total of 25 fields per film). The three additional samples in each of the four outer rings are most easily taken at the horizontal and vertical axis of the circular fields. Although this results in a slightly lower than optimal sampling rate in the first two rings, it has been chosen because of convenience. A better approximation to the optimum sampling rate could be achieved by taking two or more additional samples in the two outermost rings and only one sample for the three central strata (a total of 27 fields for each smear). The total number of bacilli per film is then calculated using the estimated mean value of one microscope field for each strata. Since the multiplication factors represent the area of each strata in terms of the

microscope field, the number of bacilli per strata can be obtained by multiplying the mean value for each strata with its associated multiplication factor. The total number of bacilli per field can then be obtained by summation of the individual strata totals (Fig. 1c). The accuracy of the estimated bacillary count depends not only on the optimization of the sampling rate within one film but also on the number of replicate fields sampled from each suspension. Theoretically, the larger the number of films examined the more closely one will estimate the true population size. However there are practical limitations of time and visual fatigue, and the increase in accuracy does not increase directly with the number of fields examined. Using the technique described above, the coefficient of variation (cv) between replicate smears was 23.6%; whereas by averaging samples taken from different areas of the smear, the cv was 20.8%. The lower cv obtained by using the traditional method is a reflection of a smaller variance which is often found in biased estimates.

It is clear from the studies reported here and those of others ([4, 5, 10]) that there are a number of difficulties to be overcome in order to obtain an accurate numerical estimation of a noncultivable mycobacterial population. The techniques of fixation, staining, and analysis described in this paper minimize these problems and will yield more accurate and reproducible estimates.

SUMMARY

Previously described methods of counting noncultivable bacteria have a number of drawbacks including unpredictable variation due to differential staining, low reproducibility between replicate sample smears, and inexact estimations of the bacillary population due to the non-normal distribution of bacilli across the counting field. A simple method is described which minimizes the disadvantages of previous methods and allows the application of a stratified sample technique which improves the accuracy of the count by partially compensating for the uneven distribution of bacilli across the counting area. This method of analysis also allows an estimation of the optimum sampling rate in each strata of the counting area and the determination of a sample variance. Different fixation and staining techniques have been compared, and the methenamine silver method which is more likely to visualize either the entire population or a constant proportion of the bacilli is recommended.

RESUMEN

Los métodos para contar bacterias no cultivables que se han usado hasta ahora tienen un número de inconvenientes que incluyen la variación impredecible debida a la tinción diferencial, la baja reproducibilidad entre duplicados y los cálculos inexactos de la población bacilar ocasionados por la distribución irregular de los bacilos en el campo de cuenta. Aquí se describe un método simple que reduce las desventajas de los métodos anteriores y permite la aplicación de una técnica de mues treo estratificado la cual mejora la exactitud de las cuentas al corregir parcialmente la distribución desigual de los bacilos en el área de cuenta. Este método de análisis también permite una estimación de la forma óptima de muestreo en cada estrato del área de conteo y la determinación de una variancia muestral. Se comparan diferentes técnicas de fijación y tinción y se recomienda el método de la metenamina argéntica como el más confiable para visualizar la población bacilar entera o una proporción constante de la misma.

RÉSUMÉ

Les méthodes décrites précédemment pour compter les bactéries non cultivables souffrent d'un certain nombre de défauts, dont une variation imprévisible due à la coloration différentielle, une reproductibilité faible entre les frottis répétés, et des estimations inexactes quant à la population bacillaire, par suite de la distribution des bacilles dans les champs d'observation, qui n'est pas normale. On décrit ici une méthode qui réduit au minimum les avantages des méthodes précédentes, et qui permet l'application d'une technique d'échantillonnage statifié améliorant la précision des énumérations en compensant partiellement pour la distribution non uniforme des bacilles dans les champs d'observation. Cette méthode d'analyse permet également l'estimation de la proportion optimale d'échantillons dans chacune des strates du champs d'énumération, de même que la détermination d'une variance pour l'échantillon. On a comparé des techniques différentes de fixation et de coloration. La méthode par la méthenamine d'argent est recommandée, car elle est celle qui est susceptible de permettre de voir dans son ensemble, soit la population entière de bacilles soit une proportion constante de celle-ci.

Acknowledgments. I would like to thank Banchayehu Guallu for expert technical assistance and Per Olcén and Robin Sayers for useful discussions. I thank Woziro Mulunesh Negash for typing the manuscript.

AHRI and its staff are financed jointly by the Norwegian and Swedish Save the Children Federations.

Patient material for the isolation of *M. leprae* was provided through the kind cooperation of the clinical staff of the All-Africa Leprosy and Rehabilitation Training Centre (ALERT), Addis Ababa, Ethiopia.

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