Relationship Between Plaque Assay and the Mouse Assay for Titrating Rift Valley Fever Virus[1] (34908)

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In the past, a number of investigators have successfully plaqued Rift Valley fever virus (RVFV) (1–6). Plaques 1 mm in diameter were observed; later, however, Runnels and Brown (7) described large plaques up to 5 mm in diameter. Undoubtedly, the above-mentioned plaques were all of the wild pantropic virus, which suggested variants in wild pantropic RVFV. It was not until recently that Boyle (8) plaqued the wild pantropic strain of RVFV and found plaques of variable size either before or after passage in mouse fibroblast tissue cell cultures. He was able to select and characterize the small-plaque and large-plaque variants of the pantropic strain of RVFV. He further demonstrated that the small-plaque variant was more virulent for mice than the large-plaque variant.

The present report describes the relationship between the *in vivo* infectivity using mice and *in vitro* plaque development in an L-cell clone. The criteria of linearity and reproducibility were met and proven. A model is also presented for estimating mouse intracerebral lethal dose ($MICLD_{50}$) from plaque-forming units (PFU). The model obtained has provided a firm basis for a large reduction in costs of routine assays for RVFV in these laboratories, due to the relative costs of mouse and tissue culture assay. The authors recommend a similar study to determine if comparable relationship holds for any virus assay system routinely performed.

Materials and Methods. Virus. The small-plaque variant of the pantropic van Wyk strain of RVFV (8) was used in this study. The origin and maintenance of this strain were described by Walker *et al.* (9).

Virus was propagated in L-DR strain, a variant of Earle's L-cell which was grown in serum (bovine) supplemented medium (9). Multiplicity of inoculum (MOI) was 0.01, cell concentration was 2×10^5 cells/ml, and the infection period was 48 hr. The tissue cells were removed by low-speed centrifugation and the virus was assayed.

Cell cultures for plaquing. A variant (L-MA) of Earle's L-cell was obtained from Dr. Donald J. Merchant, University of Michigan, Ann Arbor, Michigan. The cells were maintained antibiotic free, and a selected clonal line designated L-MA Clone 1-1 (double cloned) was established as the tissue cell line in these studies. Growth medium for L-MA Clone 1-1 was 199 medium supplemented with 0.5 bactopeptone. Cultures were grown in suspension culture (10) to produce bulk quantities that were determined to be PPLO-free by plating samples on Mycoplasma agar (Grand Island Biol. Lab., Grand Island, N. Y.). Frozen stocks (—175°) were prepared and used routinely to avoid inconsistencies in plaque efficiency due to continuous cell passage.

Plaque overlay medium. The plaquing medium was composed of two overlays. The first overlay medium consisted of two parts: (i) 1X Hanks' BSS with 1.0% Noble agar was autoclaved at 10 psi for 10 min, and (ii) 20% calf serum plus 69% 199 peptone medium containing a 1.0% solution of sodium bicarbonate. Equal volumes of both component parts were individually warmed to

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50° and then mixed; 1% Penn-Strep, 1% Mycostatin, and 1% (100 μg/ml) DEAE-dextran were then added.

The second overlay medium also consisted of two parts: (i) 1X Hanks' BSS with 1.0% Noble agar, and (ii) 77% 199 peptone medium containing a 0.5% solution of sodium bicarbonate. Equal volumes of both components were individually warmed to 50° and then mixed with, the addition of 0.007% of a neutral red solution, 1.0% Penn-Strep, and 1.0% (100 μg/ml) DEAE-dextran.

Plaque assay. Monolayers were prepared by seeding 2-oz bottles (Sani-glass Flint-Grad, Brockway Glass Co., Inc., New Jersey) with 5 ml of cell suspension at 7.5×10^5 cells/ml in growth medium. After incubation overnight at 37°, the growth medium was removed and cells were washed with 1X BSS at pH 7.6. Each bottle was inoculated with 0.1 ml of virus diluted to the desired concentration and allowed to adsorb for 1 hr at 30°. After adsorption, 5 ml of the first overlay medium at 45° was added to each bottle. When the overlay had solidified (1 hr), the bottles were inverted and incubated at 37° for 72 hr. The monolayers were then removed from incubation, and the second overlay, which contained stain for plaque observation, was applied. Plaque preparations were covered with heavy brown paper from time of inoculation until addition of second overlay to avoid photoinactivation. The plaques were counted at 1 hr and again at 24 hr. All dilutions were counted, and the PFU were calculated from dilutions having the highest number of plaques. In almost all cases, counts were made on bottles containing 50 to 150 plaques.

