A DILUTION PLATE COUNTING METHOD FOR CERTAIN STRAINS OF BACTERIUM TULARENSE[1]

T. L. SNYDER, F. B. ENGLEY, JR., R. A. PENFIELD, AND J. C. CREASY[2]

Camp Detrick, Frederick, Maryland

Received for publication May 10, 1946

Turbidimetry and titration of virulence for animals have constituted the only methods available for assaying cultures or suspensions of *Bacterium tularense*. Although these methods are generally recognized to be inadequate for the measurement of viable organisms, no dilution plate counting method has been developed for this organism. Larson (1945) has shown that the end points provided by growth from serial dilutions on glucose cystine blood agar (Francis, 1928) approximate those of mouse titrations, but this medium was not recommended for plate counts, nor have we obtained consistent results with it. Downs, Coriell, and Chapman (1946) have used successfully a surface plate counting technique employing a modification of Francis' medium in which cystine is replaced by cysteine. As a consequence of our finding that reducing agents markedly lower the minimum effective inoculum of certain strains (Snyder, Penfield, Engley, and Creasy, 1946), we have also been able to devise a plating medium which offers two advantages: it is easily prepared from common dehydrated constituents, and its transparency facilitates counting of colonies with the aid of the usual colony counters.

Broth is prepared with 2 per cent Difco bacto peptone, 1 per cent sodium chloride, 0.1 per cent glucose, and 0.1 per cent cysteine hydrochloride, and adjusted to pH 6.8 to 7.0 with sodium hydroxide. Two per cent bacto agar is added and the medium sterilized and the agar melted by autoclaving at 121 C for 15 minutes. It is dispensed into sterile 100-mm petri plates in thick layers (about 30 ml per plate). After solidification, the plates are opened, inverted, and dried for about 1 hour in a clean incubator at between 50 and 60 C.

At least three plates are inoculated each with 0.1 ml of appropriate serial tenfold dilutions of the test material in a diluting fluid consisting of 1 per cent sodium chloride and 1 per cent gelatin. The fluid is spread over the surface of the agar with a sterile, cane-shaped, glass spreader, which obviates the need for opening the plates more than slightly. Inoculated plates are allowed to stand for about 30 minutes; then they are inverted and incubated at 37 C. Colonies are counted after 3 days, at which time they are 1 mm or more in diameter.

The plates should be inoculated on the day of preparation, before excessive oxidation of the cysteine has occurred and before a few unavoidable contaminants have developed. Cysteine hydrochloride may be replaced by 0.01 per cent thioglycolic acid, but in this case 4 days of incubation are required. The conven-

[1] Studies conducted at Camp Detrick, Frederick, Maryland, between February and December, 1945.

[2] 1st Lt., SnC; 2nd Lt., SnC; T/4, WAC; and PhM3/c, USNR; respectively.

tional type of poured plate was not found satisfactory because of the extremely slow development of subsurface colonies.

The pleomorphism of *Bacterium tularense* and the presence of very small units make evaluation of the method by means of the direct microscopic count impracticable. We have used virulence titrations for this purpose, assuming an equivalence of infective and reproductive units. Forty-three comparisons were made with the highly virulent strain Schu, using 3 to 5 plates, and inoculating 6 mice intraperitoneally, with each appropriate dilution. The number of organisms per ml of original suspension was calculated from the plates in the usual manner, and from the animal titrations by the method of Stevens (Fisher and Yates, 1943). The ratios of organisms determined by plate count to organisms determined by animal titration were analyzed statistically, and gave a geometric mean ratio of 0.85 with a standard error of the logarithms of the ratios of $\pm 8.0648-10$. This mean ratio does not differ significantly from unity; hence the results provided by the two methods may be considered identical for practical purposes. Since clumps of organisms could not be observed microscopically, it is improbable that these two methods of enumeration would agree so closely unless both were dependent upon the individual organism as a unit. The standard error of the plate count, using 4 plates for each dilution, has averaged ± 9 per cent of the mean at a mean count of 150 colonies per plate.

This counting method was tested briefly with 27 other strains, but the results were not subjected to statistical analysis. Ten highly virulent strains and three strains of lowered virulence appeared very similar to strain Schu in the rate of development of colonies and in the magnitude of counts obtained with suspensions of equivalent turbidity. On the other hand, three highly virulent strains and eleven of lowered virulence either gave irregular and markedly lower counts, with considerable delay in the development of colonies, or else failed entirely to grow except with excessive inocula. The addition of blood failed to improve the results with these strains.

REFERENCES

DOWNS, C. M., CORIELL, L. L., AND CHAPMAN, S. 1946 Quantitative determinations of *Bacterium tularense* by surface plate counts. *In preparation.*

FISHER, R. A., AND YATES, F. 1943 Statistical tables for biological, agricultural and medical research. Oliver and Boyd, Ltd., Edinburgh and London.

FRANCIS, E. 1928 Symptoms, diagnosis and pathology of tularemia. J. Am. Med. Assoc., **91**, 1155–1161.

LARSON, C. L. 1945 The relative value of liquid media, glucose cystine blood agar, and mouse inoculation in the titration of *Pasteurella tularense*. U. S. Pub. Health Repts. **60**, 863–868.

SNYDER, T. L., PENFIELD, R. A., ENGLEY, F. B., JR., AND CREASY, J. C. 1946 Cultivation of *Bacterium tularense* in peptone media. *In preparation.*