JOURNAL OF BACTERIOLOGY, Jan. 1968, p. 259-261

Vol. 95, No. 1
Printed in U.S.A.

Method for Counting Rickettsiae and Chlamydiae in Purified Suspensions

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Received for publication 13 October 1967

Although rickettsiae and elementary bodies of chlamydiae can be resolved with good optical microscopes, their small size makes them difficult to see in unstained preparations. Hence, the direct counting techniques used with bacteria are not applicable. Several indirect counting techniques have been described, particularly for chlamydiae. They all have one main point in common, namely, that the count is obtained by determining the ratio between the organisms under study and reference particles such as erythrocytes, bacteria, or polystyrene spheres of known concentration (F. M. Gogolak, J. Infect. Diseases **92**:240, 1953, K. O. Smith and G. P. Manire, Proc. Soc. Exptl. Biol. Med. **100**:543, 1959, V. D. Neustroev et al., Vopr. Virusol. **4**:734, 1959). The most serious criticism of such methods is the definite possibility that organisms may be washed off in the staining procedure. Also, in the techniques involving smears, uneven distribution and margination of the organisms are variables which are not easily controlled.

The method described here, modified from that of R. C. Backus and R. C. Williams (J. Appl. Phys. **21**:11, 1950), overcomes the aforementioned objections and shows a high degree of reproducibility. A phage-free strain of *Shigella dysenteriae* was chosen as a reference organism. Stock cultures were maintained on Trypticase Soy Agar slants. A 10-ml tube of Trypticase Soy Broth (TSB) was inoculated from the stock slant and incubated for 18 to 24 hr at 37 C; 1 ml of this culture was transferred to 500 ml of TSB and incubated for 16 to 18 hr at 37 C. The resultant culture was centrifuged at 4,000 $\times$ *g* for 30 min, washed three times, and suspended in 0.1% Formalin-saline. The suspension was allowed to stand at 4 C for 24 to 48 hr, and was then tested for sterility. The bacterial concentration was determined by counting 6,000 to 10,000 cells in a Petroff-Hausser counting chamber. The suspension was adjusted to contain between 1.5×10^9 and 6×10^9 organisms per ml. Formalin-treated suspensions are stable and can be kept at 4 C for several weeks.

Strains of *Coxiella burnetii*, Nine Mile Phase I [307th guinea pig passage and 3rd egg passage (GP 307/EP 3)], Nine Mile Phase 2 (EP 88), and Henzerling Phase I (EP 22/GP 1/EP 3) were obtained as crude yolk sac suspensions in Formalin-saline from Richard A. Ormsbee of the Rocky Mountain Laboratory, Hamilton, Mont. The organisms were extracted from yolk sacs and purified with ECTEOLA according to a modification (P. Fiset and R. Silberman, Arch. Inst. Pasteur Tunis **43**:231, 1966) of the method of R. Silberman and P. Fiset (Nature **198**:699, 1963). In the final steps, the preparations were submitted to one or two ether extractions.

Equal volumes of the bacterial suspension and various dilutions of *C. burnetii* suspensions were mixed together, and an aqueous solution of acridine orange (1:1,000) was added to a final concentration of 1:20,000. After vigorous shaking, the mixture was pipetted into a DeVilbiss no. 40 nebulizer. Glass slides, thoroughly cleaned with an acetone-methanol (1:1, v/v) solution, were held approximately 6.0 cm from the orifice of the nebulizer. The preparation was sprayed evenly over the surface of the glass slide, avoiding confluency of droplets. Preheating the slides gently (about 50 to 60 C) insured rapid drying and fixation of the droplets.

The preparations were examined without further treatment in a Zeiss ultraviolet microscope, under oil immersion, with a dark-field condenser. Particles can be counted through a grid in the eye-piece, but this is extremely tedious. In the studies reported here, photomicrographs of 20 to 40 droplets of each dilution were taken on 35-mm Kodak Tri-X film and developed in Kodak D 76 developer under standard conditions. The negatives were projected onto white paper (20 by 29 cm). The organisms were checked off with a pencil and simultaneously counted with a hand tally. Figure 1 illustrates a typical droplet.

Two or three dilutions of each *C. burnetii* strain were made, and particle counts were obtained for each dilution. The cumulative results for each dilution are shown in Table 1. The χ^2 test as outlined by C. H. Goulden (*Methods of Statistical Analysis*, 2nd ed., John Wiley & Sons, Inc., New York, 1960) was applied to determine the homogeneity of the droplet suspension at the various dilutions. The *P* values obtained ranged from 0.90 to 0.9995. The composite *P* value for

