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Lab dx- microscopy

COMPLEMENTARY DOCUMENT

Comparison of various staining methods for demonstration of tubercle bacilli in sputum by direct microscopy

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INTRODUCTION

Dr Johannes Holm, Executive Director of the International Union against Tuberculosis, inquired whether it would be possible to carry out at the Tuberculosis Department, Statens Seruminstitut, Copenhagen, a comparative assay of different staining methods on smears of sputum.

The background for this inquiry was that microscopy of sputum for tubercle bacilli is the most important bacteriological method in the anti-tuberculosis campaigns run by the IUAT. In contrast to culture, microscopy can be performed on large materials with limited equipment and by personnel with a less comprehensive bacteriological education and training. It is essential for the field work of IUAT that the staining method used should be as simple as possible. We realize that the staining method is only part of the problems involved in the direct microscopy of sputum specimens, and probably not the most important. The collection of the proper material and the selection of the most suitable parts of the sputum for preparation of smears are certainly two essential aspects.

The project was discussed between Dr J. P. Bosviel (IUAT), Dr J. Bennedsen and Dr H. C. Engbaek (Statens Seruminstitut), and also with Dr A. Lind, Chairman of the Committee on Bacteriology and Immunology. It was agreed that this was a suitable project to be taken up by the Committee and that details should be set out in a Working Protocol to be sent to the Chairman. The work should be performed at Statens Seruminstitut by Dr Engbaek, in cooperation with Dr Bennedsen and Mr S. Olesen Larsen (statistician at Statens Seruminstitut).

It was stated at the meetings which methods IUAT would like examined, and Dr Bosviel provided written details concerning the Devulder and Armand methods and the Ziehl-Neelsen method in a French modification. For comparison purposes, the methods used at Statens Seruminstitut, viz. Ziehl-Neelsen and fluorescence, were also to be included.

No review of the literature was made in order to determine whether other methods than those mentioned by IUAT were preferable. Both the Devulder and Armand methods are somewhat more simplified, since decolorization and contrast staining are carried out by means of the same fluid which has alcohol as a component.

Since the methods to be compared are well-established staining

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procedures, great differences between them could hardly be expected. In such a situation, it is particularly important to determine the reproducibility of the technique used for preparing the slides. It was therefore considered essential to examine a large number of slides containing varying numbers of tubercle bacilli.

In order to conclude the experimental work and the statistical analysis of the results before the Committee meeting in September 1968, it was necessary to concentrate on the relatively few methods proposed. Four dilutions of sputum were examined by each of the methods, and ten slides were prepared from each dilution. One hundred sight fields were observed in each slide.

Other aspects, such as the addition of Tween to the staining fluid so as to make heating unnecessary, the significance of alcohol, etc., were not included in this first study, though such problems are undoubtedly of importance for the work of the IUAT. Nor has phase contrast microscopy been employed.

STAINING METHODS

The methods employed were:

1. Tan-Tiam-Hok method modified by Devulder, with heat coloration.
2. Tan-Tiam-Hok method modified by Devulder, with cold coloration.
3. Armand method.
4. Ziehl-Neelsen method (Danish version).
5. Ziehl-Neelsen method (French version).
6. Fluorescence microscopy.

For details of technique, see Appendix I.

SMEARS

Since the examination was to include smears containing a varying number of tubercle bacilli, a dilution series was made from a **strongly positive sputum** diluted by means of **sputum in which no tubercle bacilli could be demonstrated.** The positive sputum was from a new patient not subjected to treatment.

Comminution of sputum: Each of the two sputa was transferred to a round-bottomed flask containing glass beads. An amount of diluted Sauton was added so that, after careful shaking, the positive sputum had a uniform and suitable consistency for making the smears, and contained about 30 to 40 bacteria per sight field (light microscopy, magnification 800 ×). This number of bacteria (called dilution 1 : 1) was considered to be the maximum that could be counted without any particular technical difficulty. The negative sputum was treated in the same way as the positive, and adjusted to the same consistency.

Dilution series: Six $\sqrt{10}$-fold dilutions were produced from the positive sputum (1 : 1) by means of the negative sputum. Each dilution was shaken with glass beads to ensure adequate mixing before the next dilution was made.

The dilutions were 1 : 1, 1 : 3.1623, 1 : 10, 1 : 31.623, 1 : 100, 1 : 316.23, 1 : 1000. The last three dilutions were used only in a special examination with fluorescence microscopy as performed in the routine work of the Tuberculosis Department, Statens Serum-institut (dry objective, magnification 200 ×).

All the dilutions and the negative sputum were placed in the refrigerator overnight.

Preparation of smears: The smears were made on fluoro-slides marked with two circles (diameter 15 mm). Two drops of sputum were applied to the middle of each circle by means of a normal drop counter. A small wooden stick was used to spread the sputum evenly over the whole area. The slides were placed immediately afterwards on a metal rack which is adjustable to ensure that the surface is horizontal. When the smears were absolutely dry they were placed in a special incubator for drying, fixing and killing of the bacteria, according to the usual method employed at Statens Serum-institut, viz. at least 2 hours at 37° and 12 hours at 80°.

Number of slides: Slides were taken at random from each dilution for staining by the different methods. Staining by Methods 1-2-3, 4-5, and 6, was made on three different days. After staining, the slides were randomized within these three groups only, since the slides were clearly distinguishable from each other on account of the colour.

The number of slides (each with two circles) per dilution stage was:

60 for each of the first four dilution stages:	240 slides
10 for each of the three extra dilution stages:	30 ,,
60 for the negative sputum:	60 ,,
Total	330 slides

Registration: The microscopical equipment used was a Zeiss Standard GFL microscope, fitted with planapochromat × 100/nA 1.25, eye-piece 8 × kpl.; for the last three dilutions in Method 6 planapochromat × 25/nA 0.65.

One of the two circles was used for counting the **number of bacteria per sight field.** A total of 100 sight fields per smear were counted and a record made on a special form (see Fig. 1, Forms A and B) together with information concerning evaluation of the colour of the bacteria, the background colour, and the contrast. Examination of the negative slides took about 3—4 minutes and the strongly positive about one hour.