For reisolation of the virus, isolated plaques were removed by an inoculating loop of heavy-gauge wire. Single plaques were inoculated onto confluent monolayers of L-MA Cl 1-1 in 2-oz bottles. The inoculated monolayer cultures were incubated for 24 hr at 37°, after which time the medium was removed and assayed both in mice and by the plaque technique.

Mouse assay. The Fort Detrick Swiss-Webster strain of mice, weighing 6 to 8 g, was used for titrating RVFV. Mice were inoculated intracerebrally, 4 mice/dilution, with 0.03 ml of viral challenge material. Deaths were recorded for 6 days postinoculation, at which time the cumulative death percentage was >95%. Only those deaths occurring after 24 hr were used in the calculation of mouse intracerebral lethal $doses_{50}$ ($MICLD_{50}$). The probit method (11) of calculating $MICLD_{50}$ values was used.

Results. Plaque assay. Somewhat rounded, clear plaques with sharp boundaries were visible as early as 1 hr after the second overlay (which contained neutral red) was placed over the 72-hr-old culture. They were <1 to 3 mm in diameter. Statistically, there was no difference in counts taken at the third and

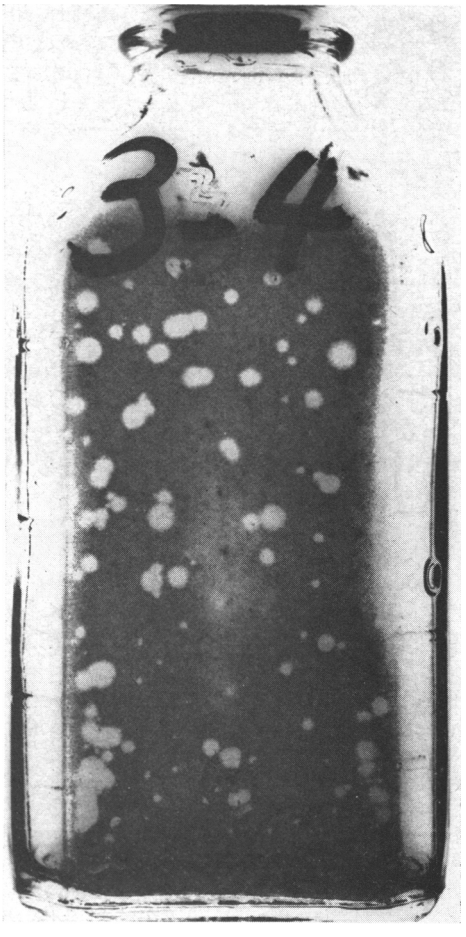

FIG. 1. Plaques of Rift Valley fever virus in L-MA Clone 1-1 tissue cells 72 hr after inoculation of virus (×2.5).

TABLE I. Relationship Between Plaque Count and Virus Concentration.

Virus and conc	Dilution	Plaques/bottle		Log_{10} PFU titer	
		No.	Av	Day 3	Day 4
48-hr Inf. Cult. $\times 10^{-3}$[a]	1	239, 242	240.5	6.38	6.38
	1/2	125, 120	122.5	6.39	6.37
	1/4	63, 63	63.0	6.40	6.38
	1/8	27, 20	23.5	6.27	6.32
$\times 10^{-4}$	1	68, 79	73.5	6.87	6.93
	1/2	25, 40	32.5	6.81	6.86
	1/4	9, 16	12.5	6.70	6.68
	1/8	1, 4	2.5	6.30	6.65
Alum precipitated $\times 10^{-2}$	1	246, 232	239.0	5.38	5.37
	1/2	142, 162	152.0	5.48	5.52
	1/4	72, 87	79.5	5.50	5.54
	1/8	43, 41	42.0	5.53	5.61
$\times 10^{-2}$	1	71, 32	52.5	4.71	4.74
	1/2	25, 14	19.5	4.59	4.66
	1/4	10, 21	15.5	4.79	4.82
	1/8	4, 3	3.5	4.45	4.45
$\times 10^{-3}$	1	255, 247	251.0	6.40	6.40
	1/2	175, 165	170.0	6.53	6.53
	1/4	104, 105	104.5	6.62	6.68
	1/8	58, 79	68.5	6.74	6.80
$\times 10^{-3}$	1	TNTC, TNTC[b]	—	—	—
	1/2	217, C.	217.0	6.34	6.35
	1/4	96, 101	98.5	6.60	6.61
	1/8	49, 60	54.5	6.64	6.71
Virus and conc	Dilution	No.	Av	Day 3	Day 4

[a] Each dilution studied was a separate virus pool which differed in treatments.
[b] Too numerous to count.

fourth day after inoculation of monolayers. The plaques were distinct, uniform in size, and easily visible with the naked eye or with an indirect lighting system if detail was desired (Fig. 1).

