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Plaque Assay for *Rickettsia mooseri* in Tissue Samples[1] (39499)

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The time-honored methods of animal or embryonated egg inoculation for quantitating the infectivity of rickettsial suspensions are cumbersome and time consuming and have inhibited the study of basic mechanisms in rickettsial diseases. However, the development of a plaque assay for rickettsiae by McDade (4) on the basis of Kordova's original brief report (3) and its refinement by Wike *et al.* (9, 10) have provided a sensitive and relatively simple procedure for the isolation and quantitation of these agents. The successful application of these techniques for the quantitative isolation of *Rickettsia rickettsii* from tick hemolymph and from guinea pig whole blood (8) showed this procedure to be useful when applied to study of rickettsial infections in animals. This paper describes the application of the plaque technique to the direct isolation of *Rickettsia mooseri* (*R. typhi*) from whole blood and other tissues of infected guinea pigs.

Materials and methods. Rickettsiae. A seed lot of *R. mooseri,* Wilmington strain, was prepared by homogenizing infected yolk sacs in sucrose phosphate glutamate (SPG) (1) solution to produce a 50% (w/v) yolk sac suspension. The homogenate was dispensed into glass ampoules which were flame sealed, quick-frozen in a dry ice-alcohol mixture, and stored at −70° until used. The rickettsiae employed for these studies, obtained from Dr. Richard A. Ormsbee of the Rocky Mountain Laboratory of the National Institutes of Health, had a passage history of 12 embryonated egg, 15 guinea pig, and 5 embryonated egg passages (12EP/15GP/5EP). A partial characterization of this seed is presented in Table I.

Quantitation of Rickettsiae. 1. Rickettsial particle counts. A modification (Fiset *et al.*, unpublished) of the method of Silberman and Fiset (6) was used to count rickettsiae in crude yolk sac suspensions.

2. Guinea pig intradermal ID_{50} titrations. Male Hartley guinea pigs of approximately 500 g (obtained from R. C. Roscrans, Hamilton, Montana) were used for all titrations. The stock egg seed was diluted with SPG and 0.1-ml intradermal (id) inoculations were employed for infection of guinea pigs. Tissue homogenates, diluted with 3.7% brain heart infusion (BHI) (Baltimore Biological Laboratories), were inoculated id in 0.2-ml amounts. Animals were bled before, and 28 days after, inoculation. Infection was determined by measuring serologic conversion using the microagglutination (MA) test (see below). All preinoculation samples displayed MA titers of ≤1:2. A titer of 1:4 or higher in the 28-day serum was considered positive and indicative of infection. The ID_{50} on the basis of serologic conversion was calculated by the method of Reed and Muench (5) and is expressed as 50% guinea pig intradermal infectious doses (GPI-DID_{50}).

3. Plaque assay technique. The primary chicken embryo (CE) fibroblast plaque assay technique employed was a modification of the procedures described by McDade (4) and Wike *et al.* (9, 10). Half-strength Dulbecco's modification of Eagle's minimal essential medium with Earle's salts (7) containing 0.1% glucose and 5% fetal calf serum was substituted for the M-199 medium and 60-mm plastic petri dishes (Falcon Plastics) were used instead of flasks. The petri dishes were incubated for 12 days at 32° in moist air containing 5% CO_2. No antibiotics were used. Tissue samples to be titrated by

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plaque assay were used undiluted (10% w/v suspensions in BHI, see below) and diluted 1:5, 1:10, 1:100, 1:1000 and 1:10,000 with BHI. Each dilution was plated in five petri dishes, 0.2 ml per dish. One hour, at room temperature with rocking every 15 min, was allowed for adsorption.

Serologic techniques. The microagglutination test (2) was used with ether-treated, "specific" particulate *R. mooseri* antigens at a concentration of 333 μg/ml. The diluent for antigen and for serum dilutions was 0.9% NaCl which contained 0.01% merthiolate and 1% nonimmune guinea pig serum.

Preparation of tissues. Whole blood and solid tissues were harvested aseptically. Solid tissues were washed free of loosely adherent materials and blood with chilled sterile physiological saline and homogenized in BHI to produce 10% (w/v) suspensions. With the exception of skin biopsies, homogenization was performed in an Omni-mixer (Sorvall). The Omni-mixer canister was immersed in a water–ice mixture and the unit was operated at maximal speed for two 1.5-min cycles separated by a 1.5-min cooling period. When skin was used for plaque assay, the area of interest was shaved, the animal was dipped in water containing 5% disinfectant (Osyl, National Laboratories), dried, treated with a depilatory agent (Nair, Carter Products), painted with 2% tincture of iodine solution, and rinsed with sterile water. Skin biopsies were obtained using an 8-mm circular cutaneous punch, the depth of the biopsy being determined by the cleavage plane between the subcutaneous fat and the subcutaneous musculature. Biopsies obtained in this manner were thoroughly disrupted in a Ten Broeck tissue grinder.