The other circle was used for determining the time of the first positive finding, which information was also recorded on the form.

Examination was always started from the left of the circle at the site of the horizontal diameter and proceeded to the right horizontally. Individual bacteria were counted. Examination of the two smears on the same slide was carried out independently.

The same person performed all the microscopical examinations and an assistant was available for help with the registration.

Microscopy of the control (negative) slides was negative in all cases. Culture of the negative sputum gave no growth. Culture of suitable

dilutions of the positive sputum showed that dilution 1 : 1 contained about 10^6 viable units per millilitre.

RESULTS

Table 1 shows a qualitative evaluation of the bacterial staining and background colour with the six methods. It will be seen that in Methods 1, 2 and 3, where methylene blue was used as contrast stain, there was a significantly larger number of slides with strong background colour than in Methods 4 and 5, and that this strong background colour resulted in poor contrast with the stained bacteria. It is characteristic for Method 6 that a good contrast could be obtained simultaneously with an impression of strong background colour.

TABLE 1
EVALUATION OF CONTRAST BETWEEN BACTERIAL STAINING AND BACKGROUND COLOUR

METHODS 1 - 2 - 3

		Background			
		1	2	3	Total
Contrast	0	0	0	0	0
	1	0	5	14	19
	2	2	90	9	101
	Total	2	95	23	120

METHODS 4 - 5

		Background			
		1	2	3	Total
Contrast	0	0	0	0	0
	1	0	1	2	3
	2	2	71	4	77
	Total	2	72	6	80

METHOD 6

		Background			
		1	2	3	Total
Contrast	0	0	0	0	0
	1	0	0	0	0
	2	0	0	40	40
	Total	0	0	40	40

Code

Background	Contrast
1 = weak	0 = none
2 = moderate	1 = poor
3 = strong	2 = good

As mentioned previously, a record was also made of the time at which the first bacillus was seen. In the majority of cases, bacteria were seen already in the first sight field. There was no difference between the methods in this respect.

Two examples are given in Fig. 1 A and B of the registered number of bacteria per sight field. One of these illustrates the most extreme

EXAMPLES OF REGISTERED NUMBER OF SIGHT FIELDS
FIGURE 1A (see also Form A)

BACTERIA

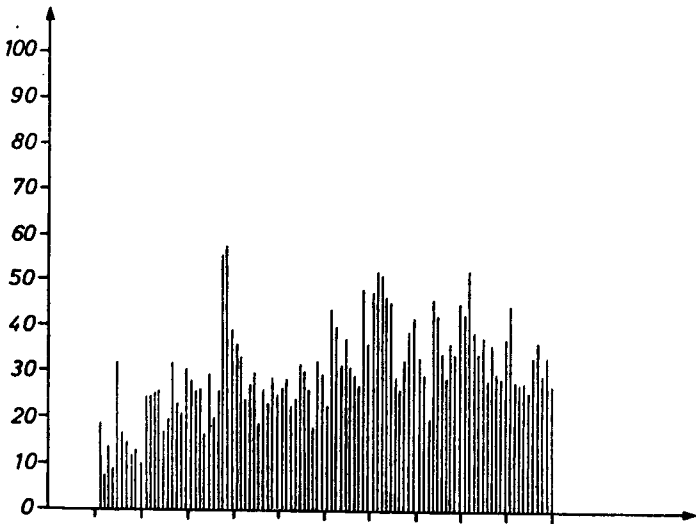

FIGURE 1B (see also Form B)

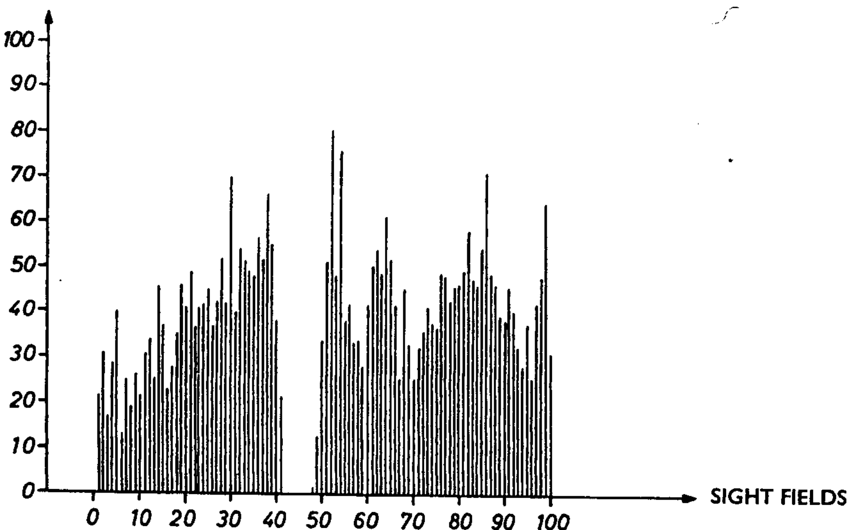

Figure 1A and B

FIGURE 1 FORM A

Statens Seruminstitut, Tuberculosis Department
IUAT PROJECT (STAINING METHODS)
Method group: 1-2-3

	1									10	11									20	21			
1		8	3	3	1	–	–	–	–	–				1	9					7				1
2				2	4				2	5				2	5				2	6				1
3				2	6				1	6				3	0				2	0				2
4				3	0				1	9				2	6				2	3				2
5				2	6				1	7				3	2				2	9				2
6				4	8				3	6				4	7				5	2				5
7				3	3				2	9				2	0				4	6				4
8				3	9				3	4				3	7				2	8				3
9				2	5				3	3				3	6				2	9				3
10		8	3	3	1					3					2					2				

	Staining of bacteria	Decolorization	Background	Contrast
Code	0 = none 1 = weak 2 = mod. 3 = strong	1 = poor 2 = good	1 = weak 2 = mod. 3 = strong	0 = none 1 = poor 2 = good

