A Microculture Technique for Observing the Early Growth of Mycobacteria

BY W. H. H. JEBB AND A. H. TOMLINSON

Public Health Laboratory, Radcliffe Infirmary, Oxford

SUMMARY: A suspension of isolated cells of Mycobacterium tuberculosis was mixed with melted, watery agar and used to prepare thin films of agar gel suspended in \( \frac{1}{4} \) in. diameter wire loops. These films were incubated in culture media and at intervals of time sample films were transferred to glass slides, fixed, stained and 'the mean count per colony' determined. Some batches of agar inhibited growth, but this inhibition was reduced by extracting the powdered agar with methanol, and was abolished by adding serum, egg or charcoal to the medium. Strain H37Rv and 5 freshly isolated strains growing in a good medium all showed lag periods of 14–17 hr. and generation times of 18–18 hr. In unfavourable media the lag extended to 10–15 days.

When a microorganism grows as clumps of cells which do not disperse it is difficult to determine its rate of growth and the duration of the lag phase. The growth of tubercle bacilli has been assessed by estimating bacterial-nitrogen (Youmans, 1946; Sattler & Youmans, 1948), but the method can only be applied when the suspensions are relatively dense. Since the introduction, by Dubos, of media giving dispersed growth it has been possible to use photometric methods to measure the rate of multiplication of tubercle bacilli (see, for example, Schaefer, 1952, and Glover, 1952), but this technique can only be employed if a surface-active agent is present in the medium and so it cannot be used to investigate the effect of such substances on the rate of growth.

Kotani, Tsujimoto, Takeuchi & Seki (1958) cultured tubercle bacilli, dispersed as single cells, in thin films of blood clotted on a microscope slide and, after incubation, stained the organisms and estimated the mean number of cells in each micro-colony. They observed a lag period of 24 hr, followed by logarithmic growth with a generation time of 80–9 hr. As this direct counting of cells in a micro-colony offers an unequivocal, though tedious, assessment of growth, a microculture technique has been developed which can be used with any growth medium.

**Principle of the method**

Organisms are dispersed in thin films of agar suspended in wire loops and a number of these loops are then immersed in medium and incubated for various periods of time. After incubation the films are transferred to slides, stained and the mean number of bacteria per colony determined.
METHODS

Organisms. Mycobacterium tuberculosis (var. hominis). Strain H37Rv was used for most of the work, but some experiments were done with recently isolated strains. Stock cultures were kept on Löwenstein-Jensen medium and for use the organism was subcultured weekly in Dubos medium (Dubos & Middlebrook, 1947).

Preparation of suspensions of isolated cells. With only minor modifications, the method of Wells (1946) was used. A medium containing 1.0% peptone; 0.5% sodium chloride and 0.75% glycerol in distilled water, adjusted to pH 7.0, was distributed in 20 ml amounts in 100 ml conical flasks, each containing fifteen 4 mm glass beads. Flasks were inoculated with 1.0 ml of a 7-day culture in Dubos medium and incubated, static, at 37° for a week. The flasks were then mounted on a machine which held them with their axes at 45° to the horizontal and which rotated each flask about its own axis at about 100 r.p.m. After a further week’s incubation on the rotator, the cultures were filtered through Whatman’s no. 4 filter paper.

Cleared, watery agar. Six grams of New Zealand agar were dissolved, at 100°, in 250 ml of distilled water; the solution was cooled to 45° and cleared by adding 7.5 g of egg albumin dissolved in 50 ml of water, heating in a steamer to coagulate the albumin, and filtering through paper. The cleared agar was dispensed in 5 ml amounts in 1 oz ‘Universal’ bottles and autoclaved.

For the later experiments, the dry agar was extracted, before use, with methanol to remove inhibitors, as suggested by Ley & Mueller (1946). In an apparatus which stirred the powder, 40 g of agar were extracted with 2 l of circulating, freshly distilled methanol for 40 hr. The agar was filtered off, washed with methanol and dried under vacuum. Evaporation of the methanol filtrate left about 1.0 g of oily material.

