QUANTITATIVE ASPECTS OF SAMPLING LEPROSY SKIN LESIONS BY THE SCRAPED INCISION METHOD

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The direct simplicity of Wade's scraped incision method (5) provides a convenient way of obtaining *M. leprae* from skin lesions for microscopic and metabolic study. The estimated number of organisms present in a lesion influences important decisions concerning clinical type, progression or regression of the disease, results of therapy, and administrative attitude as regards probable degree of infectiousness. It may be of interest that a simple modification of the habitual method of filming such samples on glass slides can improve the uniformity and sensitivity of the bacteriological examination.

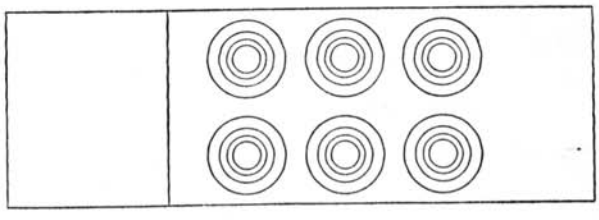
Observations which bear on this point are: the astonishing rate at which small samples are diluted when spread on glass slides; the tendency toward unequal and excessive dilution of samples in present filming methods; the art of making uniform films from samples of known and unknown volume; the natural distribution of numbers of bacilli in films that differ in thickness; and means of attaining maximal sensitivity when this feature is important.

METHODS

The variable film densities produced by present filming methods was analyzed by reviewing 29 routinely stained smears from lepromatous patients. Microscopic study was limited to identification of films in which the background of protein, cells or cell debris was uniformly spread but nowhere sufficiently dense to interfere with visualization of the bacilli. Since the density of the films corresponded with the preference arising from the study of filming methods, these were regarded as of optimal density. After measuring the diameter of each film, the macroscopic optical density of each was graded independently by two observers, who estimated also the area which each sample should have covered in order to bring all to approximately equal density. The averaged results represent the area judged to be optimal.

The art of preparing films of uniform and comparable density irrespective of the volume of the sample was investigated by stirring and conveying replicate volumes of sheep's blood from a siliconed spot plate by means of a "Gem" type (single-edged) razor blade. Spreads were made as circular films of the diameters and areas indicated in Table 1. Templates as shown in Text-fig. 1 served as guides. Direct inspection of the dried spreads revealed methods providing uniformly reproducible films.

In the spot plate used, 0.015, 0.04, 0.1 and 0.2 cc. of citrated blood yielded samples appropriate for areas of 10, 20, 40 and 80 mm^2. Evaporation during the procuring of samples was prevented by covering each spot, as a seal, with an inverted rubber stopper rim-coated with vaseline and containing a small pledget of moistened cotton within a cavity in its lower surface. Since the maintenance of blood in suspension by stirring in circular excursions promotes dehydration at the margins of droplets and



TEXT-FIG. 1. Circular templates, actual size, on which slides were laid while spreading practice samples. Areas of the respective circles: 10, 20, 40, and 80 mm².

rapidly depletes the depth of sample, a stippling motion was employed. Uniform volumes were transferrable by standard bacteriological loops and by square-cornered razor blades, but not by scalpels or instruments with more acute blade angles.

The numerical distribution of bacilli in experimental films of optimal and of maximal density was ascertained as follows: *M. leprae murium* (the smallest of mycobacteria and most difficult to demonstrate) was incorporated in undiluted blood (the densest type of sample). Razor-blade samples transferrable from 0.06 cc. of this suspension were spread on areas of 40, 20 and 10 mm². Staining and differentiation were done as follows:

1. The filmed slide was laid on a glass plate over boiling water for 30 seconds, the carbolfuchsin was applied for 30 seconds, and the smear was rinsed gently in water.
2. Differentiation was done in 4 per cent sulfuric acid (by volume) with 0.2 per cent methylene blue for 2 minutes, followed by gentle rinsing.