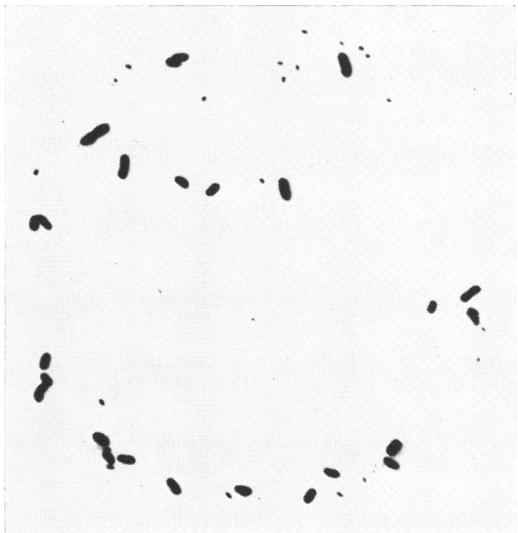

FIG. 1. *Typical droplet, as seen projected for particle counting, containing Shigella dysenteriae and Coxiella burnetii organisms.*

all three strains was 0.9995. It is evident from this type of analysis that there was a homogenous distribution of *C. burnetii* and bacteria in the sprayed droplets at the dilutions employed. The ratio of *C. burnetii* to bacterium was determined, and the concentration of the original suspension was calculated after correcting for dilution factors. The ratio of *C. burnetii* to bacterium varied from 0.906 to 2.147 in these experiments. Table 1 also shows the calculated number of *C. burnetii* per milligram (dry weight) of purified organisms. The Nine Mile strains were almost identical, whereas the value obtained for the Henzerling strain was slightly higher.

Similar results were obtained with purified suspensions of the 6BC strain of psittacosis agent (Table 2). Here the ratios ranged from 5 to 20 and the *P* values were all above 0.76, the composite *P* value being greater than 0.995.

The method shows a high degree of reproducibility, provided that the suspension of index bacteria is calibrated to contain between 1.5×10^9 and 6×10^9 organisms per ml and that the ratio

TABLE 1. *Particle counts and related chi-square values for various purified Coxiella burnetii preparations*

Strain of *C. burneti*	Dilution	No. of *C. burneti* counted	No. of bacteria counted	*C. burneti* per bacterium	No. of droplets counted	χ^2	Degrees of freedom	*P* value	No. of *C. burneti* per ml of original suspension[a]	Calculated no. of *C. burneti* per mg (dry weight) of purified material
Nine Mile, phase 1 (1 mg/ml)	1:5	2,345	1,092	2.147	39	25.58	38	.900	3.22×10^{10}	3.22×10^{10}
	1:10	463	451	1.027	19	8.96	18	.950	3.08×10^{10}	3.08×10^{10}
Nine Mile, phase 2 (1 mg/ml)	1:5	1,022	485	2.107	17	9.11	16	.900	3.16×10^{10}	3.16×10^{10}
	1:10	415	427	0.972	15	6.12	14	.950	2.92×10^{10}	2.92×10^{10}
Henzerling, phase 1 (1.32 mg/ml)	1:5	1,842	1,019	1.808	39	21.40	38	.975	5.42×10^{10}	4.11×10^{10}
	1:7.5	1,340	1,130	1.186	39	26.56	38	.900	5.34×10^{10}	4.05×10^{10}
	1:10	982	1,084	0.906	38	11.75	37	.9995	5.44×10^{10}	4.12×10^{10}

[a] Bacteria (3×10^9 per ml) mixed with equal volumes of Nine Mile dilutions; 6×10^9 bacteria per ml mixed with equal volumes of Henzerling dilutions.

TABLE 2. *Particle counts and related chi-square values for various dilutions of a purified suspension of psittacosis 6BC organisms*

Dilution of 6BC organisms	No. of bacteria counted	No. of psittacosis organisms counted	No. of psittacosis organisms per bacterium	No. of droplets counted	χ^2	Degrees of freedom	*P* value	No. of psittacosis organisms per ml of original suspension[a]
1:4	319	6,649	20.84	20	7.01	19	.994	1.25×10^{11}
1:8	365	3,699	10.13	20	11.64	19	.900	1.22×10^{11}
1:16	405	2,056	5.08	17	11.70	16	.760	1.22×10^{11}

[a] Bacteria (1.5×10^9 per ml) mixed with equal volumes of dilutions of psittacosis organisms.

of rickettsiae or chlamydiae is in the range of 1 to 20 per index organism. A higher ratio results in clumping, making the particle count difficult, whereas a lower ratio necessitates the counting of a larger number of droplets in order to obtain a significant sample. Staining the organisms in suspension and examining the sprayed material without further treatment prevents the loss of organisms likely to occur when slides are stained and washed. The use of optical microscopy instead of electron microscopy (R. C. Backus and R. C. Williams, J. Appl. Phys. **21**:11, 1950; T. T. Crocker, J. Immunol. **73**:1, 1954) favors the selection of larger droplets, allowing for a greater number of organisms to be counted. Lastly, the use of photographs makes it possible to count the particles easily and at any convenient time and also provides a permanent record.

This study was carried out under the auspices of The Commission on Rickettsial Diseases of the Armed Forces Epidemiological Board and received partial support from contract DA-49-193-MD-2277 with the United States Army Medical Research and Development Command.