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Plaque Assay for *Rickettsia rickettsii*

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A plaque technique for the assay of *Rickettsia rickettsii* is described. The method employs primary chick or green monkey kidney monolayer cell cultures with either an agarose or special Noble agar overlay. Plaques were counted in 6 days and resultant titers correlated well with LD_{50} end points obtained by a standard assay in embryonated eggs. Identification of the plaque-forming organisms was accomplished by direct observation of rickettsiae-like bodies in the monolayer lesions, inhibition of plaques by antibiotics, sensitivity of plaques to specific immune serum, and failure to cultivate other microorganisms from the infected cells. Versatility of the test was demonstrated by assaying samples of rickettsiae from several different sources commonly used in our laboratory. These included infected yolk sacs, various cell cultures, and infected guinea pig tissue. Sufficient numbers of viable rickettsiae were present in the cells of a single lesion to permit direct recovery.

Investigators working with rickettsiae have recognized a need for improved methods of assay for many years. The usual assay employing embryonated eggs has many attendant difficulties including high variability due to factors that are often difficult to control. The egg inoculation assay requires 10 or 11 days to obtain results and does not possess the advantages inherent in an enumeration procedure. Kordova (1) described a plaque technique that she applied to several species of rickettsiae but did not include *Rickettsia rickettsii*. She reported several serious limitations of the technique, including failure to obtain plaques in 16 of 22 attempts. The test required 14 to 17 days for completion, and the end points were 100-fold less sensitive than the egg-inoculation procedure. This report describes a plaque technique for *R. rickettsii* that is as sensitive as the yolk sac assay, takes much less time, and is highly reproducible.

MATERIALS AND METHODS

Organism. The organism under study was the Bitter Root (R1) strain of *R. rickettsii* in 68th passage level in chick embryo yolk sacs.

The working seeds were prepared by homogenizing the infected yolk sacs in a blendor. The homogenate was centrifuged at 6,000 rev/min for 15 min in a refrigerated centrifuge. The sediment was homogenized in a Ten Broeck grinder; after centrifugation at 1,500 rev/min, the supernatant fluid was removed and the sediment was discarded. The supernatant fluid was centrifuged at 6,000 rev/min for 15 min, and the sediment was suspended in sucrose phosphate buffer (2), dispensed in glass ampoules, and stored in a mechanical deep freeze until used. These seeds had titers of approximately $10^{7.5}$ to $10^{8.0}$ yolk sac 50% lethal doses per ml ($YSLD_{50}$/ml).

Additional sources of the organisms were obtained by infecting confluent monolayers of 24-hr primary chick fibroblasts, VERO cells (a green monkey kidney cell line), or HK cells (a hamster kidney cell line) with the working seed. Propagation of rickettsiae in these systems was initiated by (i) removing the cell attachment fluid [medium 199 (M199) containing 5% calf serum], (ii) applying 0.1 ml of working seed for 15 min at room temperature to the cells, and (iii) without removing the excess inoculum, adding 5 ml of M199 supplemented with 5% calf serum. The monolayers were then incubated in a closed system at 33.5 C until cytopathic changes were seen in 95% of cells observed under low-power magnification. The yield was harvested by suspending the cells in the supernatant fluid with the aid of glass beads and then subjecting the suspension to rapid passage through a 25-gauge needle three times. This procedure ruptured many cells and dispersed clumped cells and rickettsiae. Disruption of the cells by sonic cavitation offered no additional advantages. Rickettsiae from infected guinea pig testicles and tunica albuginea were released by first mincing the tissues with scissors and then homogenizing them, in M199 supplemented with 5% calf serum in a Ten Broeck grinder. Before the inoculum was used, the lipoidal material that collected on the surface was aspirated and discarded.

Plaque procedure. The diluent for all inocula was either cold Brain Heart Infusion (BHI) or M199 containing 5% CaS. The cell attachment fluid was removed from the 24-hr confluent chick primary monolayers, and 0.1 ml of the inoculum was applied to the cells for 15 min at room temperature with the bottles (30-ml plastic flasks; Falcon Plastics, Los Angeles, Calif.) tightly sealed. During this time, care was taken to insure that the inoculum contacted the entire sheet. Tilting the bottle at several angles immediately after

application of the inoculum dispersed the organisms. An agar overlay consisting of M199, 5% CaS, and either 0.5% agarose (special grade; Mann Research Laboratories, New York, N.Y.) or 0.15% special agar-Noble (Difco) was then applied.

