JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1979, p. 437-440
0095-1137/79/03-0437/04$02.00/0

Vol. 9, No. 3

NOTES

Simple, Differential Staining Technique for Enumerating Rickettsiae in Yolk Sac, Tissue Culture Extracts, or Purified Suspensions

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Received for publication 30 November 1978

A differential staining method employing acridine orange-stained rickettsiae and safranin-stained standardized suspensions of *Shigella dysenteriae* is described for enumerating rickettsiae harvested during various stages of the growth cycle.

The simple procedure described by Silberman and Fiset (4) for counting rickettsial bodies in purified suspensions constituted an important technical advance towards quantitation in the field of rickettsiology. Briefly, the method consisted of mixing equal volumes of a purified rickettsial suspension of unknown concentration and a standardized *Shigella dysenteriae* suspension, adding acridine orange, and spraying the mixture with a nebulizer to form small droplets on a microscope slide. Photomicrographs of droplets containing fluorescing organisms were made through a fluorescence microscope. Rickettsial content was derived from the ratio between rickettsiae and the reference shigellae, the organisms being differentiated on the basis of size in the projected films. However, when the original method was applied in our laboratories to a variety of different preparations, e.g., crude yolk sac suspensions, typhus vaccines, and the different growth phases of rickettsiae grown in tissue culture (7, 8), certain problems were encountered. In response to the different problems, various modifications of the original method were explored, and some have been incorporated into our routine procedures. This note records some of the common, more troublesome problems, means by which these problems can be minimized or avoided, and a new procedure which routinely circumvents many of the problems and which can be applied readily by the inexperienced laboratory worker.

Problems encountered in the enumeration of rickettsiae and their solutions. Most of the problems encountered fell into three major categories, which are presented below along with means found to be useful for circumventing or preventing them.

(i) Artifacts caused by diluents. Brain heart infusion broth, a common diluent for rickettsial suspensions (6), was the source of small, circular orange-fluorescing structures, not removed by filtration through a 0.22-μm membrane filter (Millipore), which developed in the droplets and which were easily confused with the minute coccoidal forms of rickettsiae characteristic of late growth phases (7, 8). Serum, from tissue culture media or other diluents, often caused clumping of the reference shigella organisms. These problems were avoided simply either by excluding brain heart infusion broth and serum from the diluents in which the rickettsiae intended for counting were suspended or by removing them by centrifugation and resuspension in a compatible diluent such as 0.067 M phosphate-buffered saline (pH 7).

(ii) Interference by host cell components. When the original method was applied to purified rickettsial suspensions, the rickettsiae and reference organisms, fluorescing a bright orange, could readily be discriminated from the faint yellow-green background either visually or in photographs on black-and-white film. However, when crude unpurified or partially purified suspensions of yolk sac or tissue culture origin were subjected to this procedure, bright yellow-green fluorescing host cell material tended to mask the organisms. Increasing the acridine orange concentration (1:6,000, final concentration) and the staining time (24 to 72 h) improved the capacity to detect the organisms by visual observation but not by photomicrography on black and white film. Accordingly, we have abandoned the practice of photographing the droplets and count the organisms directly under the microscope, by using an eye piece fitted with a grid

reticule. Exposure of a droplet to ultraviolet (UV) light excitation under a UV microscope for about 30 to 45 s before counting caused a differential loss of host cell debris background fluorescence with respect to that of the microorganisms, thereby increasing the ease with which microorganisms could be counted and, in fact, unmasking some previously obscured by the fluorescing host cell material within which they were embedded. Finally, it was found that safranin would quench most of the background host cell-derived fluorescence. Although the differential "burning out" of background fluorescence may still find application under some special circumstances, the use of safranin in the new modification of the counting method described below has, in our hands, largely eliminated the host cell background fluorescence problem.

Occasionally, small round orange-fluorescing particles of presumed host cell origin, which can be confused with stationary-phase rickettsiae (7, 8) of similar appearance, may be encountered. No entirely satisfactory method has yet been devised to eliminate this problem.

(iii) Problems of differentiating between rickettsiae and reference organisms. Under many circumstances, the rickettsiae and the reference shigellae can easily be differentiated simply on the basis of size. However, this is not always the case. For example, when cultures of the reference organism are incubated too long, "minicells" of about the same size and morphology of stationary-phase rickettsiae are produced. This problem is simply circumvented by harvesting cultures of the reference organism in early log phase of growth.

The second problem, i.e., the fact that some rickettsiae in early log phase of growth may resemble closely the reference shigellae both in size and morphology (7, 8), was more difficult to solve. For example, use of organisms with different morphology, such as staphylococci, presented problems of clumping. Finally, with the use of a microscope (American Optical model 2071) under which a given droplet could readily be observed alternately under UV illumination in the fluorescence mode and under ordinary bright-field illumination, it was found possible and practical to count in the same droplet acridine orange-stained rickettsiae in the fluorescence mode and safranin-stained shigellae in the bright-field mode, with clear discrimination between the two. Moreover, as indicated above, the safranin effectively quenches most of the background fluorescence of host cell debris.