FIGURE 1 FORM B

Statens Seruminstitut, Tuberculosis Department.
IUAT PROJECT (STAINING METHODS)
Method group: 1-2-3

	1									10	11									20	21				
1			1	3	1									2	1				3	1				1	7
2				3	1				3	3				2	5				4	6				3	7
3				4	1				4	2				4	5				3	7				4	2
4				4	8				5	6				5	1				6	6				5	5
5					0					1				1	3				3	4				5	1
6				2	8				4	1				5	0				5	4				4	9
7				3	2				3	5				4	1				3	7				3	6
8				4	7				4	6				5	4				7	1				3	8
9				3	7				2	5				4	1				4	8				6	5
10			1	3	1					3					2					2					2

	Staining of bacteria	Decolorizaton	Background	Contrast
Code	0 = none 1 = weak 2 = mod. 3 = strong	1 = poor 2 = good	1 = weak 2 = mod. 3 = Strong	0 = none 1 = poor 2 = good

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SIGHT FIELDS

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FIGURE 1 FORM A

Statens Seruminstitut, Tuberculosis Department.

IUAT PROJECT (STAINING METHODS)

Method group: 1-2-3

Row	1–5	6–10	11–15	16–20	21–25	26–30	31–35	36–40	41–45	46–50	51–55	56–60
1	8331	-----	19	7	14	9	32	17	15	12	13	10
2	24	25	25	26	17	20	32	23	21	31	18	25
3	26	16	30	20	26	56	57	39	36	33	24	27
4	30	19	26	23	29	25	27	28	22	24	31	30
5	26	17	32	29	22	44	40	31	37	31	29	27
6	48	36	47	52	51	46	45	29	28	32	39	41
7	33	29	20	46	42	34	29	36	34	45	42	52
8	39	34	37	28	36	30	29	37	44	28	27	27
9	25	33	36	29	33	27	99999					

Row	1–5	Staining of bacteria (6–10)	Decolorization (11–15)	Background (16–20)	Contrast (21–25)	x̄ (26–30)	s (31–35)	Time (secs.) at which first bact. seen (36–40)	Sight field in which first bact. seen (41–45)	46–50	51–55	56–60
10	8331	3	2	2	2	3019	1030	0	1			

Code	Staining of bacteria	Decolorization	Background	Contrast
	0 = none 1 = weak 2 = mod. 3 = strong	1 = poor 2 = good	1 = weak 2 = mod. 3 = strong	0 = none 1 = poor 2 = good

FIGURE 1 FORM B

Statens Seruminstitut, Tuberculosis Department.

IUAT PROJECT (STAINING METHODS)

Method group: 1-2-3

Row	1–5	6–10	11–15	16–20	21–25	26–30	31–35	36–40	41–45	46–50	51–55	56–60
1	131		21	31	17	29	40	13	25	19	26	21
2	31	33	25	46	37	22	28	35	46	41	49	36
3	41	42	45	37	42	52	42	70	40	54	51	49
4	48	56	51	66	55	38	21	0	0	0	0	0
5	0	1	13	34	51	80	48	76	38	41	33	34
6	28	41	50	54	49	61	51	41	25	45	33	25
7	32	35	41	37	36	49	48	42	45	46	49	58
8	47	46	54	71	38	46	39	38	45	40	32	28
9	37	25	41	48	65	30	99999					

Row	1–5	Staining of bacteria (6–10)	Decolorizaton (11–15)	Background (16–20)	Contrast (21–25)	x̄ (26–30)	s (31–35)	Time (secs.) at which first bact. seen (36–40)	Sight field in which first bact. seen (41–45)	46–50	51–55	56–60
10	131	3	2	2	2	3811	1647	0	1			

Code	Staining of bacteria	Decolorizaton	Background	Contrast
	0 = none 1 = weak 2 = mod. 3 = strong	1 = poor 2 = good	1 = weak 2 = mod. 3 = Strong	0 = none 1 = poor 2 = good

variance observed in the whole material. Fig. 2A—F shows the distributions of the mean per sight field of the ten slides stained by each of the six methods (dilution 1 : 1). The considerable variance from sight field to sight field will be discussed later and will be shown to consist of a systematic variation covering the first 30 to 40 sight fields, and a random variation.

DISTRIBUTION OF MEAN PER SIGHT FIELD OF TEN SLIDES (DILUTION 1:1)
FIGURE 2A
METHOD 1

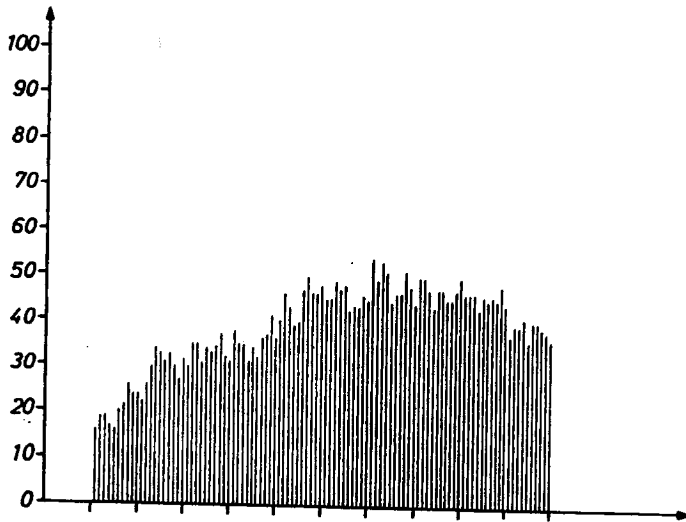
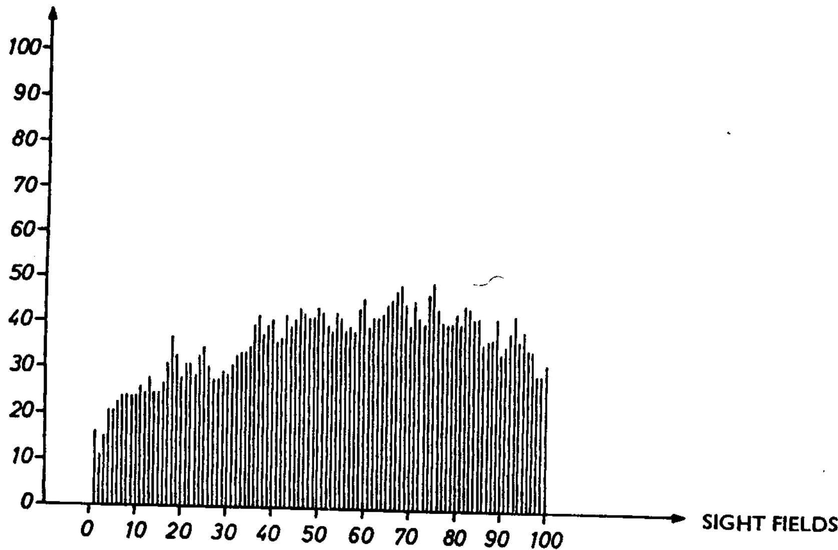


