
The Determination of the Most Probable Numbers of Streptomycin-fast Cells in Brucella Cultures and their Variability in Growing and Ageing Cultures

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SUMMARY: The method of most probable numbers (MPN) was employed in order to determine the number of resistant brucella cells at concentrations of streptomycin of 1, 10 and 100 μg/ml. The examination showed agreement with the centrifugation method (plating of the sediment of at least 100 ml. of culture together with the required concentration of streptomycin) in a range up to 1000 resistant cells/100 ml. The method showed that there exist differences in the numbers of resistant cells in different strains, and that in cultures originating from the same colony variations occur. The general trend is a relative increase of the resistant cells with the age of the culture.

Methods for determining the number of streptomycin-resistant cells in bacterial cultures have been described by many authors. Klein & Kimmelman (1946) employed broth cultures of Shigella dysenteriae. Tenfold dilutions up to 10⁻⁴ were assayed in varying concentrations of streptomycin in melted agar, the test requiring at least 42 plates. In another experiment they distributed 40 ml. of broth culture into 400 broth tubes containing 1000 units streptomycin/ml. After 48 hr., growth appeared in 5 out of 400 tubes. Yegian & Vanderlinde (1948) incubated different sizes of inocula of Mycobacterium ranae on glycerol agar with varying quantities of streptomycin; one test required at least 120 plates. English & McCoy (1951) incubated 100 tubes containing 0.3 ml. broth culture of Micrococcus pyogenes and 200 units of streptomycin. One tube out of 100 showed visible growth after 24 hr. They estimated the number of bacteria incubated in 100 tubes at 67 × 10⁸ and concluded, therefore, that one resistant cell was present in this total bacterial population. All these methods require large quantities of tubes, agar and plates, and are time and material consuming. We tried, therefore, to simplify this technique by the determination of the most probable number (MPN) of streptomycin resistant micro-organisms in a culture. This method was proposed by McCrady (1915) for the numerical interpretation of fermentation tube results. For our purposes, the determination of small numbers of streptomycin resistant cells in a large bacterial population, we used the tables published by Hoskins (1934), reprinted in Standard Methods for the Examination of Water and Sewage (1936). We expected that with this relatively simple technique it would be possible to detect the presence of streptomycin resistant organisms in brucella cultures at different stages of their incubation.
METHODS AND RESULTS

Preliminary experiments were made to determine the action of streptomycin on the whole bacterial population, and the time of the first visible growth of resistant bacteria at different levels of streptomycin. The methods employed were as follows:

**Media.** The liquid media contained, per litre, 30·0 g. dehydrated trypticase soy broth (Baltimore Biological Laboratories), 30·0 ml. glycerol and 1·0 ml. of a solution of aneurin containing 0·1 mg./ml.

**Strains.** Two strains of *Brucella melitensis*, M5 and M7, two strains of *Br. suis*, S39 and S6, and two strains of *Br. abortus*, A19 and A2308, were used. After several subcultures on the above medium 24 hr. cultures were used for the experiments described below.

*Streptomycin.* Streptomycin sulphate (Squibb) was used in all experiments.

**Technique.** All experiments were carried out in test-tubes of 20 mm. diameter. The total volume of liquid medium containing the streptomycin and the bacterial inoculum was 10·0 ml. The wide test-tubes containing this relatively small quantity of fluid were thoroughly agitated and then incubated at 37°. Small samples of tube contents were taken at intervals, serial dilutions prepared (1/40, 1/1600, 1/64,000, 1/2,560,000) and 1·0 ml. of each dilution poured to agar plates. At those stages of the experiments where the bacterial count was low the samples were diluted less, and finally the undiluted contents of the tube were poured into agar plates. Fig. 1 represents the number of micro-organisms/ml. found at different intervals within 8 hr. in contact with 1·0 μg. streptomycin/ml. Of the six strains examined five tended to grow, while one strain (A2308) after an initial rise showed a definite decrease in the number of organisms, beginning at the fourth hour.

Fig. 2 shows the behaviour of the bacteria from the tenth hour after contact with 1·0 μg. streptomycin/ml. All strains show a gradual decrease of the
A. L. Olitzki

number of bacteria; two (M7 and A2308) were completely sterile after 24 hr.; the other four strains contained many living bacteria.