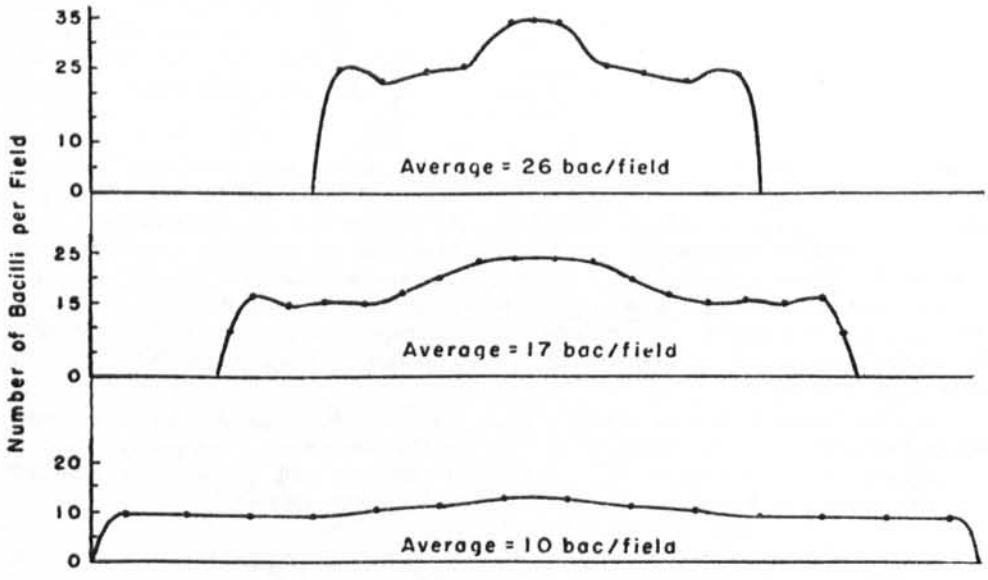
TABLE 1.—*Diameters of circles and areas of films.*

Diameter mm.	Actual area mm²	Value assigned mm²	Ratios
2.5	4.9	5	0.5
3.5	9.7	10	1
4	12.5		
5	19.6	20	2
6	28.3		
7	38.5	40	4
8	50.0		
10	78.2	80	8

Factors which influence the reliability of staining and differentiating mycobacteria have been analyzed in a recent study (3). Our inability to improve on the foregoing method probably is explained by simplification of procedure and by the fixative properties of the two reagents, carbolfuchsin and sulfuric-blue. Films actually are exposed to a fixative, then to water; to a second fixative, and again to water.

For enumerating bacilli each "field" consisted of eight squares of a checkerboard grid (Bausch & Lomb micrometer disc #31-16-15). Counts were recorded for each field while examining a continuous strip across the diameter of each film. The results, presented schematically in Text-fig. 2, represent the average distribution of counts

across two films of 10 mm² (1,820 bacilli), three films of 20 mm² (2,550 bacilli) and four films of 40 mm² (2,780 bacilli).



TEXT-FIG. 2. The numbers and distribution of mycobacteria per field after spreading of standard samples on areas of 10, 20 and 40 mm², respectively, reading from top to bottom. The base of each silhouette represents the diameter of the area covered.

EXPERIMENTAL RESULTS

The use of templates to facilitate the spreading of films on a series of doubled areas provided a startling lesson. An increase in diameter from 2.5 to 3.5 mm. increases the area from 5 to 10 mm². Since the annular space between circles of those sizes would be only 0.5 mm. wide, areas of 5 mm² were not scribed. Text-fig. 1 reveals how slightly one expands the diameter of the 10 mm² area to cover 20 or 40 mm². It is evident that slight increases in the diameter of small films may double the area and the dilution of the sample; also that the spreading of films is a means of diluting small samples at an astonishing rate. It will be recognized that uniform dilutions can be prepared only by filming each sample on an area which is proportional to the volume of the sample.

The variability and over-dilution inherent in present procedures.—In spite of the variable volumes of samples obtainable from skin incisions, present custom seems to attempt the preparation of films on reasonably constant areas. An attempt was made to estimate the extent to which this practice may result in unintentional dilution of samples. If it be assumed that the samples analyzed in Table 2 had been obtained from a restricted area in a single patient, the average concentration of sample, i. e., of bacilli, was 4.4 times less than in the "optimal" films (see methods).

The concentrations in some films were 8, 12 or 16 times more dilute than they should be in a series of samples filmed at comparable density. A further obvious effect of over-dilution is to cause very irregular distributions of samples within thin films.