When 100 ml of overlay was to be prepared, the proper amount of agar was melted in 10 ml of water at 10-lb pressure for 10 min. A 90-ml amount of medium at 56 C was added to the liquid agar which was then kept at 56 C and cooled to 40 C just prior to use. After the overlay had formed a gel over the cells, the closed bottles, without inverting, were incubated at 32 C for 6 days.

The plaques that formed were of sufficient contrast to be counted by using indirect illumination of the cell sheet while holding the bottle above a dark background. Although no stain was needed, improved contrast could be achieved by applying 1 ml of a 0.1% neutral red solution on the firm agar and permitting diffusion to the cells for 1 hr at 37 C. When semisolid agar was used, the overlay was first removed by inclining the bottle and sharply tapping the side. This caused liquefaction of the agar, which was then decanted. A 1-ml amount of neutral red solution was applied to the cells for 5 min at 37 C and the excess was decanted; after 30 min of further incubation at 37 C, the plaques could be observed without special illumination.

Isolation of rickettsiae from a single plaque was accomplished by either aspirating the cells with a curved Pasteur pipette or by removing them with a bacteriological loop. The cells were suspended in 0.2 ml of M199 with 5% calf serum and applied to another monolayer of chick fibroblasts.

RESULTS

Plaque morphology. Plaques formed by *R. rickettsii* in chick embryo (CE) monolayers after the 6th day of infection are shown in Fig. 1. The plaques were about 2 mm in diameter with relatively little size variation. Incubation beyond 6 days resulted in enlargement of the plaques to about 3 mm. Nonspecific deterioration of the entire monolayer occurred after the 8th day of incubation.

Plaques that developed under the semisolid (0.15%) special Noble agar (Fig. 1) frequently had central accumulations of cell debris which were visible macroscopically. Plaques in monolayers under firm (0.5%) agarose were opalescent, of poorer contrast, and showed no cellular accumulations in their centers. Recent experiments suggest that this observed difference in morphology is related to the kind of agar used rather than the difference in agar concentration. When CE monolayers were used, the plaques were sufficiently distinct to be counted without staining. Neutral red staining was used to improve the contrast for photographic purposes.

Plaque counts. The replicate plaque counts from the flasks similar to those shown in Fig. 1 are given Table 1. As expected, there was a direct correlation between the inoculum dilution and the plaque count. In a second experiment, twofold dilutions of a rickettsial suspension were assayed by the plaque method. The results (Fig. 2) show the direct proportionality between the plaque count and the relative concentration in the inoculum.

Plaque counts of several rickettsial suspensions prepared at different times were compared with the standard egg LD_{50} end points. Rickettsiae grown on three different substrates were used in these comparative tests. The plaque-forming units (PFU) and $YSLD_{50}$ end points were similar (Table 2), and the differences were not significant at the 5% level.

Effects of variables and procedural modifications. M199 containing 5% calf serum and BHI were compared as diluents for the plaque procedure on CE cells. Both diluents were shown to be equally suitable with two different rickettsial seeds (Table 3). The rickettsiae were

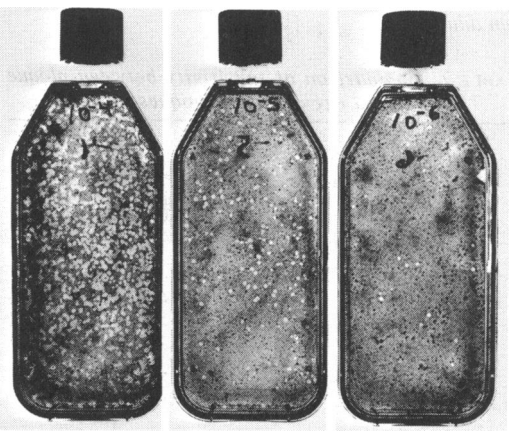

FIG. 1. *Plaques in chick primary cells due to Rickettsia rickettsii. The three 10-fold dilutions (10^{-4}, 10^{-5}, 10^{-6}) of a suspension contained $10^{8.4}$ $YSLD_{50}$/ml (log_{10}).*

TABLE 1. *Plaque counts of Rickettsia rickettsii on primary CE cell cultures*

Dilution of inoculum[a]	Plaque count/ml[b]	Suspension titer/ml
10^{-7}	2, 4, 1	2.3×10^{7}
10^{-6}	21, 20, 24	2.17×10^{7}
10^{-5}	164	1.64×10^{7}

[a] Rickettsial suspension grown in CE cell cultures containing $10^{7.5}$ $YSLD_{05}$/ml.