Fig. 3 shows the further behaviour of the four surviving strains. All of them showed on the following days in contact with streptomycin a steady increase of their bacterial count, strain M5 after a slight decrease continuing 2 days. This behaviour of the micro-organisms in the presence of streptomycin was modified under different conditions. With a greater inoculum the number of bacteria did not decrease below $10^6$/ml. and a S-shape curve resulted. When the concentration of streptomycin was 100μg./ml. the number of bacteria decreased after 4 hr. incubation.

![Fig. 2. The changes in bacterial count within 24 hr. in broth containing 1-0μg. streptomycin/ml.](image)

These experiments showed that the rate of disappearance of the non-resistant bacteria and the time of the initial growth of the resistant bacteria depend upon the concentration of streptomycin and upon the number of organisms in the inoculum. It was necessary to decide whether the bacteria resistant to a given concentration of streptomycin were resistant only to this concentration or whether they had acquired complete resistance. In an experiment carried out with three different inocula of strain M5 exposed to 1-0μg. streptomycin/ml. for 24 hr. the following results were obtained:

<table>
<thead>
<tr>
<th>Initial inocula/10 ml.</th>
<th>Surviving bacteria after 24 hr./10 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$6.5 \times 10^6$</td>
<td>$5.8 \times 10^8$</td>
</tr>
<tr>
<td>$1.3 \times 10^7$</td>
<td>$1.5 \times 10^9$</td>
</tr>
<tr>
<td>$2.6 \times 10^7$</td>
<td>$6.9 \times 10^8$</td>
</tr>
</tbody>
</table>

The figures show that the percentage of surviving bacteria increases with the increase of inoculum size. About 100 single colonies were picked and transferred to plain agar, and the resulting cultures transferred to agars
containing 1-0, 10-0 and 100-0 mg. streptomycin/ml. The results obtained with the surviving bacteria from the three inocula were very similar:

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total colonies tested</td>
<td>31</td>
<td>33</td>
<td>32</td>
</tr>
<tr>
<td>Growing on 1-0 mg./ml.</td>
<td>30</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>Growing on 10-0 and 100-0 mg./ml.</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Fig. 3. The changes in bacterial count 24–144 hr. in broth containing 1-0 mg. streptomycin/ml. Development of resistant micro-organisms.

This experiment showed that after 24 hr. the surviving bacteria were nearly all resistant to 1-0 mg. streptomycin/ml., but only about 8% were resistant to higher concentrations. All bacteria resistant to 10 mg./ml. were resistant to 100-0 mg./ml. Similar experiments were carried out with other strains exposed to different streptomycin concentrations. Most of the strains isolated at 1-0 mg./ml. were resistant only against this concentration. Of 284 strains isolated at 1-0 mg./ml. 17 were resistant against 10 or more mg./ml. When a strain tolerated the tenfold concentration then it also tolerated all higher concentrations up to 1000 mg./ml. On the other hand, all the strains
isolated from broth containing 10 and 100 μg. streptomycin/ml. tolerated all the higher concentrations up to 1000 μg./ml.

In these brucella cultures there appear to be present mainly two streptomycin-resistant variants: (1) variants partially resistant to 1 μg./ml. but not resistant to higher concentrations; (2) completely resistant variants resistant to 10 and 100 μg./ml. and higher concentrations. The following experiments were, therefore, carried out at 1-0, 10-0 and 100-0 μg./ml. to determine the MPN of the partially and completely resistant cells present in different cultures.