Relationship between plaque numbers and virus concentration. The relationship between the number of plaques detectable and the concentration of virus was determined. Starting at primary virus dilutions of 10^{-2}, 10^{-3}, or 10^{-4}, successive twofold secondary dilutions were made of the different virus preparations to test for precision and linearity of the data. The plaque assay was shown to perform equally well on each of the two virus preparations (Table I). There was excellent agreement of PFU for all secondary dilutions, and there was no real difference between the day 3 and 4 PFU readings, as indicated in the last two columns. Moreover, there is a linear relationship between the average number of plaques obtained for each virus dilution and the relative concentration of virus.

Comparison of RVFV infectivity titers obtained by $MICLD_{50}$ and plaque assay. In preliminary experiments, the comparison of RVFV infectivity titers between PFU and $MICLD_{50}$ was 1.5 logs higher for $MICLD_{50}$ (Table II). This difference was based on titrations of cultures all approximating the same degree of infectivity. In most cases, this meant that $MICLD_{50}$ were higher than PFU titers, suggesting that the $MICLD_{50}$ was somewhat more sensitive (approx 31.5 $MICLD_{50}$/PFU).

To further test the relationship and possible difference in linearity between the two

TABLE II. Comparison of Rift Valley Fever Virus by Mouse and Plaque Assay.

Assay procedure	No. of replications	Mean	Deviation	Variance[a] s^2
$MICLD_{50}$	42	8.2	0.30	0.900
Plaques	42	6.7	0.39	1.522

[a] Significant at the 6% level.

assay systems, the second in these series of experiments was performed. After low-speed centrifugation, the supernatant was (i) stored at −65°, or (ii) alum precipitated and stored at −65°. Then, in order to determine if various virus treatment affected the titers obtained by the two assay systems, samples of each of 2 stored test materials were selected at random once a week for 3 weeks and assayed at 5 different dose levels. To obtain the dose levels necessary for showing linearity, we diluted the original samples (titer) of each of the virus cultures. The dilution levels of these samples were from 10^{-1} through 10^{-5} log, which gave 5 dose levels. Subsequent dilutions were made to adjust the number of plaques per bottle. Therefore, 10^{-1} log dilution might have 5 subsequent dilutions, whereas 10^{-5} original log dilution might have only 1 or no subsequent dilutions. The means for the log PFU and log $MICLD_{50}$ were analyzed using the linear regression program to obtain calculated slopes and the correlation coefficients for the two assay techniques.

In order for this assay to be useful, a straightline relationship has to exist between dilution and titer (virus concentration) over a wide dose range. Such a relationship was demonstrated with this plaque assay (Fig. 2). There was no statistical difference between slopes over the treatments tested. Moreover, when the same treatment material was assayed in mice, no statistical change in slopes was demonstrated. In fact, there was no statistical difference between the slopes of the plaque and mouse assay system; however, the titer of the $MICLD_{50}$ was 0.7 to 1.0 log greater than the PFU. In examining the data, it does appear that the variability of the points about the lines of $MICLD_{50}$ is somewhat more than that of the PFU, although the difference in variability is not significant.

A straightline relationship was also obtained when the PFU were plotted against $MICLD_{50}$ (Fig. 3). Again no statistical difference in slope was demonstrated over the treatments when the PFU were plotted against the $MICLD_{50}$.

From this work, we concluded that the alum treatments of the virus suspensions had no appreciable effect on the plaque assay system. Therefore, all data that had been collected in our laboratory for comparing PFU and $MICLD_{50}$ could be put into one linear regression program for obtaining an estimated slope that can be used for estimating $MICLD_{50}$ from PFU (Fig 4).

The straight line shows the estimated relationship between PFU and $MICLD_{50}$; the 95% confidence limits are represented by the inner pair of curved lines and the 95% tolerance limits for a point that could be

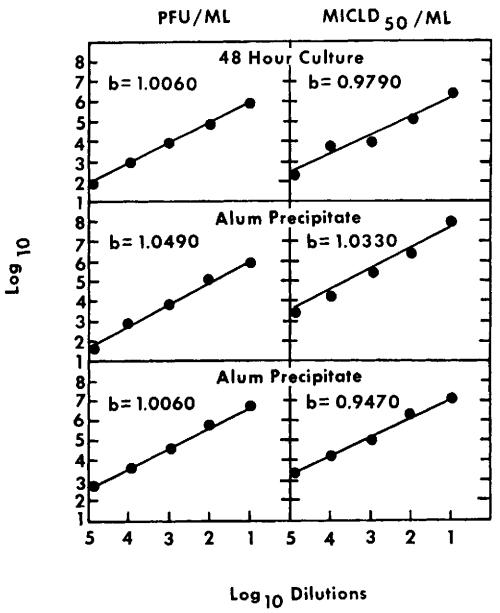

FIG. 2. Slope comparison between Rift Valley fever virus assay techniques after alum treatment.