[b] Multiple values indicate replicate counts.

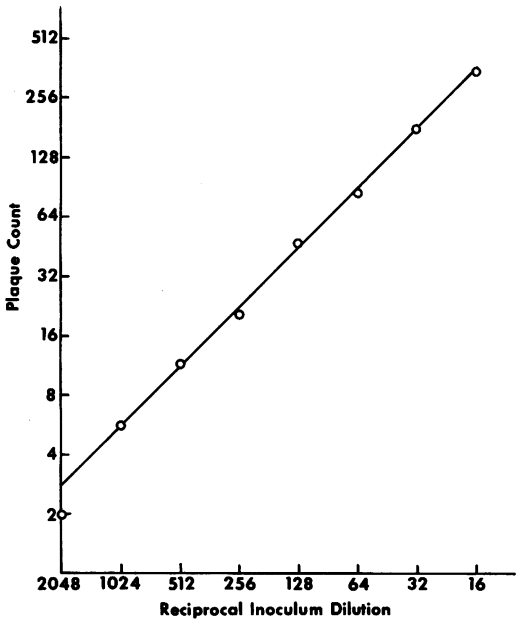

FIG. 2. *Relationship between plaque count and inoculum dilution.*

TABLE 2. *Comparison of sensitivity between plaque and egg assay techniques*

Source of rickettsiae	Lot no.	Titer (log_{10})[a]	
		Plaque (PFU/ml)	Egg ($YSLD_{50}$/ml)
L-cell cultures	1	4.4	4.1
	2	4.3	4.9
	3	5.1	5.4
	4	5.6	5.6
	5	5.4	5.4
	6	6.0	6.2
VERO cell cultures	1	4.7	5.0
	2	3.7	3.8
	3	4.8	4.9
	4	6.0	6.0
	5	5.9	6.0
	6	5.5	5.5
	7	5.3	5.5
	8	4.6	5.5
Yolk sac		7.8	7.6

[a] Mean difference was 0.18; standard deviation was 0.28.

stable for several hours at 4 C in both diluents. Rickettsiae stored at −60 C in skimmed milk could be readily assayed by the plaque method if sufficiently diluted in BHI, so that the opacity of the milk did not obscure the plaques.

Agarose (0.5%) overlays (5 and 10 ml) in the standard flasks were compared by using two seeds of *R. rickettsii*. The 10-ml overlay was apparently better; however, the difference was not statistically significant (Table 4).

In another experiment, plaques were produced under various concentrations of methylcellulose with 0.5% in M199 being optimal. The use of methylcellulose offered no advantage over the agar overlays and, because of the difficulty in handling, it was not used thereafter.

The incorporation of either 100 or 200 μg of diethylaminoethyl (DEAE) dextran per ml of 0.5% agarose or 0.5% special Noble agar overlay had no effect on either plaque size or number. The monolayers under the dextran overlays deteriorated after 6 days, which was 2 days earlier than controls without the dextran. The higher concentration of dextran affected the monolayer so severely that the plaques were difficult to differentiate.

The length of time the inoculum was allowed to contact the CE monolayers varied from 15 to 60 min. Adsorption for 15 min appeared adequate, because the plaque counts were approxi-

TABLE 3. *Comparison of diluents on the sensitivity of the plaque test*

Dilution of inoculum[a]	Seed I (counts of plaques)		Seed II (counts of plaques)	
	M199-CaS[b]	BHI	M199-CaS	BHI
10^{-5}	31; 25	47; 18	35; 56; 44	31; 25; 60
10^{-6}	5; 5	5; 3	5; 1; 3	1; 5
10^{-7}	1; 0	0; 0	1; 0; 0	0; 0; 0

[a] Suspension of *Rickettsia rickettsii* obtained from infected chick embryo cell monolayers.