**Determination of the MPN of organisms resistant to 1-0 μg. streptomycin/ml.**

To determine the MPN, 24 hr. broth cultures were diluted in tenfold steps and from suitable dilutions 1 ml. volumes were added to tubes containing 9 ml. broth, and streptomycin added in the required concentration. After preliminary experiments to determine after a 5-day incubation period the range of inocula just sufficient to give resistant organisms, three inocula around this critical limit were selected and five broth tubes seeded with each inoculum. The inocula sizes differed by a factor of 10. The MPN was determined after incubation for 5 days for an inoculum ten times greater than the largest inoculum used in the experiment, by means of the tables of Hoskins (1934).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Size of inocula</th>
<th>Positive results per 5 portions of each inoculum</th>
<th>No. of organisms per resistant MPN cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>M5</td>
<td>1.8×10⁸ 1.8×10⁶ 1.8×10⁸</td>
<td>5 0 0</td>
<td>23/1.8×10⁸ 7.8×10⁶</td>
</tr>
<tr>
<td>M7</td>
<td>5.5×10⁸ 5.5×10⁸ 5.5×10⁸</td>
<td>1 0 0</td>
<td>2/3.5×10⁸ 1.8×10⁸</td>
</tr>
<tr>
<td>S39</td>
<td>1.2×10⁸ 1.2×10⁸ 1.2×10⁸</td>
<td>4 0 0</td>
<td>13/1.2×10⁸ 9.2×10⁸</td>
</tr>
<tr>
<td>S6</td>
<td>2.6×10⁸ 2.6×10⁸ 2.6×10⁸</td>
<td>5 0 0</td>
<td>23/2.6×10⁸ 1.1×10⁹</td>
</tr>
<tr>
<td>A19</td>
<td>1.3×10⁸ 1.3×10⁸ 1.3×10⁸</td>
<td>5 0 0</td>
<td>23/1.3×10⁸ 5.7×10⁸</td>
</tr>
<tr>
<td>A2808</td>
<td>3.2×10⁸ 3.2×10⁸ 3.2×10⁸</td>
<td>1 0 0</td>
<td>2/3.2×10⁸ 1.6×10⁸</td>
</tr>
</tbody>
</table>

Table 1 shows the results of a typical experiment. There exist marked differences between the other strains and strains M7 and A2808 which, in the experiment shown in Fig. 3, did not produce resistant organisms since the inoculum was too small. The results in Table 1 explain this previous result. The strains M7 and A2808 had only one resistant cell among at least 10⁸; all the other strains had resistant cells in much smaller bacterial populations. The experiment shows that it is possible to determine the MPN of organisms resistant to a certain concentration of streptomycin when the following conditions are fulfilled: (1) the largest inoculum must be great enough to enable the appearance of resistant variants in at least one of five tubes; (2) the smallest inoculum must be small enough to enable resistant variants to appear in less than five tubes. When resistant organisms appear in all five
Streptomycin-fast cells in brucella cultures

Table 2. Variation with time of the number of cells resistant to 1.0 μg. streptomycin/ml. in cultures originating from a single colony.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age of culture (days)</th>
<th>MPN</th>
<th>No. of organisms per resistant cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>M5</td>
<td>1</td>
<td>23/1.8 x 10⁶</td>
<td>7.8 x 10⁶</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>240/2.0 x 10⁶</td>
<td>8.3 x 10⁶</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>79/1.4 x 10⁶</td>
<td>1.8 x 10⁶</td>
</tr>
<tr>
<td>M7</td>
<td>1</td>
<td>2/3.5 x 10⁸</td>
<td>1.8 x 10⁸</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2/1.7 x 10⁸</td>
<td>8.5 x 10⁷</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>27/4.0 x 10⁸</td>
<td>1.5 x 10⁷</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>22/2.0 x 10⁸</td>
<td>9.1 x 10⁷</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>13/4.0 x 10⁸</td>
<td>3.1 x 10⁷</td>
</tr>
<tr>
<td>S39</td>
<td>1</td>
<td>13/1.2 x 10⁸</td>
<td>9.2 x 10⁷</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2/2.1 x 10⁷</td>
<td>1.1 x 10⁷</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>240/8.0 x 10⁴</td>
<td>3.8 x 10⁴</td>
</tr>
<tr>
<td>S6</td>
<td>1</td>
<td>23/2.6 x 10⁸</td>
<td>1.1 x 10⁷</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9/3.0 x 10⁷</td>
<td>2.4 x 10⁶</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>13/2.0 x 10⁷</td>
<td>1.6 x 10⁶</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>13/8.2 x 10⁷</td>
<td>6.6 x 10⁶</td>
</tr>
<tr>
<td>A19</td>
<td>1</td>
<td>23/1.3 x 10⁸</td>
<td>5.7 x 10⁸</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2/3.0 x 10⁸</td>
<td>1.1 x 10⁸</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>1600/6.0 x 10⁶</td>
<td>8.8 x 10⁶</td>
</tr>
<tr>
<td>A2308</td>
<td>1</td>
<td>2/3.2 x 10⁸</td>
<td>1.6 x 10⁸</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2/1.6 x 10⁸</td>
<td>8.0 x 10⁷</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>180/1.6 x 10⁶</td>
<td>9.0 x 10⁷</td>
</tr>
</tbody>
</table>

