Simple Method For Quantitation Of Viable Mycoplasmas

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Received/Accepted

A rapid, simple, and inexpensive method for quantitation of viable mycoplasmas is described. Serial dilutions were made in sterile microtiter plates with standard microtiter equipment. The results were multiplied by a factor of 2.38 to obtain colony-forming units comparable to those obtained with the laborious glass pipette-tube dilution method.

Because of the difficulty involved in obtaining viable counts of mycoplasmas, we suggest the use of a simple method for determining colony-forming units (CFU). There are many reports in the literature describing quantitation of mycoplasma growth by studying viable counts, opacity, hemagglutination, dry weight, pH change, protein production, glucose consumption, and cholesterol consumption (1, 2, 5, 7). Although these methods appear to be adequate for many studies, they are not suitable when a simple and exact quantitation of mycoplasma growth is required. We have found that quantitation of mycoplasma growth can be done with sterile microtiter plates and standard microtiter equipment. This dilution method is not only inexpensive, rapid, and simple, but also reproducible, as compared to the laborious glass pipette-tube dilution methods frequently used.

Mycoplasma pneumoniae was obtained from the American Type Culture Collection (strain 15293), and Mycoplasma salivarium was obtained from E. D. Brastins, University of Pittsburgh. These organisms were maintained in mycoplasma broth (PPLO broth [Difco Laboratories, Detroit, Mich.] supplemented with 20% horse serum [GIBCO Laboratories, Grand Island, N.Y.]), 10% yeast extract (GIBCO), 2% glucose, and 0.004% phenol red (3). Viable counts (colony-forming units [CFU]) were determined on a similar medium containing 1% agar (Difco). Twofold dilutions of the stock mycoplasma cultures were used in this study.

CFU were determined with microtiter dilutions of the mycoplasmas. These dilutions were performed in triplicate with standard microtiter equipment in sterile flat-bottomed wells (Micro-Test II; Falcon Plastics, Oxnard, Calif.) (6). The wells were charged with 0.15 ml of unsupplemented mycoplasma broth, and fourfold dilutions were carried out with 0.05-ml standard microtiter loops. The final dilutions in the wells were 1:4, 1:16, 1:64, 1:256, 1:1,024, 1:4,096, 1:16,384 and 1:65,536. Immediately after the dilutions were made, duplicate 0.001-ml samples were collected from the 1:16, 1:256, 1:4,096, and 1:65,536 dilution wells with a calibrated bacteriological loop and inoculated on the surface of mycoplasma agar with supplements (Fig. 1). The plates were prepared with 3 ml of mycoplasma agar in petri dishes (35 by 10 mm; Corning Glass Works, Corning, N.Y.) as described above.

For purposes of comparison, we conducted dilutions similar to those described for the microtiter system with glass pipettes and screw-capped glass tubes (13 by 100 mm). These dilutions were made by adding 0.2 ml of mycoplasma suspension to 0.6 ml of unsupplemented mycoplasma broth and making fourfold dilutions in a series of tubes that each contained 0.6 ml of mycoplasma broth. Samples of the same dilutions were taken from the microtiter wells and the tubes.

The M. pneumoniae cultures were incubated in a candle jar at 37°C for 5 days. The M. salivarium cultures were incubated anaerobically for 7 days in a GasPak jar (BBL Microbiology Systems, Cockeysville, Md.) in a carbon dioxide and hydrogen atmosphere (GasPak generator) at 37°C. After the appropriate incubation, the CFU were determined with an inverted microscope at a magnification of 40 X. Only the dilutions that had 10 to 50 colonies per inoculum were counted. The numbers of CFU per milliliter of the original mycoplasma suspensions were calculated with the following formula: CFU per milliliter = microtiter dilution × loop dilution × average CFU.

Differences in both the diameters of and the volumes in the dilution tubes and microtiter wells resulted in different fluid surface tensions. As a result, the calibrated loop pickup volume was decreased in the microtiter wells, whereas the pickup volume from the 13-by-100-mm tubes was approximately the volume of the loop rating. The platinum loop (American Scientific
Dilutions

\[ \frac{1}{65536} \]
\[ \frac{1}{16384} \]
\[ \frac{1}{4096} \]
\[ \frac{1}{1024} \]
\[ \frac{1}{256} \]
\[ \frac{1}{64} \]
\[ \frac{1}{16} \]
\[ \frac{1}{4} \]

CFU

mycoplasma agar medium

0.001 ml

FIG. 1. Mycoplasma dilution procedure done with a microtiter plate and a standard 0.001-ml platinum loop.

Products, McGraw Park, Ill.) loop pickup volume was measured by determining the weight of the water that it transferred to filter paper on the balance pan. This measurement was conducted to compare the volume delivered if the sample was taken from the tube with the volume delivered if the sample was taken from the microtiter well. It was found through a series of weighings on several days that the difference between the volume delivered by the calibrated loop from the 13-by-100-mm tubes was 2.38 times greater than the volume delivered by the same calibrated loop from the microtiter wells. The weight of the distilled water delivered by the 0.001-ml calibrated loop from the 13-by-100-mm tubes was 1.070 ± 0.073 mg. Therefore, to adjust the CFU obtained from the microtiter dilution method, it was necessary to multiply these values by 2.38.

\[ M. \ pneumoniae \] viable counts were observed to be greater with the glass pipette-tube dilution method than with the microtiter dilution method. However, when the microtiter counts were adjusted using the 2.38 factor, they were similar to those obtained with the glass pipette-tube dilution system. Similar results were obtained for \[ M. \ salivarium \]. The results obtained for both methods are shown in Table 1, which also shows the microtiter dilution values adjusted with the 2.38 correction factor.

Adhesion of mycoplasmas was not a factor in the different CFU counted. Kahane et al. (5) and Feldner et al. (1) have both reported no signifi-

<table>
<thead>
<tr>
<th>Mycoplasma stock dilution</th>
<th>CFU/ml</th>
<th>[ M. pneumoniae (\times 10^5) ]</th>
<th>[ M. salivarium (\times 10^5) ]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glass Pipette-tube</td>
<td>Microtiter</td>
<td>Adjusted microtiter</td>
</tr>
<tr>
<td>1:2</td>
<td>8.96 ± 0.51</td>
<td>3.34 ± 0.19</td>
<td>7.92 ± 0.45</td>
</tr>
<tr>
<td>1:4</td>
<td>5.01 ± 0.38</td>
<td>1.79 ± 0.13</td>
<td>4.24 ± 0.31</td>
</tr>
<tr>
<td>1:8</td>
<td>2.30 ± 0.27</td>
<td>0.92 ± 0.16</td>
<td>2.20 ± 0.38</td>
</tr>
</tbody>
</table>

\( ^a \) Mean ± standard error of eight readings.

\( ^b \) Dilution of mycoplasma seed before comparisons.

\( ^c \) Microtiter dilution corrected for platinum loop factor (2.38).
cant adhesion of mycoplasmas to either glass or polystyrene in the 15 min it took to set up these studies.

This microtiter dilution method of determining CFU of mycoplasmas appears to be applicable to all mycoplasma species. It is particularly helpful in determining CFU for those mycoplasma species, such as *Mycoplasma hominis* and *M. salivarium*, that do not ferment glucose (4) and, therefore, do not allow for pH change to indicate growth. The method is easy, rapid, inexpensive, accurate, and reproducible.

**LITERATURE CITED**


