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Viable Counting of *Mycobacterium tuberculosis* in a Silica Gel Medium

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SUMMARY: A method is described in which deep counts of *Mycobacterium tuberculosis* are made in a defined medium solidified with silica gel. The method was accurate, simple and safe. The contamination rate was low. Deep counts were higher than surface counts on plates. The defined medium was superior to Kirschner medium.

The enumeration of viable bacteria is most frequently and accurately carried out on the surface or in the depths of solid medium. A surface viable count method for *Mycobacterium tuberculosis* using oleic acid + albumin agar medium was described and statistically analysed by Fenner (1951). Yegian & Budd (1951) also described a pour plate technique for mycobacteria using the same medium and they suggested that it yielded a higher count than the former method. A disadvantage of the surface count technique is the high rate of contamination, which in our hands has occasionally been as high as 15 % of the plates inoculated. In order to avoid contamination Mitchison (1950) and O'Hea (1955) described surface count methods in which the medium was distributed into small bottles and each of these was inoculated with a drop of the appropriate culture dilution. These methods are inconvenient where accurate work is required because of the large number of bottles used. Hirsch (1954) pointed out that agar has an inhibitory action on the growth of tubercle bacilli, only in part due to binding of the ferric ion. Knox (1955) described a technique for deep counts in a semi-solid medium of low agar concentration, contained in tubes. In this method optimal conditions for growth varied according to the depth of the colonies in the column of the medium and this may possibly be a source of error. Knox has not published accounts of comparisons with Fenner's method. We have investigated the use of a deep culture medium solidified with silica gel in place of agar. The counts can then be set up in narrow-necked bottles which decreases the incidence of contamination and allows the formation of a thin well-aerated layer of medium.

METHODS

Organisms. *Mycobacterium tuberculosis*; strains H37Rv and H37Ra, obtained from Dr R. J. W. Rees (Medical Research Council Laboratories, Mill Hill, London); Branch, a bovine strain, obtained from Dr A. Q. Wells (Sir William Dunn School of Pathology, Oxford); strain B.C.G., Copenhagen strain, obtained from current batches of vaccine; strain B, a variant of H37Rv highly resistant to isoniazid and catalase-negative (Barnett, Bushby & Mitchison, 1953); strains 0139 and 0704, recently isolated from patients with

pulmonary tuberculosis and sensitive to streptomycin, *p*-aminosalicylic acid (PAS) and isoniazid; strains 0942 and 0435, human strains recently isolated from patients with pulmonary tuberculosis and resistant to PAS but sensitive to streptomycin and isoniazid.

Media. Unless otherwise stated, the medium used was the 7H-3 medium described by Middlebrook, Cohn & Schaefer (1954). Since a multiplicity of media was described by these authors, the method of preparation is given:

Basal medium. KH_2PO_4 , 0.2 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.26 g.; sodium-L-glutamate, 0.1 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.1 g.; sodium citrate $\cdot 2\text{H}_2\text{O}$, 0.02 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.0001 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0002 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0002 g.; pyridoxine hydrochloride, 0.0002 g.; biotin, 0.0001 g.; glycerol, 0.4 ml.; ferrous ammonium citrate, 0.02 g.; distilled water to 100 ml. Adjust pH to 7.2 with HCl. Autoclave at 15 lb./sq.in. (120°) for 20 min.

Oleic acid-albumin complex. (a) Bovine Albumin Fraction V (Armour) 10 g.; glucose, 4 g. Dissolve in 0.85 % saline to 190 ml. (b) Oleic acid, 0.12 ml.; 0.05 N-NaOH, 10 ml. Mix (a) and (b). Seitz filter and add 20 ml. to 100 ml. of basal medium. For plates solidified with agar the 7H-3 basal medium was diluted with 100 ml. distilled water. Malachite green to a final concentration of 0.0001 % and agar to 1.4 % were added. The medium was autoclaved (15 lb./sq.in. for 20 min.) and then 20 ml. of the oleic acid albumin complex added.

