NOTES

Estimation of *Mycoplasma pneumoniae* Inoculum Size by Rate of Tetrazolium Reduction

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The rate of appearance of red-colored formazan indicating reduction of 2,3,5-triphenyltetrazolium chloride by *Mycoplasma pneumoniae* was dependent on the inoculum size. The test permits estimation of *M. pneumoniae* cells in preparations that cannot be counted by dilution methods.

During studies on the adherence properties of *Mycoplasma pneumoniae*, it was necessary to evaluate the number of living cells attached to a given surface area. Conventional dilution methods for counting the colony-forming units (CFU) or color-changing units were not feasible for this purpose, since scraping the cells off the surface either was not possible or would not provide reliable results. But if radioactively labeled mycoplasmas were used, both living and dead organisms would be counted. Therefore, we investigated the possibility of using the rate of reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to red-colored formazan by *M. pneumoniae* (2, 4) for estimation of the number of viable cells present in a given volume of medium.

A filtered (1) and frozen (-70°C) suspension of *M. pneumoniae* FH containing a known number of CFU per milliliter was used as inoculum. The indicator medium (TTC medium) consisted of liquid medium (3) containing 1% glucose, 0.05% TTC (Serva), 0.05% thallium acetate, and 1,000 U of penicillin G per ml. After inoculation and incubation, 0.6-ml samples were collected from the TTC medium at intervals of 6 to 24 h and stored at -20°C . After thawing, they were centrifuged for 2 min at $7,000 \times g$ in an Eppendorf centrifuge. All samples of one individual experiment were read together at 495 nm with a photometer (Zeiss PM4). Uninoculated TTC medium was used as a blank. For practical purposes, the transmission (percentage) rather than the absorbance was used for measurements and plotting.

Coloration of TTC medium inoculated with *M. pneumoniae* developed in a typical sigmoidal curve (Fig. 1) with a sharp linear increase between 30 and 70%. The 50% value (corresponding to 0.3 absorbance) of this curve

as determined graphically was therefore chosen as a reference point for further experiments. Appearance of TTC reduction and inoculum size showed an approximate linear relation (Fig. 2), indicating a difference of 20 to 36 h per 10-fold increase of the inoculum. This relationship was found to be reliable between 10^7 and 5×10^2 CFU per ml. Differences in the number of organisms of 0.5 $\log_{10}$ unit could be evaluated. Below that limit, however, the results were not reproducible. There was also a relationship between the time of appearance of the 50% reduction value and the number of organisms present in the medium at this coloration stage. Table 1 shows that the TTC reduction occurred when the number of organisms approached 10^7 CFU per ml, regardless of the size of the original inoculum. The time at which the reaction occurred was influenced by the incubation temperature, but not by the culture volume.

The variance of the test system was examined by setting up 10 parallel cultures inoculated with 6×10^6 and 6×10^3 CFU per ml, respectively. The results (Table 2) showed that all cultures of the same inoculum reached their 50% value with a maximal variation of 12 h.

In a typical application of the method, the specimens (e.g., cover slip fragments) with attached *M. pneumoniae* organisms are placed in wells of a 16-mm multidish plates (Linbro), covered with 2 ml of TTC medium, and incubated at 37°C . At least three or four cover slip fragments for each specimen should be used to test each experimental and control condition. The wells are examined twice a day. As soon as a distinct pink color (about 70% transmission at 495 nm) appears, a first sample is removed from each well after gentle stirring and stored frozen, and the removal time is recorded. After an interval of 6 to 16 h, a second sample is taken

and both samples are measured. The 50% reduction value of each specimen is determined graphically, and mean values can be used for further calculations. Only two samples are needed for obtaining the 50% value if the first sample is taken only after appearance of red color and a second sample is withdrawn within the next 6 to 16 h. By this method normally one sample is below and one is above the 50% value. However, if a larger amount of TTC medium is used, more than two samples may be withdrawn. Additionally, at least three different

FIG. 1. Coloration of indicator medium by TTC reduction. Inoculum at day 0. Symbols: (●—●) 10^6 CFU/ml; (●- -●) 10^4 CFU/ml.

FIG. 2. Correlation between day at which 50% reduction value was obtained and inoculum size (four parallel specimens were examined for each concentration).

TABLE 1. Relation between appearance of TTC reduction and number of *M. pneumoniae*^a

Inoculum size (CFU per ml)	50% TTC reduction (days after inoculation)	Organisms present at time of TTC reduction (CFU per ml)
1×10^6	2.2	2×10^7
8×10^4	3.5	8×10^6
1×10^4	4.6	1×10^7
1×10^3	5.6	2×10^7
1×10^2	6.8	9×10^6

^a Cultures were shaken during incubation to reduce aggregation.

TABLE 2. Variance of TTC reduction time^a

Inoculum size (CFU per ml)	Appearance of TTC reduction ^b	
	Mean value	Extreme values
6×10^6	2.4 ± 0.12	2.3–2.8
6×10^3	4.9 ± 0.17	4.7–5.2

^a Ten parallel cultures for each inoculum size.

^b Fifty percent values, days after inoculation; mean values with standard deviation are given.

concentrations of the *M. pneumoniae* suspension used in the experiment should be incubated in TTC medium in separate wells. The number of organisms in this suspension should be evaluated in CFU or color-changing units by conventional methods. A reference curve (Fig. 2) can be drawn from these known *M. pneumoniae* concentrations. The times of 50% reduction obtained for the experimental specimens can be converted into CFU color-changing units by use of this curve.

Using the method described, the time of appearance of TTC reduction in a certain volume can provide information about the size of the original inoculum. This correlation can be used to evaluate the number of living *M. pneumoniae* organisms adhering to a certain surface area, e.g., glass cover slips. Additional controls and selective inhibition of the TTC reducing potential of animal cells are necessary if the mycoplasmas tested are adhering to animal tissue, e.g., tracheal rings. For cell suspensions, the method might be helpful if strong aggregation of the organisms limits the value of dilution methods. Within its limitations, the method appears reliable and could provide a useful tool for special experimental problems.

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