

Plaque Assay for Q Fever and Scrub Typhus Rickettsiae

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The plaque assay procedure developed for spotted fever and typhus group rickettsiae is also appropriate for scrub typhus and Q fever rickettsiae. The plaque titers of suspensions of *Rickettsia tsutsugamushi* and *Coxiella burnetii* compared favorably with end points obtained by titrations in mice.

The rickettsiae have always been difficult to study because convenient titration and isolation techniques have not been available for the study of these organisms. In recent reports (3, 5), however, we demonstrated the use of a sensitive plaque test for measuring concentrations of typhus and spotted fever rickettsiae. Studies were then initiated to determine whether this assay system is appropriate for other rickettsiae. This report shows that this same plaque assay procedure has been successfully employed with *Coxiella burnetii* and *Rickettsia tsutsugamushi*, Q fever and scrub typhus rickettsiae.

MATERIALS AND METHODS

Organisms. The AD strain of *C. burnetii* used in this study was originally isolated from milk of an infected cow (2). Working seed consisted of 10% yolk sac suspensions, prepared in Brain Heart Infusion broth (BHIB), of the 10th yolk sac passage of this strain.

Several vials each of the Karp, Gilliam, and Kato strains of *R. tsutsugamushi* [20% yolk sac suspension in SP-G buffer (1)] were generously supplied by Bennett L. Elisberg, Walter Reed Army Institute of Research, Washington, D.C. These strains were in their 47th, 136th, and 89th yolk sac passages, respectively. To expand the volume of scrub typhus material, one vial of each strain was thawed, diluted 10-fold in sucrose phosphate (SP 25) buffer (6), dispensed in ampoules, and stored at -65 C. This diluted and refrozen material was used exclusively for plaquing experiments and was not used in animal tests.

Plaque assay. The plaque assay procedure used in this study was the same as that described in more detail in an earlier report (5). Twenty-four-hour chick embryo primary monolayers in 30-ml plastic tissue-culture bottles were infected at room temperature for 15 min with 0.1 ml of serial 10-fold dilutions (in BHIB) of each organism. The infected monolayers

were then covered with 5 ml of an overlay of medium 199 (5% calf serum) containing agarose at a final concentration of 0.5%. All of the infected monolayers were incubated in a closed system at 32 C. Monolayers infected with *C. burnetii* required 8 to 10 days of incubation before plaques appeared, but up to 17 days of incubation time was needed for cultures infected with scrub typhus rickettsiae. When the longer incubation period was needed, 3 ml of a second overlay was placed over the initial overlay after 10 days, and incubation at 32 C was continued for an additional 7 days. Plaques were stained by an overlay containing neutral red as described earlier (3). Each dilution was assayed in triplicate, and values were averaged. Plaque titers of three ampoules from each seed pool were determined in parallel with the animal ID₅₀ tests.

LD₅₀ and ID₅₀ determinations. Plaque titers of the various pools were compared with the animal 50% lethal dose (LD₅₀), or 50% infectious doses (ID₅₀), or both. Scrub typhus LD₅₀ values were determined by intraperitoneal (ip) inoculation of groups of 10 16- to 18-g male Swiss mice (Ft. Detrick strain) with 0.2 ml of serial 10-fold dilutions, prepared in SP 25 buffer, of the original seed materials. Deaths were recorded daily for 25 days. ID₅₀ determinations were made by challenging the survivors on the 26th day with 1,000 LD₅₀ units (0.2 ml by the ip route) of the Karp strain. LD₅₀ and ID₅₀ values were calculated by the Reed-Muench method.

The ID₅₀ for Q fever rickettsiae (4) was determined by ip inoculation of eight 16- to 18-g male Swiss mice with 0.1 ml of serial 10-fold dilutions (in BHIB) of *C. burnetii*, ranging from 10⁻⁶ through 10⁻¹¹. Twenty-two days later the animals were bled, and the complement-fixation titers of the sera were determined by using commercially prepared antigen (Lederle Nine-Mile strain of *C. burnetii*). Animals whose sera had CF titers of 1:8 or greater were considered infected. The ID₅₀ was computed by the Spearman-Kärber method. Mouse and guinea pig ID₅₀ end points are approximately equivalent (4).

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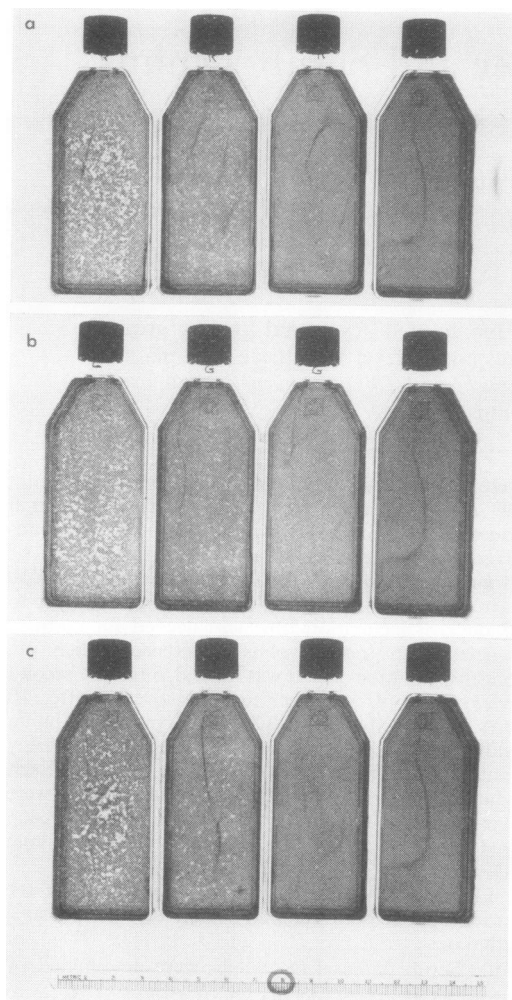


FIG. 1. Plaques formed by various strains of *Rickettsia tsutsugamushi* (17th day). (a) Left to right, 10^{-4} , 10^{-5} , and 10^{-6} dilutions of the Karp strain. At the far right is an uninfected control. (b) Left to right, 10^{-4} , 10^{-5} , and 10^{-6} dilutions of the Gilliam strain. At the far right is an uninfected control. (c) Left to right, 10^{-3} , 10^{-4} , and 10^{-5} dilutions of the Kato strain. At the far right is an uninfected control.

