J. gen. Microbiol. (1962), **29**, 97–104
Printed in Great Britain

Total Counts of Bacterial Spores using Counting Slides

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(*Received* 15 *November* 1961)

SUMMARY

Total counts were made of bacterial spore suspensions using counting slides with chamber depths of 0·02 mm. and of 0·10 mm. Sources of error were analysed. In addition to an inherent sampling error, significant variation between counts on replicate slides may result from: (i) variation in the depth of different counting chambers, (ii) variation in the fit of cover-glasses with counting chambers. The use of 0·10 mm. rather than 0·02 mm. depth slides results in a lower estimate of the total count of a spore suspension. The former slides give more reproducible counts; evidence for their greater accuracy has been obtained by a comparison of 'percentage viability' of spores determined by counting and by slide-culture techniques.

INTRODUCTION

The need to assess the accuracy of total counts arose at the outset of an investigation of factors which may affect the viability of bacterial spores. When spores are plated on a nutrient medium production of a colony depends on (1) germination of the spore and emergence of a vegetative cell, (2) survival and multiplication of the vegetative cell. These two phases may require different optimal conditions (Halvorson & Church, 1957; Hyatt & Levinson, 1957, 1959; Demain, 1958; Demain & Newkirk, 1960). A determination of the total count of a spore suspension would enable calculation of the percentage of spores giving colonies on a nutrient medium. When this percentage is low the reason may be (*a*) because a proportion of spores are not viable, (*b*) they may be viable but fail to germinate in the growth conditions supplied, or (*c*) germination may be initiated, but the environment may fail to support outgrowth and multiplication.

Microscope counts of bacteria in chambers of known depth have generally been accepted and used as the most accurate total count technique available (Glynn, Powell, Rees & Cox, 1913–14; Wilson, 1922; Wilson & Kullman, 1931; Jordan & Jacobs, 1944; Semenitz, 1951). Recently an electronic method (Kubitschek, 1958), used widely for counting blood cells, has been applied to counts of bacteria; this technique might effect a considerable saving in time and labour, but a detailed assessment of its accuracy when applied to bacteria does not appear to have been published.

In preliminary experiments using several slides (depth 0·02 mm.) the variation of replicate counts was greater than had been expected. The possible sources of variation and of error were analysed and include: (1) invisibility of spores; (2) movement of spores; (3) inaccuracy of counting the spores in a chamber and personal bias in counting; (4) sampling error due to distribution of spores in the

counting chamber; (5) technique in filling the chambers; (6) difference in depth of counting chambers having the same nominal depth; (7) variation of the *actual depth* of liquid enclosed in the counting chamber by the cover-glass. The actual depth of liquid enclosed in counting chambers with a nominal depth of 0·02 mm. has been reported to vary from about 0·0229 mm. to about 0·0297 mm. (Norris & Powell, 1961), depending mainly on the attention paid to closeness of contact between the cover-glass and the slide. Previous workers have failed to appreciate the magnitude of this error. Norris & Powell (1961) described an interferometric method for measuring the depth of liquid each time a chamber is assembled for a count.

The importance of the error due to the fit of cover-glass and slide is confirmed in this paper.

METHODS

Spore suspensions. The organisms used were several strains of *Bacillus subtilis*: Strain A, a rough variant derived from *B. subtilis* NCTC 8236; strain B, a smooth variant derived from *B. subtilis* NCTC 8236; Strain D, a laboratory strain supplied by the late Dr J. F. Powell, Microbiological Research Establishment, Porton, Wiltshire. Cultures were grown at 37° for 7 days on the surface of peptone agar containing 1 mg. $MnSO_4.4H_2O$/l. Organisms were harvested in sterile distilled water, washed several times on a refrigerated centrifuge and suspended in sterile distilled water to give a total count of about 10^{10} spores/ml. Suspensions were cooled in ice-water during this treatment, and finally stored at 0–4°. They contained about 90% of highly refractile spores; the remaining 10% consisted of spores which appeared dark by phase contrast, and some apparently 'empty' cells. 'Bright spores' only were counted.