Comparison of the acridine orange single-staining and acridine orange-safranin double-staining techniques. The reference bacterium used in this study was a Formalin-treated suspension of *Shigella dysenteriae* (ATCC 11456a) harvested during the early exponential phase of growth (3) and standardized as previously described (4). The preparations we used contained 1.36×10^9 organisms per ml. Rickettsiae used in this study were as follows: (i) *Rickettsia prowazekii* Breinl, yolk sac suspension, purified on Renografin gradients (5); (ii) *R. prowazekii* Breinl, early logarithmic growth phase (30 h), homogenized tissue culture preparation; (iii) *R. prowazekii* Breinl, mid- to late-logarithmic growth phase (40 to 45 h), homogenized tissue culture preparation; (iv) *R. prowazekii* Breinl, stationary growth phase (72 h), homogenized tissue culture preparation; (v) *R. mooseri* (*typhi*) Wilmington, crude 10% yolk sac suspension; and (vi) *Coxiella burnetii,* Nine Mile Phase 1 strain, purified by ECTEOLA (1). Rickettsial preparations were adjusted to contain between 10^8 and 10^9 organisms per ml, by either diluting with 0.9% NaCl solution or concentrating by centrifugation, as appropriate. We have found that the optimal conditions for counting are: (i) that the concentration of the reference bacterial suspension be of the order of 10^9 per ml; (ii) that 1,000 to 1,500 bacterial cells be counted; and (iii) that the ratio of rickettsiae to bacteria be in the range of 1:5 to 2:1. All rickettsial suspensions were treated with Formalin to a final concentration of 0.5% for 3 days at 4°C to decrease the hazard created by aerosolization during spraying.

The modified original single-staining technique was carried out as follows. Equal volumes of the unknown rickettsial suspension and the reference shigella suspension were carefully measured and thoroughly mixed. Acridine orange 1:1,000 (aqueous solution in 5% ethanol) was added to a final concentration of 1:6,000 (0.4 ml added to 2.0 ml of mixture). After mixing, the suspension was held at room temperature in the dark for 24 to 72 h. The suspension was thoroughly mixed, and approximately 0.5 ml was transferred to a DeVilbiss nebulizer no. 40 or 42. The suspension was sprayed onto cleaned prewarmed (60°C) microscope slides held 6 to 10 cm from the orifice of the nebulizer. The slides were gently heat fixed and examined immediately by fluorescence microscopy. Whole droplets were examined, and 1,000 to 1,500 shigellae were counted. The ratio of rickettsiae to shigellae was determined, and the rickettsial count was calculated taking into account any dilution factor. For example: (750 rickettsiae/1,000 shigellae) $\times$ (1.36×10^9) $\times$ 20 (dilution factor) = 2.04×10^{10} rickettsiae per ml.

The new double-staining technique was car-

ried out as follows in a small test tube. To 1.0 ml of a rickettsial suspension was added 0.2 ml of acridine orange. In another tube, to 1.0 ml of the shigella suspension was added 0.1 ml of safranin solution as prepared for the Gram stain (2). Both tubes were tightly covered, shaken vigorously, and stored at room temperature in the dark for 24 to 72 h. After the incubation period, 0.1 ml of acridine orange was added to the shigella suspension, and the mixture was incubated for an additional 15 min at room temperature. At this point both preparations were of equal volume. The suspensions were thoroughly mixed using a Vortex mixer. An equal volume of each suspension was transferred to a clean tube, thoroughly mixed, poured into a nebulizer, and sprayed immediately as above. This last step (mixing and spraying) should be carried out very rapidly, preferably in 15 to 20 s; otherwise, the safranin from the bacterial suspension tends to quench the fluorescence of the rickettsiae. When examined under UV light, the rickettsiae appear as bright orange, strongly fluorescent, coccobacillary to bacillary forms, easily identified regardless of size, whereas the bacteria are purplish-red and just faintly fluorescent. When the same droplet is examined under bright light, the rickettsiae are barely visible, whereas the shigellae stand out as red organisms as in the Gram stain. The rickettsiae are counted under UV light and the shigellae under bright light, and the calculations are carried out as in the single-staining technique described above. Acridine orange staining of the shigellae may be omitted without adverse effect provided an equal volume of safranin (0.2 ml) is used instead. Under these conditions the shigellae are not visible with UV light.

Table 1 presents results of comparative counts carried out on six different preparations of rickettsiae utilizing both staining techniques. There was excellent correlation between both techniques for counting either purified suspensions or tissue culture extracts prepared during the later stages of the rickettsial growth cycle when the majority of *R. prowazekii* were relatively small compared to the reference organisms. Significant deviations in favor of the new technique, however, were encountered in preparations having large, early-logarithmic-phase *R. prowazekii* or minute, purified yolk sac-grown *C. burnetii*.