[b] Medium 199 with 5% calf serum.

TABLE 4. *Effect of volume of overlay on plaque test sensitivity*[a]

Dilution of inoculum[b]	Sample A		Sample B	
	10 ml	5 ml	10 ml	5 ml
10^{-2}	TNTC[c]	TNTC	TNTC	TNTC
10^{-3}	56; 68	47; 51	40; 37	18; 32
10^{-4}	13; 7	3; 5	5; 8	3; 8
10^{-5}	1; 0	1; 0	1; 1	1; 0

[a] M199 plus 5% calf serum mixed with agarose to make an 0.5% agar overlay.

[b] Suspension of *R. rickettsii* grown in hamster kidney line cells.

[c] Too numerous to count.

mately equal for all three time periods tested (Table 5).

Attempts were made to produce plaques with *R. rickettsii* in monolayers of VERO, hamster kidney, L, and cat kidney cell lines. Only the VERO cells showed plaques. The plaques were equal in size and number of those observed on CE monolayers but were of poorer contrast. It was necessary to apply a neutral red stain to facilitate counting the plaques. The poor contrast was probably explained by the observation that there were many normal-appearing cells within the plaque boundary when examined under the microscope; the affected cells appeared rounded and granular.

Specificity of the plaques. Several observations provide substantial evidence that the plaques formed in the primary CE monolayers were a direct result of *R. rickettsii*. When cells were obtained from a plaque stained with carbol fuchsin and counterstained with malachite green, numerous intracytoplasmic rods consistent in morphology with *R. rickettsii* were seen. No such rods were seen within the cytoplasm of cells outside the plaque perimeter.

When 0.5 ml of specific immune serum (complement-fixation titer, 1:512) of Rhesus monkey origin was added to the overlay medium of each bottle, the plaque size was reduced to an average of 0.5 to 0.75 mm, and the plaque borders were no longer uniform and sharply demarcated. The plaque numbers, however, were not reduced.

Addition of a mixture of antibiotics (100 units of crystalline penicillin and 50 μg of streptomycin per ml) to the overlay reduced the plaque size to "pin points" in the three lowest inoculum dilutions tested and completely inhibited the plaque formation at the eight higher dilutions (Table 6). Chlortetracycline (0.5 μg/ml of agar) totally inhibited the formation of plaques at all inoculum dilutions.

Cell culture samples obtained from dense plaque areas were inoculated into BHI, thioglycolate broth, and a mycoplasma culture broth. The samples were also streaked on BHI-agar and mycoplasma plates. All cultures were incubated at two temperatures (32 and 37 C) and in aerobic conditions, anaerobic conditions, and under 5% CO_2 concentrations. All cultures failed to reveal the presence of other organisms. One milliliter of the inoculum used to form plaques was injected into guinea pigs intraperitoneally in a concentration of $10^{3.4}$ $YSLD_{50}$/ml diluted in isotonic saline. Additional portions of the sample were titrated in embryonated eggs

TABLE 6. *Influence of penicillin and streptomycin on plaque counts and morphology*

Dilution of a suspension containing $10^{3.4}$ $YSLD_{50}$/ml	Counts with antibiotics[a]	Counts without antibiotics
1:2	TNTC[b], PP[c]	TNTC
1:4	TNTC, PP	TNTC
1:8	5, PP	TNTC
1:16	0	367
1:32	0	190
1:64	0	98; 87
1:128	0	60; 45
1:256	0	25; 20
1:512	0	14; 11
1:1,024	0	7; 5
1:2,048	0	3; 1

[a] Potassium penicillin (100 units/ml) and streptomycin sulfate (50 μg/ml) were added to the overlay.
[b] Too numerous to count.
[c] Pin-point size plaques.