Suspensions which were not assayed immediately were shell frozen in rubber-stoppered vaccine bottles in a dry ice–alcohol mixture and were stored at −70°.

Results. Recovery of Rickettsiae from tissues of infected guinea pigs. Animals were sacrificed prior to infection and 3 and 10 days after intraperitoneal inoculation with 2.55×10^6 PFU of the *R. mooseri* seed. The tissue homogenates from these animals were assayed in the CE cell system. Homogenates from animals that had not been infected did not produce plaques in CE cell monolayers. However, after infection, plaques were readily obtained from selected tissues and the number of plaques varied with both tissue and time after infection (Table II). In addition to the results recorded in Table II, we have also been able to count rickettsial plaques from the lymph nodes, kidney, and skin following intradermal infection.

Comparison of plaque assay and GPI-DID$_{50}$. The sensitivity of the plaque assay for quantitating rickettsiae in tissues was compared to the GPIDID$_{50}$ as determined by serologic conversion. This was accomplished by simultaneous titration of samples in both CE cell cultures and in guinea pigs. The PFU was found to be a more sensitive measure of viable rickettsiae than the GPI-DID$_{50}$ (Table III). As Table III shows, the titer by plaque assay was in all cases higher

TABLE I. CHARACTERIZATION OF 12EP/15GP/5EP *R. mooseri* SEED

Character	Number per milliliter
Rickettsial particles	1.23×10^{10}
Plaque-forming units	2.55×10^{8}
Guinea pig ID$_{50}$	2.65×10^{8}

TABLE II. DETERMINATION OF RICKETTSIAL PLAQUE-FORMING UNITS IN VARIOUS GUINEA PIG TISSUES AFTER INTRAPERITONEAL INFECTION WITH 2.55×10^6 PFU OF *R. mooseri*

Tissue	PFU[a] on day after infection (days)		
	0	3	10
Spleen	$<2.50 \times 10^{2}$[b]	1.72×10^{4}	6.88×10^{3}
Myocardium	$<2.50 \times 10^{2}$	1.11×10^{3}	4.63×10^{2}
Fat pad	$<2.50 \times 10^{2}$	2.32×10^{5}	7.50×10^{2}
Whole blood	$<2.50 \times 10^{1}$	3.75×10^{2}	8.75×10^{2}

[a] PFU per gram of tissue (wet weight) or ml of blood.

[b] The minimal number of PFU per gram of tissue was 2.50×10^2 and the minimal number of PFU per ml of blood was 2.50×10^1 because of the dilution factor and the imposition of a limit of 25 PFU/ml as the minimal number which was considered significant.

TABLE III. SENSITIVITY OF PLAQUE ASSAY VERSUS GUINEA PIG INFECTIOUS DOSE FIFTY

Sample	Titer[a]		Ratio of PFU/ID_{50}
	PFU	ID_{50}	
Testicular fat pad	3.68×10^4	3.22×10^3	11.43
Spleen	6.88×10^2	3.22×10^1	21.37
Skin	2.68×10^2	1.65×10^2	1.62
Kidney	5.75×10^2	5.00×10^2	1.15

[a] Titers are expressed as the number of units per milliliter of undiluted tissue homogenate (10%, w/v, in 3.7% BHI).

TABLE IV. EXAMPLES OF INHIBITION OF PLAQUE FORMATION

Tissue	Dilution of sample	Number of PFU per Petri dish	PFU per gram of tissue or ml blood	Plaque recovery (percentage of maximum)
Skin biopsy[a]	Undiluted[a]	69.3	3,465	38.5
	1:5	25.0	6,250	69.4
	1:10	18.0	9,000	100.0
Kidney[a]	Undiluted	19.6	980	4.3
	1:5	69.6	17,400	85.7
	1:10	40.6	20,300	100.0
Whole blood	Undiluted	5.2	260	1.3
	1:10	40.3	20,000	100.0
Spleen[a]	Undiluted	80.0	4,000	13.2
	1:10	60.5	30,250	100.0

[a] Undiluted samples are 10% (w/v) homogenates of the respective tissues in 3.7% BHI.

than the titer arrived at through $GPIDID_{50}$ titrations. The relative sensitivity of the PFU to $GPIDID_{50}$ varied with the tissue sample and ranged from 21 PFU/$GPIDID_{50}$ when spleen was titrated to 1.2 PFU/$GPIDID_{50}$ when skin homogenates were used for titration.

Inhibition of plaque formation. In the course of titration of certain tissue homogenates, and in addition to the variation in PFU/$GPIDID_{50}$ with tissue noted above, a discrepancy was noted between the numbers of PFU recovered from concentrated and dilute samples. This is illustrated in Table IV, where it is shown that some undiluted tissue suspensions display fewer PFU per milliliter than the corresponding 1:10 dilution. The undiluted tissue suspensions seem to exert an inhibitory effect.

