NOTES

Improved Chicken Embryo Cell Culture Plaque Assay for Scrub Typhus *Rickettsiae*

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The plaque technique for three strains of *Rickettsia tsutsugamushi* in chicken embryo cell cultures was greatly improved by modifying the trypsinizing procedure and employing homologous chicken serum in the overlay medium.

Although chicken embryo cell cultures have been used for plaque titrations of rickettsiae for several years (3, 4, 6, 7), maintaining a sterile intact monolayer for the incubation period required by the scrub typhus group has been very difficult. Many of the monolayers begin to deteriorate rapidly as incubation is prolonged beyond 10 days, and in the only report of successful plaquing of *Rickettsia tsutsugamushi*, 16 or 17 days of incubation were needed before the plaques were distinct enough to be counted (3). For this reason, it has been difficult to titrate *R. tsutsugamushi* routinely. This report described an improved method for the preparation and maintenance of chicken embryo cell cultures which allows accurate enumeration of *R. tsutsugamushi* plaque-forming units (PFU) with a minimum number of wasted cell cultures.

Primary monolayers were prepared from 25 decapitated 10-day-old chicken embryos (Truslow Farms, Chestertown, Md.) in 25-cm² plastic tissue culture flasks (Costar, Cambridge, Mass.). Minced embryos were rinsed three times with cold Earle balanced salt solution (EBSS) and placed in 400 ml of prewarmed 0.25% trypsin (Grand Island Biological Co., Grand Island, N.Y.) in EBSS for 20 min at 37°C. After fetal bovine serum was added to terminate the trypsinization, the cells were filtered through cheesecloth, centrifuged for 10 min at 300 × g, and suspended to a concentration of 2 × 10⁶ viable cells per ml in growth medium consisting of 90% minimum essential medium (MEM) prepared in EBSS and 10% fetal bovine serum. The total yield of viable cells was approximately 1.5 × 10⁹ to 2.5 × 10⁹. Five milliliters of this suspension was dispensed into each flask, and confluent monolayers were formed in 20 h at 37°C. Growth medium was removed and inoculated with a 0.1-ml suspension of the Karp, Kato, or Gilliam strains of embryonated egg-propagated *R. tsut-*

sugamushi diluted in cold brain heart infusion broth (9) containing 20 mM L-glutamine. The rickettsiae were allowed to absorb on monolayers for 1.5 h at room temperature with gentle rocking. Five milliliters of overlay medium consisting of medium 199 prepared with EBSS plus the following components were added to each flask: 1% chicken serum, 1% fetal bovine serum, 2% tryptose phosphate broth, 5 mM L-glutamine, 2.25 g of NaHCO₃ per liter, and 0.7% agarose (Seakem, Microbiological Associates, Inc., Bethesda, Md.). Chicken serum was obtained from pullorum-free and Newcastle disease-free flocks fed an antibiotic-free diet (Truslow Farms). Following 9 days of incubation at 32°C, a second (2-ml) overlay of identical composition was placed over the initial overlay, and incubation was continued for an additional 12 days. To prevent a pH change in T-25 flasks which were not airtight, all incubation was conducted in a 5% CO₂ humidified atmosphere. Contamination was reduced by washing the outside of the T-25 caps with 70% alcohol prior to adding the second overlay and by frequently changing the needle used to dispense the overlay. Plaques were stained on days 13 through 20 by the addition of 2 ml of 1/10,000 final dilution of neutral red prepared in pH 7.4 phosphate-buffered saline. Plaques were counted and measured 6 h following neutral-red staining.

Our results are summarized in Table 1. Although small plaques were first observed by neutral-red staining with all three strains on day 13, accurate counts could not be made until after 16 days of incubation. When chicken serum was employed in the overlay, medium plaques appeared 1 day earlier than when only fetal bovine serum was present. The number of plaques increased slightly between days 16 and 20, at which time individual plaques became less distinct. The most significant difference observed

with the chicken serum was that 85% of the uninfected monolayers maintained their integrity for 20 days compared with 20% with fetal bovine serum. Although all three strains demonstrated plaque-size variability (0.5 to 1.25 mm), a larger portion of the Gilliam plaques were in the 0.5- to 0.75-mm range (Fig. 1). All plaques had irregular margins, but their centers were clear. The Kato strain produced the most distinct plaques and the only ones that could be detected without neutral-red staining. Barker et al. (1), who studied the cytopathic effects of the three strains on BS-C-1 cell monolayers, noted that the Kato strain produced the most readily distinguishable holes in the cell sheets. The number of PFU was inversely proportional to the dilution of the rickettsial suspensions. The plaque assay was found to be about as sensitive as the mouse median infectious dose (ID_{50}) or median lethal dose (LD_{50}) determinations conducted with identical dilutions of the same *R. tsutsugamushi* (Karp) inoculum (Table 2). As previously observed with other strains of mice (3), the Karp LD_{50} and ID_{50} titers were nearly identical in NIN-Nmri mice. The difference between $10^{6.7}$ PFU/ml in chicken embryo cell cultures and $10^{7.1}$ ID_{50} /ml in mice appears small if both measurements are expressed in terms of infectious units (5×10^6 for chicken embryo cells, 8.8×10^6 for mice; $1 ID_{50} = 0.7$ infectious units). The LD_{50} titer in embryonated eggs was considerably lower than the PFU or mouse ID_{50} titer.