RESULTS

The plaque morphology of the three strains of *R. tsutsugamushi* is shown in Fig. 1. Each strain produced small (1 mm), somewhat indistinct plaques that were quite similar morphologically. Plaques seldom appeared before 11 days, and usually an additional 5 to 6 days of incubation was needed before the plaques were distinct enough to be counted. Plaques formed by the AD strain of *C. burnetii* are shown in Fig. 2.

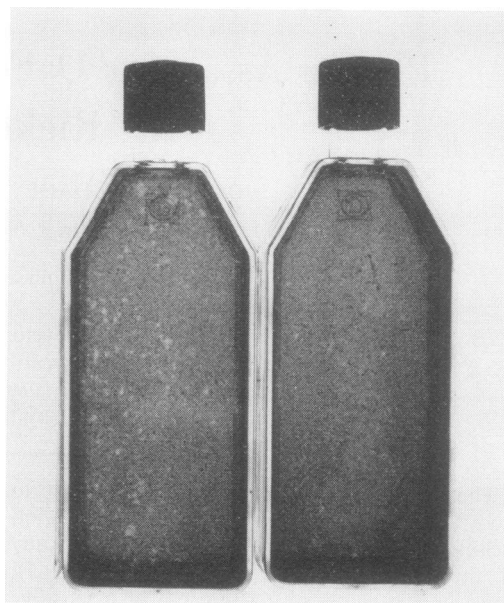


FIG. 2. Plaques of *Coxiella burnetii* (11th day), 10^{-6} and 10^{-7} dilutions.

TABLE 1. Comparison of the plaque titers of *Q* fever and scrub typhus with the LD_{50} and ID_{50} titers in Swiss mice

Organism	Titers (log 10/ml)		
	LD_{50}	ID_{50}	Plaques ^a
<i>R. tsutsugamushi</i> Karp strain	5.98	6.03	7.54
<i>R. tsutsugamushi</i> Gilliam strain	5.20	5.70	7.39
<i>R. tsutsugamushi</i> Kato strain	5.20	5.26	5.97
<i>C. burnetii</i> AD strain	ND ^b	9.75	8.4

^a Average of three repetitions with replicate test of each dilution.

^b Not done.

These plaques were also small (1 mm), but plaques were easily enumerated after 8 to 10 days of incubation. It was not possible to differentiate these organisms by plaque morphology.

A comparison of the ID_{50} titers with plaque titers is shown in Table 1. These data suggest that the plaque assay for scrub typhus rickettsiae was more sensitive than our mouse ID_{50} titration procedure. With two of the three strains (Karp and Gilliam), the plaque titer was more than one \log_{10} higher than the corresponding ID_{50} value. The plaque assay of *Q* fever rickettsiae, however,

appeared to be less sensitive than the serological ID₅₀ method. With *C. burnetii*, the ID₅₀ value was at least one log₁₀ higher than the plaque titer.

There was less than one-tenth log₁₀ difference among the plaque titers of the individual ampoules.

DISCUSSION

Results of these and previous tests (3, 5) indicate that the plaque assay system is a sensitive titration procedure for rickettsiae. We have been successful in obtaining plaques with representative species of four groups of these organisms. Moreover, we have not found a rickettsia that we have not been able to plaque. *C. burnetii* is the only rickettsia tested that gave a plaque titer lower than that by other available methods of assay (plaque titer of 8.4 compared with an ID₅₀ of 9.75). In our studies, all of the other rickettsiae tested (3, 5) gave plaque titers that were higher than titers obtained by the established assay procedure.

The plaque assay for scrub typhus rickettsiae was more sensitive than our mouse ID₅₀ determinations (Table 1), but Elisberg (*personal communication*) had previously determined the mouse ID₅₀ values for the same seed pools of scrub typhus rickettsiae and obtained mouse ID₅₀ values that were slightly higher than the plaque titers. He employed a different strain of mice in his tests (Charles River ICR certified pathogen-free Swiss mice) that could have been more sensitive to scrub typhus rickettsiae than the strain of mice we employed. Working seed of *R. tsutsugamushi* used for plaque assay tests received an extra freeze-thaw cycle when the supply of seed material was expanded (*see*

above), possibly accounting for the plaque titers being somewhat lower than the ID₅₀ values obtained by Elisberg. The fact that different diluents were employed in the plaque and ID₅₀ titrations of *R. tsutsugamushi* apparently is of minor significance since our previous studies with other rickettsiae showed that plaque titers were the same when either diluent was employed.

The plaque assay offers some advantages over the other titration procedures. It is generally faster, far less laborious, much more economical, and provides the opportunity to isolate organisms from a single plaque (5). We are unable, however, to explain why this procedure has been successful where others have failed. In a few preliminary studies, several procedural modifications were included in the assay, but no single factor could be identified as critically important to successful plaque formation.

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