Bacterial and fungal contamination. Because antibiotics are not included in the medium employed for rickettsial plaque assay, contamination by bacteria and fungi is a potential problem. In addition to the contaminants randomly introduced during the plaquing procedure, certain tissues presented particular problems. Skin, for example, routinely showed bacterial contamination in low dilutions. Spleen and kidney homogenates, on the other hand, sporadically yielded a few fungal colonies. The contamination of skin homogenates with bacteria could at times interfere with the quantitation of rickettsiae, when low numbers of rickettsiae were present. With strict attention to aseptic technique, however, bacterial and fungal contamination was a relatively small problem in the more dilute samples.

Discussion. This study has shown that it is possible to obtain *R. mooseri* plaques directly from infected guinea pig tissue homogenates, that the number of plaques varies from tissue to tissue at a given time after infection, and that the number of plaques varies in a given tissue at different times after infection. These results show that the chicken embryo fibroblast plaque assay method is capable of detecting rickettsiae and enumerating the relative number of rickettsiae in guinea pig tissues. Because the plaquing efficiency of rickettsiae is less than 1 and because multiple factors may contribute to this inefficiency, the reliability of the method requires scrutiny.

A simple method has been devised for determining the absolute rickettsial body count of purified suspensions (2). However, no reliable method has yet been devised to quantitate the absolute concentration of viable rickettsiae. Instead, it has been necessary to quantitate viability in terms of infectivity for animals or cells, and infectivity values vary with the system employed. For example, the ratio between PFU titers in CE cells and the 50% guinea pig infectious titers as determined by serological conversion appears to vary with the source of the inoculum. Simultaneous titrations of *R. mooseri* from stock yolk sac seeds have shown the guinea pig infectivity titrations measured by serologic conversion to be as sensitive (see Table I) or more (10) sensitive than plaque titrations. However, the plaque titration is the more sensitive measure of rickettsiae in homogenates of guinea pig tissues (see Table III). In addition to this variability between systems, we found that recovery of rickettsiae from some tissues depends upon the dilution of the tissue homogenate. This suggests that tissue homogenates may contain a factor(s) that differentially inhibits plaque-forming capacity.

The presence of antirickettsial antibodies in the tissue homogenates could, possibly, be responsible for the guinea pig ID_{50} being less sensitive than the plaque titration when rickettsiae in tissue homogenates are being titrated. Antibody has been shown to be capable of reducing infectivity for mice (C. L. Wisseman, Jr., D. W. Krause, I. B. Fabrikant, and P. A. Mackowiak, manuscript) but does not inhibit plaque formation in the chicken embryo fibroblast plaque assay (12). Two observations suggest that the inhibitor of plaque formation observed in these experiments is not antibody: (1) The inhibition is lost upon dilution of the tissue suspension containing the rickettsiae; and (2) competition by host cell fragments, presumably membranes, has been shown by others in these laboratories to be capable of reducing plaque titers (12). The high (10%) tissue content of the homogenates suggests the presence of a high concentration of host cell membrane fragments. The influence of host cell membrane concentration on guinea pig infectivity is unknown. Other undiscovered inhibitory factors may also be present in crude animal tissue homogenates.

Because the number of plaques recovered from a given tissue varies with dilution, it is necessary to prepare the tissue homogenates in a standardized fashion. Others have demonstrated (11) that the removal of host cell membranes from suspensions containing rickettsiae removes the membrane-associated inhibitory effect. Indeed, in some preliminary experiments in the present study (data not shown), mixing 10% normal guinea pig kidney homogenate with a rickettsial suspension reduced the number of recoverable plaques. Low speed centrifugation, which undoubtedly contained host cell membrane fragments, retained some inhibitory activity. This suggests that the development of techniques to remove guinea pig cell components selectively from the suspensions containing the rickettsiae would result in an increase in the efficiency of the plaque assay for rickettsiae from these tissues.

The observed increase in recovered PFU with dilution of tissue homogenate suggests that the effect of inhibitory factors in the homogenates decreases with reduction in concentration more rapidly than the capacity of rickettsiae to form plaques. From a practical point of view, therefore, the highest dilution of a standardized tissue homogenate that yields plaques should be chosen to calculate the relative plaque titer of the tissue.

The variation between tissues as to capacity for inhibition of plaque formation (see Table III) makes comparison of relative number of PFU in one organ as compared to another of questionable validity. However, under standardized conditions, the comparison of the number of PFU recovered from an organ of one animal as compared to the number of PFU recovered from the same organ of similarly infected animals has been shown to be a highly reproducible value (J. R. Murphy, C. L. Wisseman, Jr. and P. Fiset, in preparation).

Summary. A plaque assay using primary CE fibroblasts was shown to be sensitive for the direct isolation and quantitation of the relative number of *R. mooseri* from infected guinea pig tissues.

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