A liquid medium modification of the 7H-3 medium was also used, in which glycerol was omitted from the basal medium and 100 ml. water, 0.025 % Tween 80 (Atlas Powder Co.), 0.2 % glucose and 0.5 % bovine albumin were added.

Kirschner Medium (Mackie & McCartney, 1953) was used in one experiment with the following modifications. Penicillin was not added to the horse serum; the pH of the medium used for agar plates was adjusted 6.7 with N-HCl; the medium used with the silica sol was prepared at double concentration and its pH was adjusted to 7.2 with N-HCl.

Silica sol. A 2.6 % sol was prepared according to the method described by Smith (1951), using Zeo-Karb 225 (Permutit Co. Ltd., London, W.4) as the cation-exchange resin. The sol was sterilized by autoclaving at 15 lb./sq.in. for 15 min. and was stored in c. 100 ml. volumes in Pyrex flasks at 4° . It remained usable for at least a week.

Diluent. Sterile distilled water or 0.1 % bovine albumin fraction V in distilled water were used in different experiments.

Use of silica gel solidified medium

The medium to be solidified with silica gel was distributed with aseptic precautions in 3 ml. amounts into 2 oz. sterile medical flats with screw-caps. The bottles were incubated at 37° for 2 days as a sterility check and then could be stored, when necessary, for several months at 4° . Dilutions of cultures or of tissue homogenates were then added, usually in 0.2 ml. volumes from 1 ml. graduated pipettes. These operations were carried out inside an inoculation hood using a Pumpett automatic pipette control (Shandon,

London). Accurate counts were obtained using 40-fold dilution steps. After inoculation of all the bottles on any day, 3.2 ml. of the silica sol were added to each, the contents were mixed and the bottles immediately laid flat on the bench, so that a layer of medium about 3 mm. deep covered one of the larger surfaces. Gelling began within 5 min. and was complete after *c.* 1 hr., during which period the bottles were left undisturbed. They were incubated for 4 weeks at 37° and colonies were then counted as in pour plates. When the number of colonies was between 600 and 2000/bottle an approximate count could be obtained by counting within a series of thin-ruled areas at intervals along the bottle. Less accurate counts up to 10⁵ colonies/bottle could even be made with a plate microscope containing a square of known area in the eye-piece focal plane.

For surface counts on plates the method of Fenner (1951) was used. Dropping pipettes were calibrated both with a micrometer screw gauge and by measuring the volume of a large number of drops. Counts were made after 4 weeks of incubation.

RESULTS

Comparison of deep and surface counts

In the first experiment (Table 1) 10-day cultures of strains grown in 7H-3 + Tween + albumin medium were diluted 1/10⁵ in sterile distilled water and this dilution was inoculated on to plates and into bottles. The counts on

Table 1. *Surface and deep viable counts of Mycobacterium tuberculosis using 7H-3 medium*

χ^2 tests the conformance of the counts on the strains H37 Rv, H37 Ra, Branch and B.C.G. to a Poisson distribution.

Strain	Surface plate counts			Silica gel deep counts		
	No. of replicate drops	Mean count/drop	Viable units/ml.	No. of replicate bottles	Mean count/bottle	Viable units/ml.
H37 Rv	6	13.3	6.67×10^7	5	141	7.05×10^7
H37 Ra	9	6.3	3.17×10^7	4	65	3.25×10^7
Branch	6	31.3	1.57×10^8	4	343	1.71×10^8
B.C.G.	18	3.17	1.58×10^7	5	481	2.41×10^7
0139	10	29.9	1.50×10^8	5	32	1.60×10^7
B	10	2.4	1.20×10^7	5	1	5.00×10^6

$\chi^2 = 43.01$, D.F. = 35,
 $P = 0.5-0.3$

$\chi^2 = 11.98$, D.F. = 14,
 $P = 0.7-0.5$

silica gel media of strains H37 Rv, H37 Ra, Branch and B.C.G. were slightly higher than the surface plate counts. The χ^2 indicates, in both methods, that the scatter of the individual counts followed a Poisson distribution. However, with strains 0139 and B, the silica gel counts were far lower than the surface plate counts. Growth of these two strains on both types of media was extremely poor, the colonies being minute even after 4 weeks of incubation. This finding suggested that they were particularly exacting in their growth requirements.

