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Comparison of Two Methods For Enumeration of Mycoplasmas

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Two methods of viable cell counts were evaluated for *Mycoplasma hominis* and *Ureaplasma urealyticum*: color change unit$_{50}$ and colony-forming unit. The color change unit$_{50}$ method gave higher estimates of cell numbers; furthermore, the color change unit$_{50}$ values correlated better with the DNA content of the cell pellet and the published genome sizes.

Enumeration of mycoplasmas presents several difficulties. The small size of mycoplasmas (ca. 0.3 to 0.5 μm in diameter [14]) rules out total cell counts by direct light microscopy, whereas the need to prefix, wash, and concentrate the organisms precludes counting by electron microscopy. Such a small size together with relatively low populations (10^7 to 10^9 per ml) usually prevent light scattering or absorbance methods from detecting cell growth. For these reasons, populations are most often measured by viable cell counts. The method of determining colony-forming units (CFU) per ml is most commonly employed. However, Purcell et al. (8) used a series of 1:10 dilutions of cultures in broth with a metabolic indicator to estimate titers of *Ureaplasma urealyticum*. The dilution of the last tube to show growth was taken as the number of color change units (CCU). Good correlation between CCU and CFU was reported for both *U. urealyticum* (8) and *Mycoplasma hominis* (6). More recently, Ford (4) modified the CCU method to make a 50% endpoint determination, expressing the titer as CCU$_{50}$. This method has been used in at least two other laboratories (5, 11) but has not gained wide acceptance. We compared the results of the CFU and CCU$_{50}$ methods and related these to calculated and experimentally determined DNA content.

The sources of *U. urealyticum* T960 and *M. hominis* ATCC 14027 (11) and the preparation of bromothymol blue broth and genital mycoplasma agar (11) have already been described. Inocula of 1% (vol/vol) of late-logarithmic-phase cultures were introduced into large volumes of B broth in silicone-coated flasks. The inoculated cultures were incubated at 35°C. *M. hominis* cells were collected when the medium pH was between 6.9 and 7.0 and *U. urealyticum* at pH 7.1 to 7.2, i.e., at the end of exponential growth.

A sample of each culture was taken for population determinations by both the CFU and the CCU$_{50}$ methods (11). The basis of the CCU$_{50}$ is the establishment of a dilution of the culture that would inoculate 50% of the test wells. According to the Poisson distribution, this occurs at an average of 0.7 "hits" (or organisms) per well. (Assuming that a well that demonstrates metabolic activity [growth] has received at least one viable cell [one or more hits], a well not showing growth, therefore, has received no viable cells [zero hit]. If x is equal to the average number of viable cells per well, i.e., the average number of hits per well, then from Poisson distribution: P(o) class = $1 - 0.5 = e^{-x}$ and x = 0.6931 or ca. 0.7.) Therefore, all CCU$_{50}$ titers were corrected by multiplying by 0.7.

The remainder of each culture was collected by centrifugation at 20,000 × *g* for 20 min at 4°C. The supernatant fluids were removed, and each cell pellet was washed in sterile 0.85% (wt/vol) NaCl and then resedimented at 17,750 × *g* for 20 min at 4°C. After lysing the organisms in the washed cell pellets with 1% (wt/vol) sodium dodecyl sulfate, DNA was assayed by the modified diphenylamine method (10). The crude lysate was extracted with amyl acetate, and the absorbance of the extracted material was determined at 600 nm. Calf thymus DNA was used as a standard.

Table 1 shows the titers obtained with the two methods for establishing viable cell number. Since the CCU$_{50}$ method has certain similarities with most-probable-number determinations, data from replicate tests gave a skewed rather than a normal distribution curve. For such a distribution, the log mean is more appropriate than a simple arithmetic mean. By the CFU methods, values approximated a normal distribution, and the arithmetic and log mean values

TABLE 1. Determination of mycoplasma populations based upon CFU and CCU_{50} methods

Species	Trial	CFU			CCU_{50}			Probability[c]
		No. of replicates[a]	Log mean no. of organisms per ml of culture	SD[b]	No. of replicates[a]	Log mean no. of organisms per ml of culture	SD[b]	
U. urealyticum	1	10	7.53	0.064	1	8.04		<0.001
	2	10	7.47	0.039	10	7.95	0.31	0.001
	3	10	7.17	0.013	10	7.76	0.23	<0.001
M. hominis	4	10	8.95	0.062	10	9.35	0.23	<0.001
	5	10	9.03	0.018	9	9.20	0.18	0.059

[a] Number of independent determinations. Each CFU value was the mean of two counts. Each CCU_{50} value was the result of a single test.