Wire loops. Loops were prepared and mounted as follows: a twist was made from three strands of stainless steel suture wire, no. 40 S.W.G. This 3-ply wire was wound round a 3/4 in. former; the resulting circular loop was distorted into an ellipse; the free ends were cut off short and fused into the narrowed end of a piece of glass rod. The rod was pushed through a hole punched in the cap of a 1 oz. screw-capped bottle (Fig. 1). A few drops of water were placed in the bottle, the cap with the loop fitted was put on, and the whole autoclaved.

Preparation of films. A bottle of agar was melted, cooled to 46°, and to it were added 5 ml of the suspension of isolated cells, previously warmed to 46°. The loops were dipped into the mixture, withdrawn and gently rocked, to obtain an even thickness of film and then returned to their bottles and left

Fig. 1. A mounted wire loop suspended in a culture bottle.
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horizontal for an hour to allow the agar to set. The organisms were kept at 46°C for not more than 15–20 min.

Culture of organisms in agar films. The medium was dispensed in 10 ml. amounts in 1 oz. ‘Universal’ bottles. For each experiment a sufficient number of bottles was prepared to provide two cultures for each sampling time, together with two or three extra, because a film occasionally floated off its loop during incubation. A batch of agar films was prepared, each loop was transferred to a bottle of medium, incubated at 37°C and, after various periods of time, pairs of loops were removed from each medium being tested, and the films of agar mounted and stained.

Mounting and staining of films. The loop with its film of agar and a little adherent medium was pressed against a clean microscope slide and the film was gently detached with a glass needle. The slide was then flooded with absolute alcohol which was left to evaporate and when the film had dried it was stained for 1 hr. in a bath of carbol-fuchsin at 45°C; it was washed, decolorized in 5% sulphuric acid for 30 min. and, after a further wash, was lightly counterstained with methylene blue.

Counting of the cells in each bacterial unit. The slides were examined microscopically, using a 2 mm. oil-immersion objective and a ×10 eye-piece. The microscope was modified so that the fine-focus knob and the stage-traverse could be manipulated by the left hand, while the right hand was free to operate the switches of a series of electric tally-counters.

Two films were examined for every sampling time and from the counts of c. 100 colonies, in each film, the weighted arithmetic means of the number of cells per colony was computed—to be referred to as the ‘mean count per colony’.

Media. The basal medium of Dubos & Middlebrook (1947) was used alone and with various additions referred to below. Kirchner medium was prepared by the method of Vollum (1952).

RESULTS

Efficacy of technique for preparing suspensions of isolated cells

The fluid which came through Whatman no. 4 filter-paper was just faintly turbid and counts, performed by the dilution technique using Kirchner medium, were of the order of 10^7–10^8 viable cells/ml., as Wells (1946) had found. Counts performed on films prepared from 22 such suspensions showed that over 90% of the bacterial units were single cells and the mean count per unit ranged from 1.03 to 1.29 cells. From the proportion of single cell units remaining after several days' incubation it was apparent that over 95% of the cells were viable.

Precision of the mean count per colony. When agar films were incubated in media containing serum, cell division commenced after about 20 hr. and counts could be performed until the majority of colonies contained about 10 cells. When the colonies became bigger than this, the overlapping of cells made accurate counting impossible. A typical protocol is given in Table 1.
Since a different film and, of course, a different tube of medium, was used for each sampling time it was important to assess the reproducibility of growth in replicate preparations. Ten films were prepared from the same suspension, transferred to ten tubes of Dubos basal medium +10% horse serum, incubated for 48 hr. and the mean count per colony for each film was determined. The average was 8-08 cells/colony; the range was 2-88-3-50, and the standard error 0-20. In less favourable media the variation was greater.

Table 1. Specimen protocol: the growth of Mycobacterium tuberculosis H37Rv in films of unpurified agar suspended in Kirchner medium

<table>
<thead>
<tr>
<th>Time (hr.)</th>
<th>0</th>
<th>16</th>
<th>24</th>
<th>40</th>
<th>49</th>
<th>64</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of colonies</td>
<td>185</td>
<td>182</td>
<td>197</td>
<td>96</td>
<td>83</td>
<td>3</td>
<td>18</td>
<td>18</td>
<td>27</td>
<td>42</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>16</td>
<td>88</td>
<td>8</td>
</tr>
<tr>
<td>Mean count per colony</td>
<td>1-21</td>
<td>1-20</td>
<td>1-27</td>
<td>1-96</td>
<td>2-88</td>
<td>5-01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2 shows that in Dubos basal medium supplemented with 10% (v/v) horse serum, or 0-5% (w/v) bovine plasma albumin fraction V, Mycobacterium tuberculosis began to grow rapidly after a lag of about 24 hr. but that in the basal medium alone no growth occurred in 3 days. A long series of experiments showed that, in the unsupplemented medium, growth rarely commenced in under 5 days, sometimes occurred erratically between the tenth and twentieth days, sometimes there was no sign of growth after 80 days. It is remarkable that occasionally rapid growth started after a lag of 15 days.