The preparation of films of uniform density and dilution.—The art of making uniform, properly-diluted films cannot be acquired while con-

TABLE 2.—*Analysis of 29 routine skin scrapes with respect to variations in optical density and dilution.*

Approximate area covered mm^2	Area judged to be optimal mm^2	Number of samples per category (3)	Degree of over-dilution	
			Actual (4)	Accumulative[a]
40	5	2	×8	16
	10	6	4	24
	20	7	2	14
	40	1[b]	0	
	80	1[c]	2	2
60	5	1	×12	12
	10	1	6	6
	20	1	3	3
	40	0		
	60	1[b]		
80	5	1	×16	16
	10	3	8	24
	20	3	4	12
	40	1	2	2
	80	0		
Totals:		29		127/29
Average degree of over-dilution:				4.4×

a Product of column (3) × column (4).
b Two of optimal density; all others were considered more dilute than optimal.
c One film with excessive blood and multiple bedding.

tending with the variable volumes and turbidities of samples from scraped incisions. It may be acquired, however, by brief practice with an instrument which transfers constant volumes of samples from small droplets of blood or of serum blackened with India ink. As each film dries, the imperfections caused by improper methods are apparent to the eye.

This convenient method is justified by the fact that in dried films the numerical distribution of bacilli tends to coincide with that of filming

proteins and cells or pigments.[1] Distribution of these components depends largely upon the flattened form of liquid films or hemispherical form of droplets during the early stages of evaporation. The central peaking of bacterial distribution which results from spreading small amounts of liquid over broad, predetermined areas has already been illustrated (2). When smaller samples—e. g., 0.00022 cc. from a 0.7 mm. standard platinum loop—are allowed to dry while spreading to the natural limits of the droplets, the bacilli are distributed in uniform numbers in a central plain surrounded by a rim in which they are several times more numerous. These distributions may be designated as Type A and Type B, respectively.

The more conveniently prepared Type B films cannot be used for samples from scraped incisions. The bedding of tissue components in multiple layers gives rise to two dilemmas. During decolorization of carbol-fuchsin-stained films with 3 per cent HCl-alcohol, cells and bacilli are lost. Differentiation with dilute sulfuric acid containing methylene blue preserves and retains cells and bacilli more successfully (3), but excessive numbers of cells interfere with accurate examination. Flatter distributions, intermediate between Types A and B (i. e., between flattened and hemispherical), can be achieved conveniently. They appear ideal for general purposes and for dealing with the present problem.

By transferring aliquots of blood from 0.04 cc. in the spot plate, the following observations became clear:

1. If the razor blade is used to deposit a series of blood droplets on a slide without spreading, constant volumes are transferred and uniform areas are covered. The films, however, exhibit Type B rims and are too dense for microscopic study.

2. If transferred droplets are expanded to 10 mm^2 by simply tracing the perimeter of that area, their surfaces assume the form of a rainbow; the blood dries in a plaque of uniform optical density.

3. With a little practice, uniform spreads may be made on twice this area, or 20 mm^2. This is accomplished by employing a rapid circular motion to expand each droplet, by withdrawing the blade from the center of the area, and by giving the slide a tap or tilt to ensure an even redistribution of the blood cells.

4. If one attempts to spread on 40 mm^2 the supply of blood which accompanies the blade, it is found to be inadequate; no amount of practice will produce uniform films. On intermediate areas, the small droplet of liquid following the blade cannot be redistributed, and it leaves a mound of cells wherever the blade is withdrawn from the slide.

5. If one now repeats the procedure described under #3 above, but employs different volumes of sample, it is observed that the crucial point

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[1] Such methods also have been used to evaluate the merits of protein films which bind mycobacteria to diagnostic slides during Ziehl-Neelsen staining; also to identify the fixative or disruptive effects of reagents on the integrity of cells and protein films (3).

is to cease spreading while the supply of liquid following the blade is just adequate to produce a shallow, rainbow form of liquid film and to permit a uniform redistribution of the cells.

As a practical test of this matter, four different volumes of blood (yielding samples appropriate for 10, 20, 40 and 80 mm^2 areas) were placed in the spot plate, stirred behind a screen, and made accessible to Operator A. Three of the first four spreads were made on the proper areas; thereafter deception was impossible. Operator B had the same success. Operator C, elected because of having had no part in the exploration of such methods, and coached only by the foregoing text, succeeded with two of the first four samples, and uniformly thereafter. Subsequent discussion revealed that the spreading of unknown samples on appropriate areas was facilitated by: (*a*) recognizing the size of droplets formed on the slide before spreading, and (*b*) not attempting to spread to a doubled area if in doubt concerning the amount of liquid.

In dealing with the continuous variation in volumes of material obtainable from skin incisions, the size of the droplet prior to spreading and the termination of spreading before exhausting the supply of liquid will be the only clues.