[b] Standard deviation of the log mean counts.

[c] Analysis of data according to the *Statistical package for the social sciences* (7) was done at the University of Alberta Computing Centre. Probability was calculated by the *t* test as a separate variance estimate of the probability that CFU and CCU_{50} values were identical.

showed agreement. Although the CCU_{50} determinations showed greater standard deviation than the colony counts, the number of CCU_{50}s were higher. The *t* test indicated that this observation was significant for all three trials with *U. urealyticum* and at least one of the two trials with *M. hominis*. Least-square analysis of variance also supported this conclusion (data not shown).

To establish which estimate of the viable cell count was more valid, total cellular DNA of the washed cell pellet was calculated per CFU and per CCU_{50} and compared with the amount of DNA anticipated by the published genome size (Table 2). For both *U. urealyticum* and *M. hominis*, the value obtained by the CCU_{50} method gave better agreement than the estimate based upon CFUs. That is, a single CFU of either species represented enough DNA for four to seven genome equivalents, whereas a CCU_{50} represented between one and three genome equivalents. These calculations were made on the assumptions that all cells were collected by centrifugation. In fact, the supernatant fluids together contained about 0.5% of the CCU_{50} present of *M. hominis* or almost 16% of those of *U. urealyticum*. Despite sonication, these values might include cell clumps. Because these losses are minimal we did not correct the data.

TABLE 2. DNA content of the two species of mycoplasmas

Species	DNA ($g \times 10^{15}$)[a]		Genome equivalents[b]	
	CFU	CCU_{50}	CFU	CCU_{50}
U. urealyticum	2.8	0.88	3.5	1.1
	3.4	1.1	4.3	1.4
	5.7	1.5	7.1	1.9
M. hominis	3.1	1.5	3.7	1.8
	3.1	2.2	3.7	2.6

[a] All values represent the mean of two determinations. Column sequence represents the population sequence presented in Table 1.

[b] The molecular weight of the *U. urealyticum* genome was reported as 4.8 ± 10^8 (2); the molecular weight of the *M. hominis* genome has been reported as 5.1×10^8 (3) and 5.3×10^8 (13) for strain H39 and 4.5×10^8 (1) for strain PG21 (ATCC 23114). An average of 5.0×10^8 was used for further calculations. Genome equivalent, DNA (per viable unit) × [Avogadro's number/genome molecular weight].

Obviously, if each organism behaved as an independent unit and showed the same capacity for growth in liquid medium as on solid medium of similar composition, as in the present study, the number of CFU and CCU_{50} per ml should show substantial agreement. The present work agrees with earlier reports that broth is more sensitive than agar for the detection of both *M. hominis* (12) and *U. urealyticum* (11). Furthermore, an examination of logarithmic-phase cultures of *U. urealyticum* serotypes 1 through 8 showed that the CFU per ml of a given strain ranged from 7% to 71% of the CCU_{50} (J. Robertson, unpublished data) and, therefore, that the better growth in broth is not a phenomenon related only to strain T960. Although extra copies of the genome may be present in mycoplasma as in bacteria (9), the calculated values of between one and three copies calculated per CCU_{50} are closer to our expectations for a cell in late-logarithmic growth than the 4 to 7 copies per CFU. Since the amount of DNA in the cells of the two species is similar and compatible with the published values, a major error in the CCU_{50} determinations is unlikely. In our view, then, the use of the CFU procedure may underestimate the true numbers of both species and for *U.*

urealyticum, particularly, should be replaced by the more easily performed and more accurate CCU_{50} methodology.

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