As the erratic growth in unsupplemented medium might have been caused either by a deficiency in the medium or by an inhibitor in medium or agar, the following alternative supplements were tried, including starch and charcoal which are known to neutralize certain inhibitors in culture media (Pollock, 1949; Hirsch, 1954).

(a) Slurries of egg yolk and egg white. Eggs were hard-boiled, yolk and white were separated and each macerated with its own weight of water in a mechanical blender. The slurries were tested separately by adding 1.0 ml. of each to 9.0 ml. of basal medium before autoclaving.

(b) Lysed red blood cells. One millilitre of packed, thrice-washed, sheep red cells were lysed in 90 ml. of water, and 1.0 ml. of this was added to 9.0 ml. of basal medium.

(c) Starch. 1.0 ml. of a 1.0% (w/v) solution of starch was added to 9.0 ml. of basal medium.

(d) Charcoal. A 1.0% (w/v) suspension of Norit charcoal (Harrington) was autoclaved and 1.0 ml. added to each 9.0 ml. of basal medium.

(e) Crude mycobactin was used because Marks (1954) found that it stimulated the growth of tubercle bacilli in a serum-agar medium. By using the
first stage of the procedure described by Francis, Macturk, Madinaveitia & Snow (1958) a crude extract of *Mycobacterium phlei* was prepared and dispersed in Dubos basal medium.

![Graph](Image)

**Fig. 2.** The growth of *M. tuberculosis* H37Rv suspended in films of unpurified agar. ×—×, in Dubos basal medium; —○, in basal medium +10 % (v/v) horse serum; +—+, in basal medium +0-5 % (w/v) bovine plasma albumin fraction V.

The mycobactin had no effect, but the other five supplements promoted growth (Table 2) and the fact that starch and charcoal were effective suggested the presence of an inhibitor in the medium or the agar, rather than a nutritional deficiency.

**Table 2. The growth of Mycobacterium tuberculosis in films of unpurified agar suspended in Dubos basal medium plus various supplements**

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Lysed Serum</th>
<th>Egg yolk</th>
<th>Egg white red cells</th>
<th>Starch</th>
<th>Charcoal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation Mean count per colony no. (hr.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 48</td>
<td>1-11</td>
<td>5-15</td>
<td>6-61</td>
<td>3-58</td>
<td>—</td>
</tr>
<tr>
<td>2 48</td>
<td>1-12</td>
<td>6-88</td>
<td>—</td>
<td>—</td>
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<tr>
<td>3 96</td>
<td>1-16</td>
<td>—</td>
<td>—</td>
<td>6-09</td>
<td>—</td>
</tr>
<tr>
<td>4 96</td>
<td>1-20</td>
<td>7-5</td>
<td>—</td>
<td>—</td>
<td>5-00</td>
</tr>
</tbody>
</table>

Since tubercle bacilli will grow in liquid media without serum, provided the inoculum is not too small, and since agar is known to contain inhibitors of growth (Dubos & Middlebrook, 1947) it was thought that the agar might be responsible for the erratic growth in unsupplemented media. When agar of batch no. 1, which had been used for all the work so far, was extracted as described in Methods and used for the preparation of films, consistent growth occurred in the basal medium. This change from erratic to consistent growth.
showed that the agar contained an inhibitor similar to that found by Ley & Mueller (1946). When untreated agar of batch no. 2 was used, growth in the basal medium alone was less erratic than with agar of batch no. 1, but there was less improvement after extraction with methanol. It appeared, therefore, that the methanol-soluble material in the agar was not the only inhibitor present but it seemed worth while to use extracted agar for all subsequent experiments. When serum or bovine plasma albumin fraction V was present in the medium rapid growth occurred in all batches of agar tested, whether or not they had been extracted with methanol.