Dilutions of stock suspensions. 100- to 500-fold dilutions of stock suspensions were prepared in distilled water sterilized by filtration through a 5/3 sintered glass filter, and shaken for 5 min. to ensure adequate dispersion.

Counting slides. Two types of slides were obtained from Messrs Hawksley and Sons Ltd., 17 New Cavendish Street, London, W. 1.

(1) Helber slide with central annulus and Thoma ruling. Nominal depth of chamber 0·020 mm. ± 0·001 mm ('0·02 mm. slide').

(2) Haemocytometer slides according to the British Standard Specification 748:1953 with Improved Neubauer ruling ('0·1 mm. slide'). Nominal depth of chamber 0·100 mm. ± 0·001 mm. The dimensions of one slide of each type were checked at the National Physical Laboratory. Using Johnson's (1952) procedure the mean depth of the 0·02 mm. slide was 0·0215 mm. ± 0·0003 mm., that of the 0·1 mm. slide was 0·1001–0·1003 mm. ± 0·0003 mm. (depth measured over two rulings). The observed volume of the counting chamber was 7·5% greater than the nominal volume of the 0·02 mm. slide and 0·25% less than the nominal volume of the 0·1 mm. slide. Twelve slides of type 1 and six of type 2 were used.

Cover-glasses for 0·02 mm. slides were 19 × 23 mm. area, 0·5 mm. thick, those for 0·1 mm. slides were 22 × 23 mm. area, 0·45 mm. thick.

Filling the counting chambers. 0·02 mm. slides. A dropping pipette (Cook & Yousef, 1953) was rinsed out with the suspension, and a drop placed on the central plateau of the slide. The coverglass was then lowered into position. The

effect of pressing down the edges of the cover-glass to ensure contact with the slide was significant, and is considered later. 0·1 *mm. slides.* The cover-glass was wrung to the supporting surfaces of the slide, a drop of spore suspension from the pipette was allowed to flow under the cover-glass.

Storage of filled counting slides. Sedimentation of spores and counting five replicate slides could require up to 2 hr. Evaporation was prevented by sealing the edges of the 0·1 mm. slides with DPX mounting fluid (British Drug Houses, Ltd.) and by storage of all slides in the presence of moist cotton wool.

Counting technique. A phase-contrast microscope was used (C. Baker Ltd.), with phase plate type TA-0·25λ, ×40 objective, ×10 binocular eyepieces and blue filter. Spores in 50 or 100 small squares (area 0·0025 mm.^{2}) were counted.

RESULTS

Error due to invisibility of spores. Their refractile nature makes spores readily visible by the phase-contrast microscope. Using a ×40 objective (numerical aperture 0·7) the depth of field is less than 0·001 mm.; the counting chambers were 20 or 100 times this depth, therefore sedimentation of spores is essential. It was observed to occur within half an hour in both types of slide. Counts were made after sedimentation, and the error due to invisibility of spores was judged to be negligible.

Movement of organisms. Spores are non-motile, Brownian movement practically ceased after sedimentation.

Error in counting spores on a slide. Three experiments were made with 0·02 mm. slides stored for up to 7 hr., and four with 0·1 mm. slides stored for up to 3½ hr. Five counts were made during the storage, and the coefficient of variation of replicate counts of spores in a single, filled chamber, was calculated. For 0·02 mm. slides, coefficients of variation ranged from 0·79 to 1·81%: for 0·1 mm. slides the values ranged from 0·54 to 1·14%.

Error due to personal bias in counting. Replicate counts of the same slides were made by direct microscopic examination and from photomicrographs. The photographic count was 98% of the visual count, and had a lower coefficient of variation.

Sampling error due to distribution of spores in the counting chamber. 'Student' (1907) showed that under ideal conditions the occurrence of yeast cells on the squares of a haemocytometer slide followed a Poisson distribution. Conditions which must be met before the distribution of the bacterial cells will be random are (1) the cells should not repel one another, or else there must be sufficient space so that the repelling effect will be negligible; (2) the volume of the cells, relative to the volume of the liquid in which they are suspended, should be small; (3) there should be no clumping of the cells (Stearman, 1955).