Cohn, Oda, Kovitz & Middlebrook (1954) showed that occasional strains of *Mycobacterium tuberculosis* are very exacting in their nutritional requirements and that isoniazid-resistant strains require a high iron content, pyruvic acid or catalase for their growth. The effect of adding silica sol on the iron concentration and the pH value of the medium was therefore examined. The addition of the sol, which had a pH value of 3.02, decreased the pH value of the medium

Table 2. *Surface and deep viable counts using 7H-3 and Kirschner medium*

Strain	Medium	Surface plate counts			Silica gel deep counts		
		No. of replicate drops	Mean count/drop	Viable units/ml.	No. of replicate bottles	Mean count/bottle	Viable units/ml.
H37 Ra	7H-3	9	7.34	3.67×10^7	5	90.2	4.51×10^7
	Kir.	5	3.41	1.70×10^7	5	39.2	1.96×10^7
H37 Rv	7H-3	17	3.53	1.76×10^7	6	42.2	2.11×10^7
	Kir.	7	1.43	7.15×10^6	6	43.7	2.18×10^7
B	7H-3	9	16.79	8.38×10^7	4	144	7.20×10^7
	Kir.	6	2.17	1.08×10^7	3	2.3	1.16×10^6
0704	7H-3	13	3.77	1.88×10^7	5	48.8	2.44×10^7
	Kir.	8	5.38	2.68×10^7	5	51.4	2.57×10^7
0942	7H-3	18	6.78	3.39×10^7	6	80.8	4.04×10^7
	Kir.	3	7.00	3.50×10^7	6	78.4	3.92×10^7
0435	7H-3	12	8.25	4.12×10^7	4	231	1.16×10^8
	Kir.	12	7.33	3.66×10^7	5	210	1.05×10^8
7H-3 medium		$\chi^2 = 70.22$, D.F. = 72, $P = 0.91$			$\chi^2 = 15.16$, D.F. = 25, $P = 0.95-0.90$		
Kirschner medium		$\chi^2 = 103.9$, D.F. = 35, $P = 0.001$			$\chi^2 = 34.49$, D.F. = 22, $P = 0.05-0.02$		

Analysis of variance

	Degrees of freedom	Mean square	Variance ratio	P
Between methods	1	11.4955	94.8	< 0.001
Between media	1	17.5275	144.6	< 0.001
Between strains	5	16.5896	136.9	< 0.001
Methods-media interaction	1	0.0260	0.2	> 0.2
Methods-strains interaction	5	8.4747	69.9	< 0.001
Media-strains interaction	5	8.0724	66.6	< 0.001
Error	5	0.1212	—	—

from 7.25 to 6.83 and removed by adsorption approximately half of the ferrous ions. In a second experiment the ferrous ammonium citrate concentration was therefore increased to 0.05% and the pH value of the medium used for the plates adjusted to 6.8 by the addition of N-HCl. In this experiment Kirschner medium and 7H-3 medium were compared, both as plates solidified with agar and as silica gel deep cultures. The results together with an analysis of variance are shown in Table 2. The χ^2 on the counts in 7H-3 medium indicated conformity with a Poisson distribution, but those in Kirschner medium failed to conform. Counts by the silica gel method were significantly

higher than those on plates and they were also significantly higher in 7H-3 medium than in Kirschner medium. The highly significant interactions between methods and strains, and between media and strains indicates that the differences between the counts by the two methods and in the two media depended on the choice of strain. Strain B grew very poorly both on plates and in deep cultures of Kirschner medium. The analysis of variance was therefore repeated with the omission of the results using this strain, but the same terms were still significant.