TABLE 5. *Effect of adsorption time on plaque assay sensitivity*

Dilution of inoculum[a]	Adsorption time[b]					
	15 min		30 min		60 min	
	Counts	Mean	Counts	Mean	Counts	Mean
10^{-5}	55; 50; 45 60; 35; 53	50	44; 35; 46 45; 57; 56	47	53; 52; 52 54; 39; 40	48
10^{-6}	3; 1; 6 4; 8	4.4	10; 5; 3; 5; 2	5.0	4; 1; 5 0; 1	2.2
10^{-7}	0; 1; 0 1; 0		2; 0 0; 0; 0		0; 1 0; 0; 0	

[a] Partially purified rickettsial suspension. Yolk sac source containing 7.4 ($\log_{10}$) $YSLD_{50}$/ml.
[b] Interval between application of 0.1-ml inoculum and addition of the overlay.

and by the plaque method on CE cells. The febrile and scrotal responses in guinea pigs and the mortality pattern in eggs were typical of reaction normally produced by *R. rickettsii*.

DISCUSSION

The usefulness of plaque assay techniques based on the ability of organisms to produce focal lesions in monolayer or suspensions of cells in agar has been well documented with many viruses. Plaque procedures are considerably more versatile as research tools than quantal assays based on the presence or absence of signs of infection or death in a host. Accordingly, the development of a plaque assay of *R. rickettsii* would facilitate research with this organism.

Examination of several variables and procedural modifications showed little or no effect on the numbers of plaques formed. M199 containing 5% calf serum and BHI gave similar results as rickettsial diluents. Ten-milliliter agar overlays produced counts that were slightly higher than those under 5-ml overlays, but the difference was not statistically significant. Plaques formed equally well under agar, agarose, and methylcellulose. The incorporation of 100 to 200 μg of DEAE dextran per ml of agar or agarose did not alter the size or number of developing plaques. Results from the experiments in which the adsorption time was varied indicated that rickettsiae attached to the cells within 15 min or that adsorption could take place after the overlay was applied.

Several experimental approaches were used to establish the specific relationship between the observed plaques and *R. rickettsii*. The drastic reduction of plaques by the incorporation of antibiotics into the overlay virtually eliminates the possibility that the lesions were caused by some unknown, contaminating virus. The failure to recover organisms from the inoculum by aerobic as well as anaerobic culture techniques tends to eliminate bacteria from consideration.

Because mycoplasma are frequent contaminants of embryonated eggs and cell cultures, are sensitive to many antibiotics, are sometimes difficult to isolate, and have been known to cause plaques in cell cultures (3), organisms of this group were more difficult to eliminate as agents responsible for the observed plaques. Evidence which suggests that the plaques were not caused by mycoplasma is as follows. (i) The plaques were produced by an organism which is highly sensitive to a combination of penicillin and streptomycin. Most known mycoplasma are not sensitive to either antibiotic and *R. rickettsii* is known to be sensitive to streptomycin. (ii) When samples from growth studies in cell culture were assayed by both the plaque test and by embryonated egg inoculation, the PFU and $YSLD_{50}$ end points were similar. If mycoplasma were responsible for the plaques, one would have to postulate that there is an uncommon virulence of mycoplasma for eggs that mimics the pathogenicity of *R. rickettsii*, or that growth of mycoplasma and rickettsiae in the cell cultures proceed at the same rate and to equivalent yields. (iii) When an inoculum that produced plaques was injected into guinea pigs, the animals showed typical signs of *R. rickettsii* infection leading to death. Further, a suspension of testicular tissue including the tunica albuginea taken at autopsy produced plaques and was lethal for eggs to the same titer. If the plaques had been produced by mycoplasma, one would also have to argue that the mycoplasma infected the guinea pigs and was found in the testicular tissue in the same concentration as the rickettsiae.

Additional evidence that suggested that the plaques were formed specifically by *R. rickettsii* was based on the presence of rickettsia-like bodies in the cytoplasm of stained cells within the perimeter of the plaque. Moreover, immune serum taken from a monkey infected with *R. rickettsii* caused a reduction in plaque sizes when incorporated into the overlay medium.

The results strongly suggest that the plaques observed in these experiments were formed by *R. rickettsii*. As an assay procedure, the plaque test is more economical, requires less time, has less variability, and is more versatile than the conventional assay procedure involving embryonated eggs. Recent studies in this laboratory have already adapted this technique to testing the organism's sensitivity to antibiotic discs. Recovery of the organism from single plaques makes it possible to perform genetic studies with *R. rickettsii* that were not previously possible. Experiments are under way to develop a neutralization test based on plaque inhibition.

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