To evaluate the reproducibility of the double-staining technique on a particular suspension, six sets of counts were carried out independently by four investigators (*R. mooseri* suspension, Table 1) on four different days using both the acridine orange single-staining technique and the acridine orange-safranin double-staining technique. The average difference observed between the six paired counts for this suspension was 0.13×10^{10}. The paired t test was used to establish statistical significance, and a value of $P < 0.05$ was obtained.

The double-staining technique described here

TABLE 1. *Comparative enumeration of rickettsiae in chicken embryo yolk sac and tissue culture extracts using single staining with acridine orange and double staining with acridine orange and safranin*

Organism	Origin	Stain[a]	No. of shigellae	No. of rickettsiae	Ratio: Rickettsiae/shigellae	Rickettsial dilution factor	Rickettsial concn (per ml)
R. prowazekii Breinl	Yolk sac, purified, Renografin	A	1,045	803	0.768	1:20	2.09×10^{10}
		B	1,028	787	0.765	1:20	2.08×10^{10}
	Early-log-phase (30 h) TC[b]	A	2,010	110	0.055	None	0.75×10^{8}
		B	1,983	154	0.078	None	1.06×10^{8}
	Mid-late-log-phase (40–45 h) TC[b]	A	1,051	258	0.245	None	3.33×10^{8}
		B	1,019	242	0.237	None	3.22×10^{8}
	Stationary-phase (72 h) TC[b]	A	1,037	516	0.497	None	6.8×10^{8}
		B	1,005	516	0.510	None	6.9×10^{8}
R. mooseri Wilmington	Crude yolk sac	A	1,106[c] (6,641)	1,967[c] (11,802)	1.77	1:5	1.21×10^{10}
		B	1,017[c] (6,103)	1,995[c] (11,968)	1.96	1:5	1.33×10^{10}
C. burnetii Nine Mile Phase 1	Yolk sac, purified, ECTEOLA[d]	A	1,035	1,345	1.30	1:10	1.77×10^{10}
		B	1,000	1,750	1.75	1:10	2.38×10^{10}

[a] A, Acridine orange single stain; B, acridine orange-safranin double stain.
[b] Tissue culture.
[c] Mean of six counts by four individuals on different days. Parentheses indicate sum of these six counts.
[d] Reference 1.

has several advantages. (i) It permits easy identification and enumeration of rickettsiae in all stages of the rickettsial growth cycle. This is of particular importance during the early phase of growth, when rickettsiae are sometimes as large as the reference organisms. (ii) It allows differentiation between rickettsiae and shigellae when the former are closely associated with the latter. This was especially useful when counting very small rickettsiae such as *C. burnetii,* which were often not picked up by using the original single-staining method. (iii) It permits an accurate and reproducible means of enumerating purified rickettsiae, rickettsiae in crude yolk sac suspensions, or rickettsiae from tissue culture extracts, since the presence of safranin in the spraying mixture even for brief periods tended to suppress background fluorescence that was present in all but the purified suspensions. (iv) Finally, it is a simple procedure easily applied by the inexperienced worker.

We acknowledge the excellent technical assistance provided by Anna D. Waddell, Mary Lee Cremer, and Maybritt Doelp.

This study received financial support from contract no. DADA-17-71-6-0007 with the U.S. Army Medical Research and Development Command, Office of the Surgeon General, Department of the Army.

LITERATURE CITED

1. **Fiset, P., and R. Silberman.** 1966. Purification des rickettsies au moyen déchangeurs d'ions. Arch. Inst. Pasteur Tunis **43**:231-236.
2. **Hucker, G. J., and H. J. Conn.** 1927. Further studies on the methods of gram staining. N.Y. State Agr. Expt. Sta. Tech. Bull. no. 128.
3. **Ormsbee, R., M. Peacock, R. Gerloff, G. Tallent, and D. Wike.** 1978. Limits of rickettsial infectivity. Infect. Immun. **19**:239-245.
4. **Silberman, R., and P. Fiset.** 1968. Method for counting rickettsiae and chlamydiae in purified suspensions. J. Bacteriol. **95**:259-261.
5. **Weiss, E., J. C. Coolbaugh, and J. C. Williams.** 1975. Separation of viable *Rickettsia typhi* from yolk sac and L-cell host components by renografin density gradient centrifugation. Appl. Microbiol. **30**:456-463.
6. **Wike, D. A., R. A. Ormsbee, G. Tallent, and M. G. Peacock.** 1972. Effects of various suspending media on plaque formation by rickettsiae in tissue culture. Infect. Immun. **6**:550-556.
7. **Wisseman, C. L., Jr., and A. D. Waddell.** 1975. In vitro studies on rickettsia-host cell interactions: intracellular growth cycle of virulent and attenuated *Rickettsia prowazekii* in chicken embryo cells in slide chamber cultures. Infect. Immun. **11**:1391-1401.
8. **Wisseman, C. L., Jr., A. D. Waddell, and D. J. Silverman.** 1976. In vitro studies on rickettsia-host cell interactions: lag phase in intracellular growth cycle as a function of stage of growth of infecting *Rickettsia prowazeki,* with preliminary observations on inhibition of rickettsial uptake by host cell fragments. Infect. Immun. **13**:1749-1760.