The preparation of dilutions on glass slides is not of interest in the bacteriological examinations of leprosy lesions, and deserves but brief consideration. Spreading of a suitable aliquot on twice a given area represents a 2× dilution of the sample. A proper method of preparing such dilutions presents no difficulties. The transfer instrument is used to bring in one transfer of diluent and then the sample. There is now sufficient liquid to complete the spreading on twice the original area and to make uniform films. Three aliquots of diluent yield smooth films on four times the given area. The filming of enlarged areas with a given sample likewise is feasible. With a transfer volume appropriate for 10 mm^2, smooth films can readily be made with two aliquots on 20 mm^2 and with four on 40 mm^2.

Effect of film thickness on efficiency and sensitivity.—As emphasized by Wade (5) in his first communication concerning material obtained from scraped skin incisions, minimal spreading of the material contributes to sensitivity as regards the finding of bacilli when they are few. This principle is correct, though subject to the law of diminishing returns. In order to ascertain the degree to which multiple bedding of cell and tissue components may obscure bacilli or increase losses from the surface of films, comparisions were made of the numbers of bacilli demonstrable in films which were two times and four times as thick as the baseline provided by the thinnest uniform spreads. While theoretical ratios between the numbers of bacilli observable in films spread on 40, 20 and 10 mm^2 should be 10: 20: 40, the actual average counts of bacilli per field proved to be 10: 17: 26 (Text-fig. 2). Although the films of increased thickness were less efficient (revealing, respectively, 85% and 65% of the expected

numbers of bacilli), those made four times the baseline density had increased the possibility of finding bacilli by a factor of 2.6×.

The relative concentrations of mycobacteria to be expected in different portions of uniform films also are illustrated in Text-fig. 2. It will be noted that the films on 40 mm² afforded an astonishingly even distribution of the bacteria: 13 per field at the center and 9 near the margins. Films on 20 and 10 mm² exhibit increasing accentations of combined Types A and B distributions. The central plateaus in the silhouettes on 20 and 10 mm² are of especial interest when finding bacilli is difficult. These areas revealed, respectively, concentrations of bacilli which were 2.3 and 3.5 times greater than the average numbers in the baseline films.

Errors due to blood in samples.—In order to ascertain if it might be practical to compensate for the variable amounts of blood which dilute the tissue components in material from scraped incisions, blood was diluted in serum to concentrations of 12.5, 25, 50 and 75 per cent. Different volumes of each suspension and of undiluted blood were filmed on appropriate areas: 10, 20, 40 and 80 mm². Comparison of properly spread samples against such standards prior to staining permits recognition of the approximate proportion of blood in that sample.

Corrections for 12.5 and 25 per cent blood would be only 1.1 and 1.3× the numbers of bacilli seen; while corrections for 50 and 75 per cent blood would be 2× and 4×, respectively. Since 80-90 per cent blood cannot be distinguished from undiluted blood, samples which appear to contain more than 75 per cent should be replaced by a re-scrape during the collection of samples.

DISCUSSION

Major sources of error in sampling scraped-skin incisions are known to be: the varying ratios of blood to tissue component, and the impossibility of drawing known or reproducible volumes of the sample. In view of the small multiplication factors required to compensate for modest proportions of blood, and the practicability of replacing bloody samples, corrections for blood seem less important (and less convenient) than the preparation of more uniform films.

As judged by present observations, the usual filming methods cause the samples to be diluted in variable, often extreme, degrees. Because of irregular distribution of components, the concentration of bacilli depends largely on the portion of film examined. An excessive number of fields must be examined to obtain a useful measure of the number of bacilli.

The crux of the problem lies in the fact that the spreading of small samples on glass slides causes dilution at astonishing rates, and that uniform dilution requires spreading on areas which are proportional to volumes. Since volumes cannot be standardized, the simplest expedient is to let the volume of the sample obtained determine the area on which it should be filmed. This can be accomplished, and uniform distributions

produced, if spreading is stopped while there remains sufficient fluid to permit a natural redistribution of the suspended components. In practice this means that the small amount of cellular paste obtainable from slight, inactive, or retrogressive lesions should be pressed directly onto the slide without spreading, or that the tip of the scalpel should simply be "dunked" into a micro-droplet of water; that minimal samples from more succulent lesions should be confined to areas of the order of only 5 mm² (2.5 mm. diameter); that as long as a sample floods the area already covered, it should be expanded as far as may be feasible. The template (Text-fig. 1) serves only for practice or inquiry with reproducible samples. The actual art, as at present, would be practiced free hand.