FIGURE 2B
METHOD 2



DISTRIBUTION OF MEAN PE
(DILUTION 1 : 1)
FIGURE 2C
METHOD 3

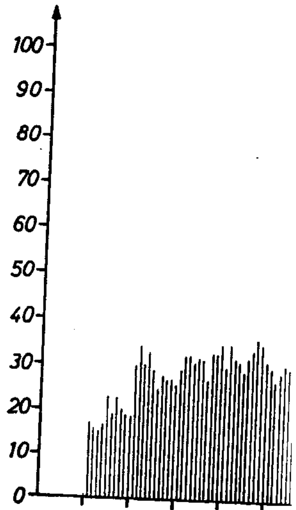
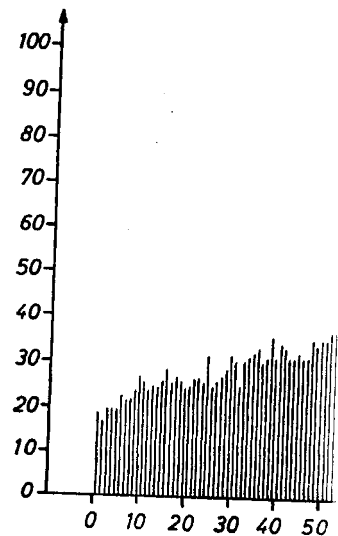


FIGURE 2D
METHOD 4



DISTRIBUTION OF MEAN PER SIGHT FIELD OF TEN SLIDES (DILUTION 1 : 1)
FIGURE 2E
METHOD 5

BACTERIA

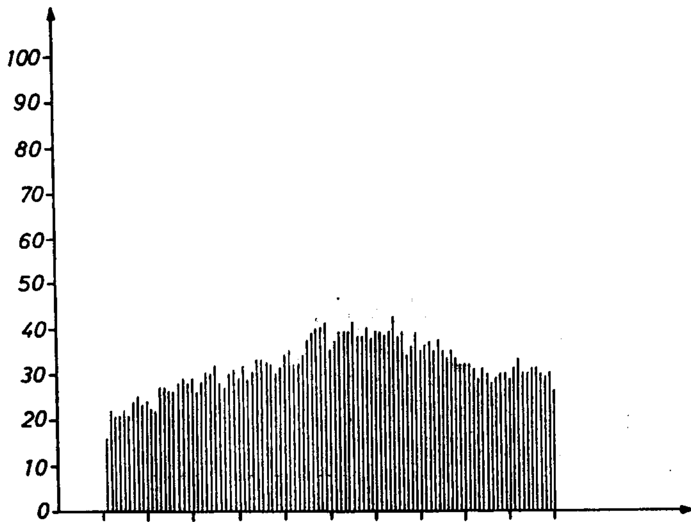
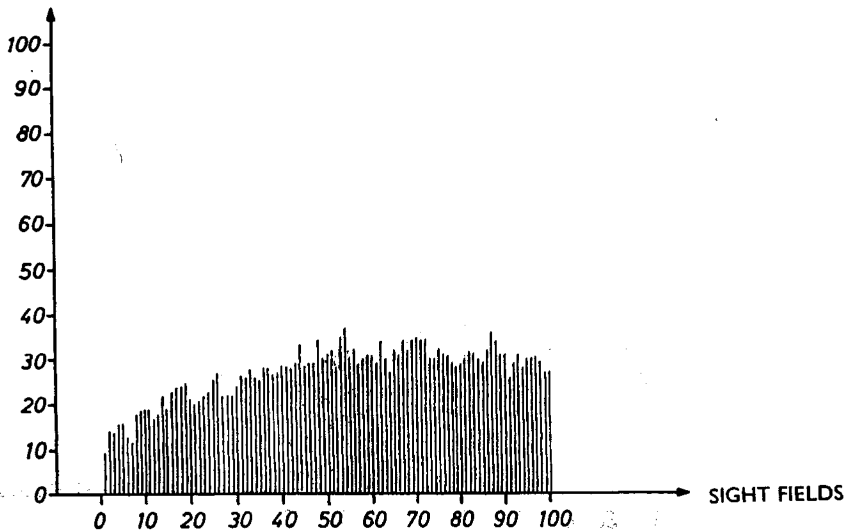


FIGURE 2F
METHOD 6



The average and v
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TABLE 2
RESULTS OF COUN

Method	Dilution 1 : d	A
Devulder (heat) Method 1	1 : 1	3[illegible]
	1 : 3.1623	1[illegible]
	1 : 10	[illegible]
	1 : 31.623	[illegible]
Devulder (cold) Method 2	1 : 1	36
	1 : 3.1623	10
	1 : 10	3
	1 : 31.623	1
Armand Method 3	1 : 1	32
	1 : 3.1623	10
	1 : 10	2
	1 : 31.623	0
Ziehl-Neelsen Danish Method 4	1 : 1	30
	1 : 3.1623	9
	1 : 10	3
	1 : 31.623	1
Ziehl-Neelsen French Method 5	1 : 1	31.
	1 : 3.1623	9.
	1 : 10	3.
	1 : 31.623	1.
Fluorescence Method 6	1 : 1	27.
	1 : 3.1623	8.
	1 : 10	2.
	1 : 31.623	1.
	1 : 100*	3.[illegible]
	1 : 316.23*	0.[illegible]
	1 : 1000*	0.[illegible]