Chicken serum was required for adequate plaque formation by scrub typhus. Although serum from flocks fed a diet supplemented with antibiotics did allow plaque formation by *R. tsutsugamushi*, it was found to inhibit plaque formation by members of both the spotted fever and typhus groups of rickettsiae. Although rickettsiae are generally highly susceptible to broad-spectrum antibiotics, some reports have indicated that *R. tsutsugamushi* can grow in the presence of low levels of certain antibiotics in cell culture (2, 7, 8). However, since some interference of scrub typhus growth in cell cultures has been reported (2), no antibiotics were employed in cell culture media, the feed of chickens furnishing the fertile eggs, or the chicken serum used in the plaque assay of *R. tsutsugamushi*.

The effect of reduced trypsinization is more difficult to evaluate. However, reducing trypsinization to 20 min from the customary 30 min and prompt termination of the action of trypsin by the addition of fetal bovine serum may have contributed to the stability of the monolayers. Enhanced plaque formation observed with our modification was attributed to the stability of the chicken embryo cell cultures. The monolay-

FIG. 1. Plaques at 19 days in chicken embryo cell cultures by strains of *R. tsutsugamushi* diluted in brain heart infusion broth containing 20 mM L-glutamine. Cells were maintained in E-199 medium prepared with 1% chicken serum, 1% fetal bovine serum, 2% tryptose phosphate broth, 5 mM L-glutamine, 2.25 g of $NaHCO_3$ per liter, and 0.7% agarose. Photographed with Kodak ortho type 3 film and a 55-mm lens. (A) Karp strain, (B) Kato strain, and (C) Gilliam strains. $\times 4$.

ers remained uniform and intact until clearly defined plaques were visible. Uninfected-cell culture controls could be maintained intact for 21 days with a single refeeding. Therefore, the number of infected-cell cultures used in each exper-

TABLE 1. *Plaque formation by various strains of R. tsutsugamushi in chicken embryo culture*

Medium E-199 plus:	Survival rates of monolayers (%) ^a	First identification of plaques (day)	Size range (mm) on day:		Titer (PFU/ml) on day:	
			16	18	16	20
1% Chicken serum, 1% fetal bovine serum	85	13	0.5–1.25 (Karp)	0.5–1.25 (Karp) 0.5–1.25 (Kato) 0.5–1.25 (Gilliam) ^b	8 × 10 ⁵ (Karp)	5 × 10 ⁶ (Karp) 3 × 10 ⁷ (Kato) 10 ⁷ (Gilliam)
5% Fetal bovine serum	20 ^c	14	0.75–1.00 (Karp)	— ^d	5 × 10 ⁵ (Karp)	— ^d

^a Uninfected-cell culture monolayers remaining intact for 20 days.^b Approximately half of the plaques were at the lower size range.^c Majority deteriorated by day 14.^d Chicken embryo cell cultures could not be routinely held longer than 18 days on this medium.TABLE 2. *Characterization of R. tsutsugamushi (Karp) yolk sac inoculum*

Host	Amt	Titer (log 10/ml) ^a
Chicken embryo cell culture	PFU	10 ^{6.7}
Mouse (intraperitoneal)	LD ₅₀	10 ^{7.1}
Mouse challenge ^b (intraperitoneal)	ID ₅₀	10 ^{7.1}
Embryonated egg	LD ₅₀	10 ^{4.7}

^a Mean of three determinations.^b None of the survivors resisted a challenge dose of 500 LD₅₀ given 28 days later.

iment could be reduced to that required for statistical significance with a minimum amount of waste.

A recent publication by Oaks et al. (5) described a plaque assay for scrub typhus in irradiated L cells. The relative merits of the two methods cannot be evaluated at this time. Our method, however, has the advantage that it can be applied to procedures that require primary cell cultures and may be useful for other obligately intracellular bacteria or viruses that need long incubation periods.

The method here described makes scrub typhus titration more convenient and accurate and more conducive to applications requiring quantitative infectivity measurements, cloning, or iso-

lation of *R. tsutsugamushi* in cells free from adventitious agents.

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