Table 2 shows that the MPN varies with age of culture. The general trend seems to be that in older more dissociated cultures the MPN of resistant variants increases. This increase of resistant cells was very marked in the cultures of the strains S39, A19 and A2308.

**Determination of the MPN of resistant cells at 10 and 100 μg. streptomycin/ml.**

Using different inoculum sizes at a constant concentration of streptomycin it was not possible to determine the minimal inoculum necessary to give resistant strains in streptomycin concentrations of 10 and 100 μg./ml. since resistant organisms appeared in many instances in smaller inocula, while relatively large inocula did not give resistant strains. A typical experiment is recorded in Table 3 which shows that, using only one tube for each inoculum, it is impossible to find a clear limit between inocula large enough and those too small to give rise to resistant variants. There exists, therefore, only the possibility of determining the MPN of resistant organisms in the total bacterial population. Since all the strains isolated at 10 μg./ml. were also resistant to all the higher concentrations all the following experiments were carried out at 100 μg./ml. To determine this number the method for the calculation of small numbers of coliform bacteria was used; 10-0, 1-0 and 0-1 ml. of 24 hr. broth cultures were inoculated into broth tubes containing...
Table 3. The appearance of resistant forms in cultures exposed to 10 and 100μg. streptomycin/ml. for 10 days at 37°

<table>
<thead>
<tr>
<th>Streptomycin (μg./ml.)</th>
<th>Strain</th>
<th>No. of organisms inoculated/10 ml. (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>10.0</td>
<td>M5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>S39</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>S6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A19</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A2308</td>
<td>0</td>
</tr>
<tr>
<td>100.0</td>
<td>M5</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>M7</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>S39</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>S6</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>A19</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>A2308</td>
<td>.</td>
</tr>
</tbody>
</table>

0 = no appearance of resistant organisms.
R = resistant organisms appeared.

100μg. streptomycin/ml. For the inoculation of 10.0 ml. of culture, tubes containing 10.0 ml. broth + 200μg. streptomycin/ml. were used in order to bring the final concentration to 100μg./ml. Five tubes were used for each size of inoculum. The results are summarized in Table 4 which shows that not all strains were equally able to produce completely resistant variants. Considerable quantitative differences existed and under the conditions of the experiment strain M5 was only once able to produce a resistant variant with an inoculum of 10.0 ml. Strain A19 also showed irregular behaviour; it produced resistant variants with an inoculum of 10.0 ml. in two tubes, but did not produce resistant variants in any of the 5 vol. of 10.0 ml. The ability of the strains M5 and A19 to produce resistant variants was therefore re-examined using larger volumes of broth cultures. Five volumes of 100 ml., 5 of 10 ml. and 5 of 1 ml. were used; concentration streptomycin 100μg./ml. To the 100 and

Table 4. The MPN of brucella cells resistant to 100μg. streptomycin/ml. determined for 100 ml. of 24 hr. broth cultures

Five replicate tubes were inoculated with varying amounts, to give three tenfold dilutions.

<table>
<thead>
<tr>
<th>Volume (ml.)</th>
<th>10.0</th>
<th>1.0</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>No. of positives in 5 portions</td>
<td>MPN (resistant cells/100 ml.)</td>
<td>Total no. of organisms/100 ml.</td>
</tr>
<tr>
<td>M5</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M7</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>S39</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S6</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>A19</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>A2308</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>
Streptomycin-fast cells in brucella cultures

10 ml. volumes equal volumes of broth + 200 \mu g. streptomycin/ml. were added to bring the final concentration to 100 \mu g./ml. This experiment is summarized in Table 5. It is interesting to note that the figure obtained in this experiment for the strain M5 was the same as obtained previously (cf. Table 4). The figure for A19 was about twice that obtained in the previous experiment. The MPN of cells resistant to 100 \mu g./ml. remained fairly constant at the different periods of incubation, although in this case also the general trend seemed to show an increase of resistant cells with the age of culture (Table 6).