* denotes magnification 20
$\bar{x}$ denotes average of the
Variance within slides is a
Variance between slides is
the 10 slides. If there was
within slides divided by 10
f denotes the number of d

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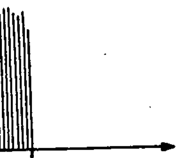

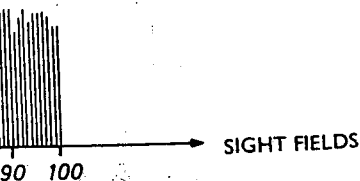

The average and variance of the counts per sight field were calculated for each slide (100 sight fields). Ten such averages and variances were obtained for each method and dilution. The averages of these ten values are shown in Table 2 under the headings "average" and "variance within slides". The variances between slides, also shown in Table 2, are those calculated from the ten average counts per method per dilution.

TABLE 2
RESULTS OF COUNTING OF BACTERIA PER SIGHT FIELD

Method	Dilution 1 : d	Average $\bar{x}$	n	Variance within slides $\overline{s_w^2}$	f	Variance between slides $\overline{s_b^2}$	f
Devulder (heat) Method 1	1 : 1	38.87	1000	163.35	990	8.71	9
	1 : 3.1623	10.73	1000	28.55	990	0.37	9
	1 : 10	3.75	1000	9.18	990	0.24	9
	1 : 31.623	1.30	1000	2.93	990	0.038	9
Devulder (cold) Method 2	1 : 1	36.27	1000	142.94	990	5.57	9
	1 : 3.1623	10.22	1000	21.16	990	1.28	9
	1 : 10	3.23	1000	7.24	990	0.15	9
	1 : 31.623	1.24	1000	2.95	990	0.079	9
Armand Method 3	1 : 1	32.85	1000	137.37	990	11.80	9
	1 : 3.1623	10.03	1000	27.46	990	1.54	9
	1 : 10	2.92	1000	7.20	990	0.28	9
	1 : 31.623	0.99	1000	2.57	990	0.099	9
Ziehl-Neelsen Danish Method 4	1 : 1	30.61	1000	63.93	990	30.76	9
	1 : 3.1623	9.96	1000	15.86	990	1.35	9
	1 : 10	3.27	1000	5.89	990	0.31	9
	1 : 31.623	1.04	1000	1.74	990	0.012	9
Ziehl-Neelsen French Method 5	1 : 1	31.77	1000	71.72	990	7.23	9
	1 : 3.1623	9.87	1000	17.08	990	1.65	9
	1 : 10	3.39	1000	5.86	990	0.16	9
	1 : 31.623	1.10	1000	2.27	990	0.12	9
Fluorescence Method 6	1 : 1	27.08	1000	76.14	990	9.80	9
	1 : 3.1623	8.89	1000	13.40	990	1.78	9
	1 : 10	2.77	1000	3.57	990	016	9
	1 : 31.623	1.17	1000	1.89	990	0.061	9
	1 : 100*	3.04	250	6.88	240	0.68	9
	1 : 316.23*	0.89	250	1.35	240	0.079	9
	1 : 1000*	0.34	250	0.42	240	0.018	9

* denotes magnification 200 ×.
$\bar{x}$ denotes average of the 10 averages (i.e. average of 1000 sight fields).
Variance within slides is a pooled estimate of the variance from sight field to sight field.
Variance between slides is a variance calculated from the 10 average values (n = 100) of the 10 slides. If there was no slide variation it should have a magnitude of the variance within slides divided by 100. It can be seen clearly that this is not the case.
f denotes the number of degrees of freedom.

No obvious dilution effect can be observed in the average counts, i.e. the proportions between the average counts correspond for a given method to the dilution factor. Thus, with the technique used, it was possible to count up to an average of 40 bacteria per sight field without any noteworthy loss of bacteria.

The dilution factor often encountered in culture experiments with tubercle bacilli was not evident in the present examinations. This is because individual bacteria were counted, and also because the mechanical treatment resulted in such a degree of comminution that it was possible to count the individual bacteria in the clumps. Further comminution on account of dilution would therefore have no effect on the bacterial count.

The variances **within** slides from sight field to sight field are due to a number of causes. It is inevitable that the random distribution of the bacteria on the slides has had some effect. Further causes are clumping of the bacteria, and also the heterogeneity of the smear with its possible influence on the staining. A systematic component of the variance within slides is the obvious increase in bacterial density from the periphery to the centre of the smear, as can be seen from the distributions of the mean values for the six methods shown in Fig. 2A—F. This effect can also be observed for the other dilutions by comparing Tables 2 and 4 (see below).

Under ideal conditions, i.e. when only the random distribution is involved, a Poisson distribution would be expected. In that event, the variance would equal the mean value. Table 3 shows the variances within slides ($\overline{s_w^2}$) divided by the average values ($\bar{x}$) (as shown in Table 2) for each method and dilution. The figure 4.20, for example, in Table 3 is the proportion 163.35/38.87. The relative precision of the figures in this table is about 5 per cent, no account being taken, however, of the systematic component of the variance. As the figures do not vary around one, it can be seen that the observations do not follow Poisson distributions. Furthermore, there are differences both between the methods and between the dilutions, the least diluted smears having the highest values for the ratio $\overline{s_w^2}/x$.