Agreement with a Poisson distribution was tested by calculating the Index of Dispersion (χ^2) of numbers per square on each slide (Fisher, 1948). The χ^2 values were grouped and the observed frequency of occurrence compared with that expected from a Poisson distribution. The results for strain D spores are shown in Table 1. For 60 counts of strain D spores total $\chi^2 = 3008{\cdot}3$ (60×49 degrees of freedom). Determination of $\sqrt{2\chi^2} - \sqrt{(2n-1)}$ and reference to the Normal Deviate table showed that the probability (P) of obtaining a total χ^2 equal to or greater than

this observed value was 0·185. In counts of strain A spores closer agreement between expected and observed frequencies was obtained. For 60 counts (60×49 degrees of freedom) the corresponding probability was 0·742. There was no evidence

Table 1. *The distribution of χ^2 values for counts of spores of* Bacillus subtilis *(strain D)*

Counting chambers 0·10 mm. depth, or 0·02 mm. depth. Each value of χ^2 is derived from counts of spores in 50 squares on one slide. (χ^2 distribution for 49 degrees of freedom from Fisher & Yates, 1955).

χ^2	Expected frequency	Observed frequency
<28·942	0·6	0
29·942 –30·872	0·6	0
30·872 –33·931	1·8	2
33·931 –36·819	3·0	3
36·819 –40·530	6·0	2
40·530 –43·3665	6·0	6
43·3665–48·335	12·0	13
48·335 –53·6695	12·0	15
53·6695–57·0785	6·0	5
57·0785–62·037	6·0	7
62·037 –66·338	3·0	5
66·338 –71·405	1·8	1
71·405 –74·9185	0·6	1
>74·9185	0·6	0
	60	60

Table 2. *Variation between counts on replicate slides*

Number of replicate slides for each experiment, $\geqslant 5$, Series 1, cover-glass *pressed down* on to slide. Series 2, cover-glass *lowered* on to slide.

	Spore suspension *B. subtilis* strain	Mean number of organisms per square	Coefficient of variation (%)
Series 1. Depth of slides = 0·02 mm.	D	1·305	23·1
	A	2·395	12·2
	A	4·058	14·0
	A	4·186	22·0
	B	4·292	14·4
	A	5·136	14·5
	D	5·676	16·5
Series 2. Depth of slides = 0·02 mm	D	2·372	12·1
	D	2·936	11·2
	B	4·752	15·0
	D	5·992	8·6
	A	6·030	7·7
	A	6·423	6·2
	A	6·920	8·3
	A	7·580	8·6
	A	10·550	10·9
Series 3. Depth of slides = 0·10 mm.	D	4·578	5·8
	D	5·471	5·6
	A	6·236	10·3
	D	7·304	5·5
	A	7·364	9·2
	D	7·825	6·2
	A	9·033	3·6

that distribution of spores in the counting chambers differed significantly from a Poisson distribution.

Variation in technique of filling a slide. A single 0·02 mm. slide was used to count five replicate samples from a spore suspension. With an average of 4·56 spores per square the coefficient of variation was 4·9%. A comparable coefficient of variation, 5·1% is shown by five replicate counts recorded in his table 1 by Wilson (1922).

Between slides variation. Coefficients of variation of counts on replicate slides are recorded in Table 2. The highest coefficients of variation were obtained when 0·02 mm. slides were used with the cover-glass pressed firmly down on to the slide. Use of 0·1 mm. slides gave the lowest values; the variation between counts on these slides was therefore compared with that attributable to sampling errors.