Table 5. The most probable numbers of brucella cells resistant to 100 \mu g. streptomycin/ml. determined for 1000 ml. of broth cultures of strains M5 and A19

Five portions of each volume, 100, 10 and 1 ml., were seeded.

<table>
<thead>
<tr>
<th>Volume (ml.)</th>
<th>100-0</th>
<th>10-0</th>
<th>1-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>No. of positives resistant organisms/1000 ml.</td>
<td>Total no. of organisms/1000 ml.</td>
<td>No. of organisms/resistant cell</td>
</tr>
<tr>
<td>M5</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A19</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6. Variation in the number of cells resistant to 100 \mu g. streptomycin/ml. in cultures originating from a single colony

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age of culture (days)</th>
<th>MPN</th>
<th>No. of organisms/resistant cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>M7</td>
<td>3</td>
<td>4.0/6 x 10^{10}</td>
<td>1.5 x 10^{10}</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.0/4 x 10^{8}</td>
<td>4.0 x 10^{7}</td>
</tr>
<tr>
<td>A19</td>
<td>3</td>
<td>4.0/3 x 10^{10}</td>
<td>7.5 x 10^{9}</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>23.0/12 x 10^{8}</td>
<td>5.2 x 10^{8}</td>
</tr>
<tr>
<td>A2308</td>
<td>3</td>
<td>1.8/25 x 10^{8}</td>
<td>1.4 x 10^{10}</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>27.0/31 x 10^{8}</td>
<td>1.1 x 10^{10}</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.5/8 x 10^{7}</td>
<td>1.8 x 10^{7}</td>
</tr>
</tbody>
</table>

Agreement between the determination of the MPN with other methods

The figures obtained by the MPN method were compared with the results of direct observation of resistant cells when large quantities of culture were plated out in the presence of 100 \mu g. streptomycin/ml. The method was compared with the centrifugation method in which the whole bacterial growth from 100 ml. of culture was collected on a refrigerated centrifuge and poured in plates containing 100 \mu g. streptomycin/ml., and with a simple plating method in which only 2-0 ml. of culture were removed and plated with the same concentration of streptomycin. A comparison was made between the growth of a strain known to be resistant to 100 \mu g. streptomycin/ml., starting from an inoculum of about 5 cells/100 ml., and the growth of the resistant cells surviving from a 48 hr. non-resistant culture to which 50 % (v/v) of fresh broth and 100 \mu g. streptomycin/ml. were added.
Table 7 shows that the results of the MPN method agree with those obtained by the centrifugation method until the upper limit of 1600 cells/100 ml. of broth culture is reached. The direct plating method does not give reliable results so long as the number of resistant cells is lower than 100/100 ml. With

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time (hr)</th>
<th>2 ml. culture</th>
<th>the sediment of 100 ml. culture</th>
<th>Determination of MPN/100 ml. broth culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>S38</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>4 2 0 22</td>
</tr>
<tr>
<td>non-resistant</td>
<td>10</td>
<td>0</td>
<td>23</td>
<td>5 1 0 33</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0</td>
<td>26</td>
<td>5 2 0 40</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2</td>
<td>59</td>
<td>5 1 0 33</td>
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<td></td>
<td>22</td>
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<td>78</td>
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<td>278</td>
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</tr>
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<td></td>
<td>22</td>
<td>265</td>
<td>5,560</td>
<td>5 5 5 .</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>46,000*</td>
<td>960,000*</td>
<td>5 5 5 .</td>
</tr>
</tbody>
</table>

* By the aid of dilutions.

an increase of number of resistant organisms above 1000 the direct plating methods without or with suitable dilutions is the method of choice. The experiment presented in Table 7 also shows that in the non-resistant S39 strain, resistant bacteria were present in the culture at the time when streptomycin was added and that these bacteria multiplied slowly after a prolonged lag phase which lasted about 14 hr.

REFERENCES


(Received 13 August 1951)