It is known from experience that the variance of counts often depends both on the level of the counting and on the square of that level, i.e. the ratio $\overline{s_w^2}/x$ tends to increase as a linear function of $\bar{x}$. The figures in Table 3 (i.e. the relation between the variance within slides and the averages) can be described as follows:

Devulder and Armand: $\overline{s_w^2} \sim 2.14\bar{x} + 0.058x^2$
Ziehl-Neelsen : $\overline{s_w^2} \sim 1.79x + 0.005x^2$
Fluorescence : $\overline{s_w^2} \sim 1.43x + 0.033x^2$

TABLE 3
PROPORTION BETWEEN VARIANCE WITHIN SLIDES AND AVERAGE VALUES FROM TABLE 2
$\overline{s_w^2}/\bar{x}$: f = 990

Method	Dilution			
	1 : 1	1 : 3.1623	1 : 10	1 : 31.623
1	4.20	2.66	2.44	2.25
2	3.94	2.07	2.24	2.38
3	4.18	2.74	2.46	2.59
4	2.09	1.59	1.80	1.67
5	2.26	1.73	1.73	2.06
6	2.81	1.51	1.29	1.62

As an example of the use of this formula, taking Method 1, dilution 1 : 1, where $\bar{x} = 38.87$:

$$2.14 \times 38.87 + 0.058 \times 38.87^2 = 171.66$$

It will be seen that this lies near the empirical value of 163.35.

However, as mentioned above, and as shown in Fig. 2A—F, the counts increase from the periphery of the smear up to a certain level which is generally maintained for the remaining sight fields counted. In order to get an impression of the importance of this phenomenon and its effect on the variances, calculation of the average values and

variances was repeated
are shown in Table 4. H
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of the systematic effect.

TABLE 4
RESULTS OF COUNTING
WITH THE FIRST 36 SIGHT

Method	Dilution 1 : d	Average $\bar{x}$
Devulder (heat) Method 1	1 : 1	44,52
	1 : 3.1623	12.19
	1 : 10	4.14
	1 : 31.623	1.37
Devulder (cold) Method 2	1 : 1	41.00
	1 : 3.1623	10.97
	1 : 10	3.42
	1 : 31.623	1.34
Armand Method 3	1 : 1	35.82
	1 : 3.1623	11.06
	1 : 10	3.26
	1 : 31.623	1.09
Ziehl-Neelsen Danish Method 4	1 : 1	33.16
	1 : 3.1623	10.62
	1 : 10	3.52
	1 : 31.623	1.07
Ziehl-Neelsen French Method 5	1 : 1	34.54
	1 : 3.1623	10.78
	1 : 10	3.67
	1 : 31.623	1.18
Fluorescence Method 6	1 : 1	30.59
	1 : 3.1623	9.73
	1 : 10	3.08
	1 : 31.623	1.31

For further details, see Table 2.

Table 5 (analogous to Tab
and dilution between the
son with Table 3 shows h
come to the Poisson distri
a certain dependence on
may still be present, an
larger variance than the fi
one may take $\overline{s_w^2} \sim 2.38\bar{x}$
for Method 3. The large v
on the background of the
teria could not be counte
colour (see Fig. 1B). The
described roughly by the

variances was repeated, excluding the first 36 sight fields. The results are shown in Table 4. Here it will be seen that all the average values are larger than in Table 2. A graphical analysis has shown that the relative increase in the counting level from the first 36 to the last 64 fields is independent of method and dilution. However, there was large variation from slide to slide with respect to this relative increase. The variances within slides are generally smaller in Table 4 than in Table 2. This was to be expected, since the variances in Table 4 do not reflect the systematic effect of the increase in the counts for the first 36 sight fields. It can also be seen that it is the variances pertaining to the dilution 1 : 1 that show the largest relative decrease. This is also in agreement with the elimination of the systematic effect.

TABLE 4
RESULTS OF COUNTING OF BACTERIA PER SIGHT FIELD WITH THE FIRST 36 SIGHT FIELDS EXCLUDED FROM EACH SLIDE

Method	Dilution 1 : d	Average $\bar{x}$	n	Variance within slides $\overline{s_w^2}$	f	Variance between slides $\overline{s_b^2}$	f
Devulder (heat) Method 1	1 : 1	44,52	640	101.01	630	8.39	9
	1 : 3.1623	12.19	640	26.57	630	0.92	9
	1 : 10	4.14	640	8.57	630	0.22	9
	1 : 31.623	1.37	640	2.96	630	0.062	9
Devulder (cold) Method 2	1 : 1	41.00	640	107.61	630	7.94	9
	1 : 3.1623	10.97	640	19.06	630	1.87	9
	1 : 10	3.42	640	7.56	630	0.17	9
	1 : 31.623	1.34	640	3.07	630	0.12	9
Armand Method 3	1 : 1	35.82	640	123.33	630	16.66	9
	1 : 3.1623	11.06	640	27.00	630	1.44	9
	1 : 10	3.26	640	7.81	630	0.47	9
	1 : 31.623	1.09	640	2.93	630	0.15	9
Ziehl-Neelsen Danish Method 4	1 : 1	33.16	640	48.99	630	32.54	9
	1 : 3.1623	10.62	640	15.90	630	1.41	9
	1 : 10	3.52	640	5.96	630	0.37	9
	1 : 31.623	1.07	640	1.81	630	0.026	9
Ziehl-Neelsen French Method 5	1 : 1	34.54	640	53.36	630	7.10	9
	1 : 3.1623	10.78	640	15.78	630	2.35	9
	1 : 10	3.67	640	6.30	630	0.28	9
	1 : 31.623	1.18	640	2.25	630	0.20	9
Fluorescence Method 6	1 : 1	30.59	640	54.95	630	13.14	9
	1 : 3.1623	9.73	640	12.18	630	1.88	9
	1 : 10	3.08	640	3.49	630	0.26	9
	1 : 31.623	1.31	640	2.10	630	0.074	9

For further details, see Table 2.

Table 5 (analogous to Table 3) shows the relation for each method and dilution between the values of $\overline{s_w^2}$ and $\bar{x}$ from Table 4. Comparison with Table 3 shows how much closer the distribution has now come to the Poisson distribution. As regards the first three methods, a certain dependence on the dilution (the level of the counting) may still be present, and Method 3 seems to have a somewhat larger variance than the first two methods. As a general description, one may take $\overline{s_w^2} \sim 2.38\bar{x}$ for the first two methods and $\overline{s_w^2} \sim 2.74\bar{x}$ for Method 3. The large variance in Method 3 must be considered on the background of the fact that in certain sight fields the bacteria could not be counted because of the very strong background colour (see Fig. 1 B). The data for the last three methods can be described roughly by the relation $\overline{s_w^2} \sim 1.56\bar{x}$.