The lag period and rate of early growth

Films of purified agar, inoculated with bacilli, were incubated in basal medium, alone, and supplemented with 10% horse serum, or with the slurry of egg yolk. Figure 8 shows that in the basal medium after a lag period of 70 hr. logarithmic growth began with a generation time of 23 hr. There was little difference between the effects of the two supplements, and in each instance the lag period was about 14 hr. followed by growth with a generation time of 14.9 hr.

Strain H87 Rv, dispersed in agar films, was grown in three media: (a) the basal medium plus 10% (v/v) horse serum; (b) Dubos liquid medium (i.e. basal

![Graph](image-url)
medium plus bovine plasma albumin fraction V, glucose and Tween); and (c) Kirchner medium. The results (Fig. 4) showed that the medium had little effect on either the duration of the lag phase or the rate of growth.

It will be noticed that in Figs. 3 and 4 there are points for values of log $N = 0.90$ which lie to the right of the line and which appear to suggest a deceleration of growth, but this displacement is only a consequence of the counting technique. Until the mean count per colony exceeded 8 there were very few colonies of more than 10 cells, but later the large colonies could only be recorded as ‘10 or more cells’ and therefore the mean count per colony would rise asymptotically to 10. The technique gave accurate counts for about the first three generations. At later stages of growth useful information can be got by recording the proportion of the original cells which have grown to large colonies.

![Graph showing growth of M. tuberculosis H37Rv](image)

Fig. 4. The growth of *M. tuberculosis* H37Rv suspended in films of extracted agar. +——+ in Dubos basal medium +10 % (v/v) horse serum; O——O, in basal medium + 0.5 % (w/v) bovine plasma albumin fraction V, 0.5 % (w/v) glucose and 0.5 % (v/v) Tween 80; △——△, in Kirchner medium.

Freshly isolated strains. Four strains of *Mycobacterium tuberculosis* isolated from sputum and one strain from urine were grown by the agar film technique in the basal medium plus 10 % (v/v) horse serum. With each strain the lag period was 15–17 hr. and the generation times varied between 18 and 18 hr., but the rates of growth were not related to the lengths of time (8–7 weeks) which had elapsed before growth was visible in the primary culture.
DISCUSSION

To observe the growth of single cells seems to be the most satisfactory way of assessing the lag phase and early growth of an organism, such as a mycobacterium, which grows in clumps. Slide culture and the present technique have the advantage over the observation of single cells in a hanging-drop culture in that they facilitate the observing of many cells and the estimation of a mean value for the lag period and the generation time. The agar film technique has a further advantage over slide culture, especially for a pathogenic organism, in that the slide which is examined has not been contaminated by immersion in the culture fluid. The present technique is open to the criticism that it is not possible to be sure that agar is without effect on the growth of the bacteria, but since the growth in the presence of serum is at least as rapid as that observed by other workers, the agar does not appear to inhibit under these conditions.

The present estimate of the generation time, c. 15 hr., accords well with the 14.4 hr. given by Youmans & Youmans (1949) for growth in Proskauer and Beck's medium supplemented with 10% beef serum. Gutiérrez-Vázques (1956) reported generation times for Mycobacterium tuberculosis varying from 16 to 29 hr. depending on the medium and Whalen & Mallmann (1955) found 21 hr. to be the generation time on charcoal agar.

Given a knowledge of the rate at which tubercle bacilli can grow under favourable conditions it is interesting to speculate as to why primary cultures from pathological material frequently take so long to become visible. A single viable organism growing with a generation time of 15 hr. could produce $10^9$ cells in 3 weeks, or over $10^7$ cells if the generation time were 20 hr., so that, even if this rate of growth were not maintained for many days, visible colonies might be expected in 3 weeks or less, as in fact happens with over 50% of the specimens of tuberculous material submitted to routine culture. About 10% of routine isolations are not, however, apparent before 6 weeks so that these organisms must either grow more slowly on first isolation or else have a very long lag period. The observation that cells can begin to divide after a delay of as much as 15 days supports the suggestion that a long lag period can occur. In the present work, the addition of serum or egg abolished the long lag but it apparently does not do so in primary cultures.

REFERENCES


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