Sensitivity is not an issue when dealing with active lepromatous lesions. Nevertheless, it is of concern, however, for confirming a difficult diagnosis, for judging the results of therapy, or for making decisions regarding parole or probable infectiousness. Employing the observations available from the clinical samples at hand, Table 3 was assembled in order to derive some concept of the extent to which sensitivity might be increased on special occasions. It will be noted that by confining the

TABLE 3.—*The potential increases of sensitivity if the clinical samples had been spread as films of baseline or diagnostic thickness.*[a]

Thickness of uniform films[a]	Over-dilution of clinical samples	Increased numbers of bacilli which might be demonstrated			
		(f)	Across entire films[b]	(f)	In central plateaus[b]
Baseline	Average, 4×	(1.0)	4.0	(1.3)	5.2
	Extreme, 16×	(1.0)	16.0	(1.3)	20.8
Diagnostic	Average, 4×	(2.6)	10.4	(3.5)	14.0
	Extreme, 16×	(2.6)	41.6	(3.5)	56.0

a Baseline—thinnest films of uniform character (as shown in Text-fig. 2 on 40 mm²). Diagnostic = 4 times thicker (e. g., on 10 mm²).

b (f) = relative numbers of bacilli per field as shown in text and in Text-fig. 2. Results = overdilution × the respective factors.

search for extracellular bacilli to the central plateaus of the densest type of film shown in Text-fig. 2, sensitivity might exceed that in the average clinical films studied by 14×, and that in the thinnest clinical films by 56×. If these data are at all representative of the unnecessary degree to which samples may be diluted by present filming methods, it may be seen

why concentration methods (1, 4) are more sensitive and why recourse to sections and histologic study is so often necessary.

A second means of attaining maximal sensitivity does not depend upon the natural distributions of dispersed cells and bacilli as shown in Text-fig. 2, but upon examining under oil immersion all cell clusters or clumps which are visible under low power. These clusters, wherever situated, are samples of tissue, and they should never be disregarded.

SUMMARY AND CONCLUSIONS

1. The problem of obtaining more useful and convenient estimates of the numbers of *M. leprae* in material from scraped skin incisions has been investigated by demonstrating: (*a*) that the spreading of small samples on glass slides causes very rapid dilution; (*b*) that the areas filmed should be proportional to the volume of samples; (*c*) that variability in present practical results may often be due to attempts to spread the variable samples on standard areas; and (*d*) that this practice results in over-dilution and in irregular distribution of components within the films.

2. A study of the art of preparing uniform films on slide areas which are proportional to unknown volumes of sample leads to the suggestion that each sample be spread to the maximal limit which permits a natural redistribution of the cellular components before drying begins. Enumerations reveal that this modification of habit results in a predictable distribution of bacilli.

3. Increased sensitivity, when desired, may be achieved by increasing the thickness of film to 4 times that recommended for routine purposes. Such films may be on the order of 14 times more sensitive than average films, and 56 times more sensitive than the thinnest clinical films which were available for analysis in this study. A second means of increasing sensitivity is to examine all tissue fragments and cell clusters in each film.

RESUMEN Y CONCLUSIONES

1. Se ha investigado el problema de obtener cálculos más útiles y convenientes de los números de *M. leprae* en el material procedente de incisiones cutáneas raspadas, demostrándose que: (*a*) la dispersión de muestras pequeñas en portaobjetos ocasiona dilución muy rápida; (*b*) la zona abarcada debe ser proporcional al volumen de las muestras: (*c*) la variabilidad de los actuales resultados prácticos puede deberse a menudo a tratarse de esparcir muestras variables en zonas fijas; y (*d*) este sistema da por resultado sobredilución y distribución irregular de los componentes dentro de las laminillas.

2. Un estudio del arte de preparar placas uniformes en zonas de portaobjetos que guarden proporción con los volúmenes incógnitos de la muestra lleva a la indicación de que se esparza cada muestra a su límite máximo, lo cual permite una redistribución natural de los componentes celulares antes de que comience la desecación. Las numeraciones revelan que esta modificación de lo habitual da por resultado una distribución predecible de bacilos.

3. Puede obtenerse mayor sensibilidad, cuando se desea, aumentando el grueso de la película al cuádruple de lo recomendado para fines corrientes. Esas películas

pueden mostrar una sensibilidad de un tenor 14 veces mayor que las películas corrientes y 56 veces mayor que las películas clínicas más delgadas que había disponibles para análisis en este etudio.

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