Effect of diluent

The use of distilled water as a diluent was investigated by carrying out viable counts on a $1/10^5$ dilution of 10-day cultures grown in 7H-3 + Tween + albumin liquid medium, with distilled water or 0.1 % bovine albumin as diluents. The results of one of these experiments, with the H37Rv strain, is shown in Table 3. There was no decrease in the count over a 24 hr. period. A second experiment with strain B yielded similar results. One can therefore conclude that the results of the first two experiments were not influenced by the use of distilled water as a diluent, particularly as counts were always set up within 15 min. of dilution.

Table 3. *Viable counts on strain H37Rv after contact with distilled water or 0.1 % bovine albumin*

Time	Diluent	
	0.1 % bovine albumin	Distilled water
	log ₁₀ number of viable units/ml.	
0	*	7.81
15 min.	7.85	7.85
30 min.	7.87	7.82
2 hr.	7.97	7.83
4 hr.	7.94	7.84
8 hr.	7.96	7.85
24 hr.	7.97	7.80

* Contaminated.

Further use of the silica gel method

We have used the silica gel method for the past two years in over 3000 counts of cultures and of homogenates of animal tissue. The method has been convenient, accurate and associated with a contamination rate of less than 1 %. Comparisons of 7H-3 medium and the oleic acid medium used by Fenner (1951) have shown that counts were the same, within experimental error, but colonies were slightly larger in the 7H-3 medium.

DISCUSSION

Surface viable counts of *Mycobacterium tuberculosis* on plates suffer from four disadvantages: (1) The contamination rate is high, particularly in certain laboratories where fungal spores are prevalent. (2) Closely spaced colonies tend to coalesce and are difficult to count. This necessitates the use of closely spaced

dilution intervals and numerous replicates. Alternatively, counts can be read with a plate microscope when the colonies are small, but the counts then tend to be low. (3) Plates must be poured the day before or the same day as the counts are carried out. (4) The opening of plates to read the counts may be a source of laboratory infections. Contamination is greatly reduced by seeding small bottles of medium with single drops, but the accuracy of this method is diminished by the coalescence of colonies which cannot be differentiated by the use of a plate microscope.

The deep counts of *Mycobacterium tuberculosis* in silica gel medium have the following advantages. The contamination rate is low, and there is no coalescing of colonies so that accurate counts can be made on bottles containing large numbers of colonies and wide dilution intervals are possible. The medium can be prepared well in advance of its use and the bottles need not be opened for counting. The dilution of drugs or other substances carried over in the inoculum is accurately known. The two disadvantages are that deep colonies have a less characteristic colonial morphology than surface colonies and it is more difficult to subculture from deep colonies. However, these disadvantages are of no great importance in the majority of experiments.

Deep counts in silica gel medium were higher than those on plates. Malachite green was used in the 7H-3 medium plates, but not in the silica gel bottles because it has been recommended as a procedure to decrease contamination. Although it remains possible that this dye may have been responsible for the lower counts, it seems unlikely as Cohn *et al.* (1954) stated that the concentration used was not inhibitory. Furthermore, phenol red was used in plates and bottles in Kirschner medium and the methods-medium interaction in the analysis of variance in the second experiment was not significant. This suggests that the incorporation of Malachite green in the 7H-3 medium did not contribute to the lower counts on the plates.

Miles & Misra (1938) found that the χ^2 distribution of surface counts was an indication of the quality of the medium. The results of the analysis of variance in Expt. 2 on the size of the counts and the values of χ^2 suggest that 7H-3 medium is superior to Kirschner medium.

Although the sterile distilled water used in these experiments had no bactericidal activity over a period of 24 hr., this cannot be accepted as a generalization to other laboratories for, as Wilson (1935) has shown, the bactericidal activity of distilled water from different stills varies.

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