Seven groups of replicate counts (four of strain D spores, three of strain A spores) on 0·1 mm. slides were analysed. χ^2 for each group of replicates was combined, $\Sigma\chi^2 = 47{\cdot}1846$ (35 degrees of freedom) corresponding to a probability of 0·05–0·10. This is comparable with the variation between corrected counts in replicate 0·02 mm. slides reported by Norris & Powell (1961). Since the probability of Poissonian distribution of strain D spores was 0·185 the variation between counts on replicate 0·1 mm slides was probably not significantly greater than the variation due to sampling. Using *Bacillus stearothermophilus* spores and 0·1 mm slides, seven groups of replicate counts were made by a second operator. For this series of counts $\Sigma\chi^2 = 30{\cdot}752$ (31 degrees of freedom) corresponding to a probability of 0·3–0·5.

For the second series of experiments in Table 2 $\Sigma\chi^2 = 121{\cdot}77$ (38 degrees of freedom), corresponding to a probability less than 0·001. Clearly when either method of fitting the coverglass was used the variation between counts on replicate 0·02 mm. slides was significantly greater than that attributable to sampling.

Variation in fit of cover-glasses to slides. The 0·1 mm. slide has the following advantages over the 0·02 mm. slide, (1) the former is an 'open' type of chamber, to which the cover-glass can be firmly fitted before the spore suspension is introduced, whereas the 'closed' type of Helber chamber requires introduction of the suspension before application of the cover-glass; (2) the error introduced by variable fit of the cover-glass will be proportionally less in the case of the slide with the greater depth.

Using 0·02 mm. slides, counts with the cover-glass pressed firmly down on to the supporting edges of the slide were compared with those on slides with the cover-glass lowered into position. Four to eight slides were prepared by each method and the experiment was repeated 6 times. Typical results were:

mean count with cover-glass pressed down (method 1)—

5·136 spores/square (95% fiducial limits 4·212–6·059);

mean count with cover glass lowered (method 2)—

6·924 spores/square (95% fiducial limits 6·209–7·693);

$$\frac{\text{count by method 1}}{\text{count by method 2}} = 74{\cdot}2\%.$$

From the whole series of experiments the average ratio of these counts was 75·5%.

Comparison of counts by 0·02 mm. and by 0·1 mm. slides. Six slides of each type were used to estimate the count of a spore suspension. Four such experiments were

performed. In three of the experiments the same spore suspension was counted in both types of slide, therefore the mean number of spores per square of the 0·1 mm slide would be expected to equal 5 times the mean number per square of the 0·02 mm. slide. In the 4th experiment the concentration of the suspension counted by the 0·02 mm. slide was 5 times that counted on the 0·1 mm. slide. When the count by 0·1 mm. slide was expressed as a percentage of the count by 0·02 mm. slide the values obtained were 69·9%, 85·6%, 75·4% 63·4%; mean 73·6%.

0·1 mm. slides gave more reproducible counts which were lower and likely to be more accurate than counts by 0·02 mm. slides. An attempt was made to confirm this conclusion by an investigation of the percentage viability of a spore suspension (1) by comparison of total count with viable count estimated by colony production, (2) by slide culture of the spores.

Determination of percentage viability

Method 1. Total counts were made using 0·1 mm. slides. 'Bright' (highly refractive) spores only were counted, since the small proportion of 'dark' spores were less easily visible in the counting chamber. The percentage of bright spores initially present in the suspension, determined in the slide culture experiments, was taken as 94·5% based on the fourth experiment (Table 3). The count of bright spores was multiplied by a factor of 100/94·5 to give the total count shown in Table 3. Viable counts were performed by a spread plate technique. In the first experiment four dilution series were used, and six plates prepared from the final dilution in each series. In the second experiment eight plates were prepared from each of five dilution series.