TABLE 5
PROPORTION BETWEEN VARIANCE WITHIN SLIDES AND AVERAGE VALUES FROM TABLE 4
$\overline{s_w^2}/\bar{x}$; f = 630

Method	Dilution			
	1 : 1	1 : 3.1623	1 : 10	1 : 31.623
1	2.27	2.18	2.07	2.16
2	2.63	1.74	2.21	2.29
3	3.44	2.44	2.40	2.69
4	1.48	1.50	1.69	1.69
5	1.54	1.46	1.72	1.91
6	1.80	1.25	1.13	1.60

The variances **between** slides are not affected in any systematic way by the exclusion of the first 36 sight fields. It will be seen that these depend to a great extent on the level of the counts, whereas there is no clear difference between the six methods. Since there are only nine degrees of freedom, these variances may be expected to show a high degree of random variation. Assuming the mean values per slide to follow a normal distribution (and this is only an assumption), not all the variances pertaining to a given dilution agree too well with each other. However, no alarming discrepancies can be seen. In fact, the variances between slides are almost proportional to the square of the counting level. If there were no true variations between the slides, we would expect $s_b^2 \sim \overline{s_w^2}/n$, where n is the number of sight fields per slide. In this connection, it is most reasonable to take the values for $\overline{s_w^2}$ from Table 4. It will be seen that $\overline{s_w^2}/n$ contributes only little to the magnitude of s_b^2. Only for the dilution 1 : 31.623 is this contribution of any particular importance.

A tendency to proportionality between the variances between slides and the square of the counting level can hardly be explained by the heterogeneity of the suspension used for the smears. It would seem to indicate either a variation in the volume of the suspension actually used for the smear, or a variation from slide to slide in the percentage of the bacteria actually stained by the staining method, or both.

To explain the variation between slides by a volume effect, i.e. a variation in the volume of bacterial suspension actually applied to the slide, one would have to assume a relative precision of about 10 per cent in measuring out the volume. The amount placed on the slide would then range from about 80 per cent to 120 per cent of the volume prescribed. An error of this magnitude seems greater than could reasonably be expected.

No obvious explanation can therefore be offered for the large variations between slides.

A final comparison of the six methods must be based on all four dilutions. Therefore, for each method an estimate of the number of bacteria per sight field (dilution 1 : 1) based on all four dilutions was calculated as a weighted average of the averages (Table 2) per dilution multiplied by the dilution factor. The results of these calculations are shown in Table 6.

The standard error of the number of bacteria in Table 6 is estimated to be 0.67 (except for Method 6 (200 ×), where it is 17.43). The

TABLE 6
ESTIMATED NUMB
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BETWEEN THE SIX

No. of bacteria
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31.64
31.44
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Conclusions regar

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TABLE 6
ESTIMATED NUMBER OF BACTERIA PER SIGHT FIELD (DILUTION 1 : 1) BASED ON ALL FOUR DILUTIONS, AND ESTIMATED RELATIONSHIP BETWEEN THE SIX METHODS

No. of bacteria	Proportion between number of bacteria by two methods						
	Method	1	2	3	4	5	6
37.10	1		1.08	1.17	1.18	1.15	1.32
34.34	2	0.93		1.09	1.09	1.07	1.22
31.64	3	0.85	0.92		1.01	0.98	1.12
31.44	4	0.85	0.92	0.99		0.98	1.11
32.20	5	0.87	0.94	1.02	1.02		1.14
28.20	6	0.76	0.82	0.89	0.90	0.88	
301.68	6 (200 ×)	8.13	8.78	9.53	9.60	9.37	10.70

standard error of the proportion between two methods is about 0.03, the exact figure depending somewhat on the number of bacteria. In the comparisons with Method 6 (200 ×), it is about 0.50. A difference between two methods is therefore significant if the figures in Table 6 are >1.06 or <0.94.

It will be seen from Table 6 that, on the basis of decreasing staining effect, the methods examined can be divided into four significantly different groups, viz. Method 1, Method 2, Methods 3, 4 and 5, and Method 6. As regards Methods 4, 5 and 6 (particularly Method 6), the decolorization was more prolonged than in the first three methods. It will also be seen that the "best" method (No. 1) was only about 30 per cent better than the "worst" (No. 6).

However, comparison of the variances within slides ($\overline{s_w^2}$) shows that Methods 4, 5 and 6 have the lowest values. Whether this is due to the prolonged decolorization and/or the weaker background colour cannot be determined (contrast staining is not carried out in Method 6). It should be mentioned that the stronger background colour when methylene blue is used undoubtedly has contributed considerably to the larger variances in Method 3 (see Tables 2 and 4), and that a strong background colour gives a poor contrast with the stained bacteria (Table 1).

It is evident from comparison of the results with Methods 6 and 6 (200 ×) that the advantage of the fluorescence method is not the staining method but the lower magnification which facilitates examination of a greater area. The conversion factor for the number of bacteria per sight field with the two magnifications is about ten times.

Conclusions regarding the counting method

1. In this assay, every effort was made to produce a sputum suspension of very uniform consistency, so that the smears were reproducible, both as regards thickness and distribution of bacteria. It is clear that a smear produced in this way is an artificial product that cannot be compared with an ordinary smear from a sputum specimen.

2. Counting of the bacteria in a large number of sight fields across the area of the slide revealed that, in spite of all the above-mentioned efforts, there were systematic variations **within** each slide.

3. However, examination of the four dilutions showed that there was no obvious dilution factor.

4. The results also show that the variance **between** slides is decisive for the accuracy of comparison between various staining methods.

5. The reasons for such variances are not evident, but, apart from the variance within slides, it cannot be explained entirely by differences in the volume actually applied to the slide.

6. No false positive results were registered in the present study.

On the basis of these conclusions, the following alterations in technique can be recommended for use in the subsequent project:

1. On account of the importance of the variance between slides, efforts should be concentrated on improving the reproducibility of the slide.

2. Since there is no obvious dilution factor, it is sufficient to count one dilution stage with an average of 30—40 bacteria per sight field.

3. Fewer sight fields can be counted, but these must be spread over the whole area, e.g. every fourth sight field and a total of 25 sight fields across the circle.

4. The number of slides must be increased considerably for any effect on the accuracy to be achieved. Comparison of more sputum specimens should probably be preferred to an increase in the number of slides with one sputum.

FINAL CONCLUSIONS

As mentioned previously, smears produced in routine work have a far greater inhomogeneity than those used in the present assay, both from the point of view of thickness and the distribution of the bacteria. Therefore, with a reasonable content of tubercle bacilli distinction could hardly be made in routine work between the methods, the "best" of which (under the experimental conditions of this study) revealed only about 30 per cent more bacteria than the "worst".

In comparing the methods, particular attention should be paid to the size of the variances within slides, since it could be feared that a large variation in this study would be even larger in the more inhomogeneous routine smears. Thus, it could be expected that the large variances would compromise the demonstration of tubercle bacilli in smears with a smaller content of bacteria, even though in this study the method showed a higher bacterial count per sight field.

Taking such considerations into account in the evaluation, we would be diffident in recommending Method 1 in the form proposed, despite its larger average and its advantage as regards the simplified staining technique.

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APPENDIX

Staining metho

METHOD 1: TAN-
DEVULDER — HEA

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2. Wash in running ta
3. Cover the slides w
4. Wash in running ta
5. Dry.

METHOD 2: TAN-T
DEVULDER — COL

1. Place the slides in
without heating, **for** a
2. Wash in running ta
3. Cover the slides wi
4. Wash in running ta
5. Dry.

Solution 1 (Kinyoun

Basic fuchsin :
Phenol :
Ethanol 95% :
Distilled water :

Solution 2 (Gabett)

Methylene blue :
Sulphuric acid 60%:
Absolute ethanol :
Distilled water :

METHOD 3: ARMA

1. Cover the slides wit
utes on hot plate (75°)
2. Wash in running ta
3. Cover the slides wit
4. Wash in running ta
5. Dry.

Carbolfuchsin

Basic fuchsin
Ethanol 96%

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For smaller laboratories, Method 5 (possibly Method 4) can be recommended, and for larger laboratories Method 6 (200 ×).

These recommendations must be taken with reservation because of the limited number of staining methods examined.

APPENDIX I

Staining methods

METHOD 1: TAN-TIAM-HOK METHOD MODIFIED BY DEVULDER — HEAT COLORATION

1. Cover the slides with Solution 1 (Kinyoun) and heat for **10 minutes** on hot plate (about 75°).
2. Wash in running tapwater.
3. Cover the slides with Solution 2 (Gabett) for **5 minutes.**
4. Wash in running tapwater.
5. Dry.

METHOD 2: TAN-TIAM-HOK METHOD MODIFIED BY DEVULDER — COLD COLORATION

1. Place the slides in a bath (in basket) in Solution 1 (Kinyoun), without heating, **for at least 3 hours.**
2. Wash in running tapwater.
3. Cover the slides with Solution 2 (Gabett) for **5 minutes.**
4. Wash in running tapwater.
5. Dry.

Solution 1 (Kinyoun)

Basic fuchsin	:	40 g
Phenol	:	80 ml
Ethanol 95%	:	200 ml
Distilled water	:	1000 ml

Solution 2 (Gabett)

Methylene blue	:	10 g
Sulphuric acid 60%	:	200 ml
Absolute ethanol	:	300 ml
Distilled water	:	500 ml

METHOD 3: ARMAND METHOD

1. Cover the slides with carbolfuchsin solution and heat for **10 minutes** on hot plate (75°).
2. Wash in running tapwater.
3. Cover the slides with Armand solution for **2 minutes.**
4. Wash in running tapwater.
5. Dry.

Carbolfuchsin

Basic fuchsin	:	20 g
Ethanol 96%	:	200 ml

Phenol liqu.	:	100 ml
Distilled water	:	1800 ml

Armand solution

Ethanol 95%	:	500 ml
Saturated aniline solution (buffered)	:	300 g
Nitric acid (for analysis)	:	200 g
Methylene blue	:	15 g

METHOD 4: ZIEHL-NEELSEN (DANISH)

1. Cover the slides with carbolfuchsin* and heat for **3 minutes** on hot plate (75°).
2. Wash in running tapwater.
3. Decolorize in two lots of sulphuric acid (25%) for **6 minutes** altogether.
4. Wash in running tapwater.
5. 96% ethanol for **3 minutes.**
6. Wash in running tapwater.
7. Sulphuric acid for **a few seconds.**
8. Wash in running tapwater.
9. Contrast stain with 3/4 per cent picric acid for **15 seconds.**
10. Wash in running tapwater.
11. Dry.

METHOD 5: ZIEHL-NEELSEN (FRENCH)

1. Cover the slides with carbolfuchsin* and heat for **15 minutes** on hot plate (about 75°).
2. Wash in running tapwater.
3. Sulphuric acid (25%) for **3 minutes.**
4. Wash in running tapwater.
5. 96% ethanol for **3 minutes.**
6. Wash in running tapwater.
7. Sulphuric acid for **3 minutes.**
8. Wash in running tapwater.
9. Contrast stain with picric acid (3/4 per cent) for **15 seconds.**
10. Wash in running tapwater.
11. Dry.

METHOD 6: FLUORESCENCE

1. Place the slides in small baskets and submerge in auramine-rhodamine solution for **9 minutes** at 50°.
2. Wash in running tapwater.
3. Decolorization: Submerge the baskets successively into the following baths: (a) 25% sulphuric acid, (b) 25% sulphuric acid, (c) 96% ethanol, (d) 25% sulphuric acid. Each submersion lasts **3 minutes** and the baskets are agitated constantly. Between each bath wash the slides in running tapwater.
4. Dry by hot air.

* See Armand method