Table 3. *The percentage viability of* Bacillus subtilis *strain D spores, as determined:* (1) *by comparison of total, and colony counts,* (2) *by slide culture.*

Method 1

Time of storage of suspension (days at 4°)	Total count per ml.	95% fiducial limits (as % of mean)	Viable count per ml.	95% fiducial limits (as % of mean)	% viability
135	$3{\cdot}130 \times 10^7$	±9·91%	$2{\cdot}899 \times 10^7$	±6·90%	87·52
175	$1{\cdot}938 \times 10^7$	±4·14%	$1{\cdot}615 \times 10^7$	±3·46%	83·3

Method 2

	Number of replicates	Initial % 'bright' spores	95% fiducial limits (as % of mean)	% viability	95% fiducial limits (as % of mean)
175	1	—		83	
176	4	—		73	
213	2	—		88·0	
221	5	94·46	±1·25	88·87	±2·07
225	2	94·42		91·7	
227	2	92·25		91·2	

Method 2. Slide cultures were prepared according to the technique described by Postgate, Crumpton & Hunter (1961). When inoculated on to peptone agar and incubated at 37°, spores of *Bacillus subtilis* strain D showed a rapid fall in refractive index followed by swelling and outgrowth of the vegetative cell. The

percentage of spores showing outgrowth or swelling after incubation at 37° for 3 hr. was recorded as percentage viability. On further incubation growth appeared to proceed to microcolony formation, and no lysis of outgrown cells was observed.

The results are shown in Table 3.

DISCUSSION

The tolerance of ±0·001 mm. quoted by the manufacturers is ±5% of the depth of 0·02 mm. slides. Norris & Powell (1961) found no serious errors in the chambers they examined; the depth of one of our slides was reported to be at least 5% greater than the nominal depth. The demonstration of a significant systematic error depending on the fit of the cover-glass with the 0·02 mm. slide agrees with the observations of Norris & Powell (1961). These workers reported that a minimum depth of 0·02288 mm. of liquid was enclosed in slides with a mean depth of 0·0204 mm., i.e. the actual depth of the slide was at least 12·4% less than the depth of liquid enclosed between slide and cover-glass.

The tolerance of 0·001 mm. is 1% of the depth of 0·1 mm. slides; the error of the slide tested was less than this. In our experiments total counts of spores by 0·1 mm. slides were 15–36% lower than those by 0·02 mm. slides with coverglasses firmly pressed into position. In view of Norris & Powell's findings, the closer fit between cover-glasses and 0·1 mm. slides and the smaller significance of this source of error in relation to the depth of the slide, it seemed likely that use of 0·1 mm. slides would give a more accurate count. This was confirmed by the correlation between percentage viability of *Bacillus subtilis* spores determined by counting experiments and by slide culture. If 0·02 mm. slides had been used the resulting estimates of viability would have been about 61, and 57%, i.e. 26% lower than estimates based on the use of 0·1 mm. slides.

The variation between counts of replicate slides was greater between 0·02 mm. slides than 0·1 mm. slides. Greater variation occurred when the cover-glass was pressed down on to the slide, than when it was merely lowered. The χ^2 test for variation between counts of *Bacillus subtilis* spores on 0·1 mm. slides gave a probability of 0·1–0·05. Since the probability of Poissonian distribution of strain D spores was 0·185 the use of replicate slides does not appear to have significantly increased the variation. Two total counts by 0·1 mm. slides are shown in Table 3; the first was based on counts of about 390 spores on each of four slides, the second on 230 spores on each of 10 slides. The replication used for the second count has reduced the 95% fiducial limits to about ±4% of the mean count. Unless systematic errors can be shown to be small in relation to this variation there is probably no value in further replication to increase the precision of this determination.

Errors due to the other factors investigated are relatively small; if there is a tendency of organisms to form clumps the distribution will not be Poissonian, and sampling errors will be increased. Norris & Powell concluded that other errors resulted from the adherence of organisms to solid surfaces, and advised the use of a detergent and a low surface/volume ratio of suspensions to minimize this effect. We have noted a drop in total count of spore suspensions after placing in clean glass bottles and storing for 24 hr. at 4°, presumably due to adsorption of spores on to the glass. For this reason a fresh spore suspension of suitable concentration was prepared for each series of counts, and replicate samples removed within a few hours.

The use of 0·1 mm. slides for total counts of vegetative bacteria has not been investigated; these organisms may not sediment sufficiently rapidly for the technique to be practicable.

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