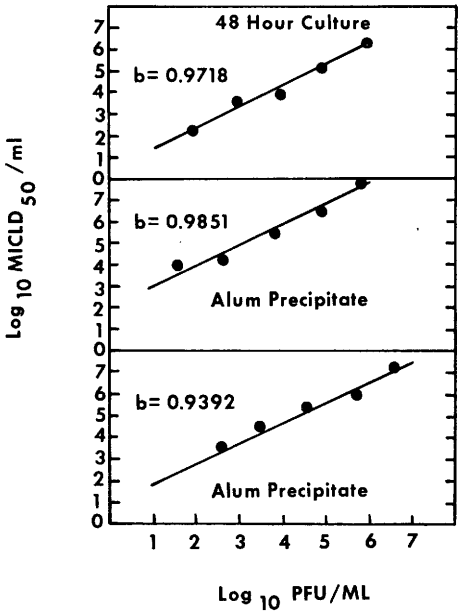

FIG. 3. Relationship of PFU to $MICLD_{50}$ after alum treatment.

determined by a single plaque assay and a single mouse assay is represented by the outer pair of curved lines.

If one uses the model to estimate the $MICLD_{50}$ infectivity (potency) of test material for which the plaque assay gives 6.5 $\log_{10}$ PFU, the estimate will be 7.1 with 95% tolerance limits of approximately 6.0 to 8.3 $\log_{10}$ $MICLD_{50}$. Obviously, the present slope is restricted to 8.5 $\log_{10}$ PFU, thus restricting the range in which $MICLD_{50}$ can be estimated unless the line is extrapolated to account for higher PFU values.

Re-Isolation of virus. RVFV was readily re-isolated from plaques; a single plaque of the small-plaque RVFV inoculated into monolayer produced 10^{-3} to 10^{-5} $TCID_{50}$/plaque. No plaques were seen in the absence of virus inocula, nor was virus recovered from clear areas of the infected agar surface. The re-isolated virus was neutralized by specific hyperimmune sheep serum.

Discussion and summary. RVFV produced plaques that were well defined and characteristic of the small-plaque variant in monolayer cultures of L-MA Clone 1-1 tissue cells under a double agar overlay. Plaque counts made on days 3 and 4 were not statistically

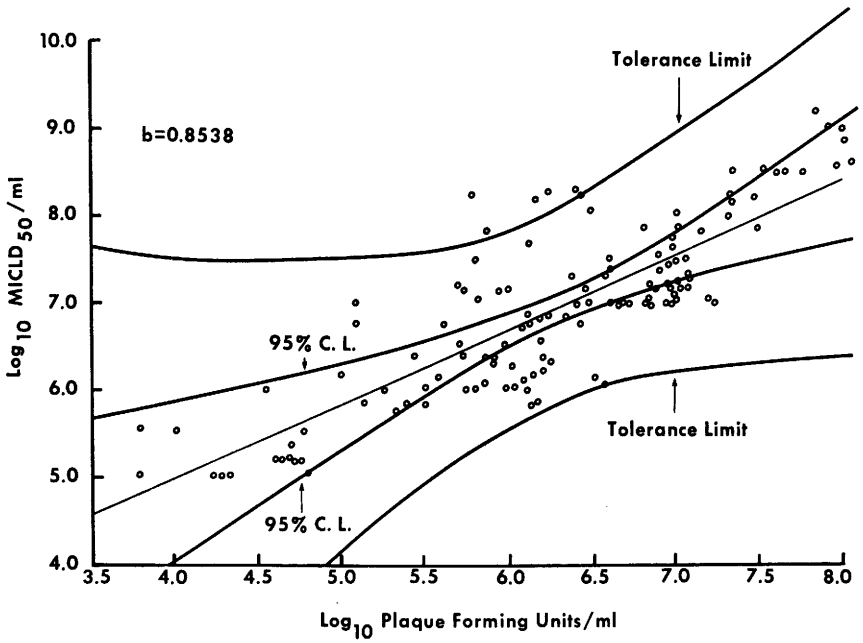

FIG. 4. Model for estimating $MICLD_{50}$. The straight line shows the estimated relationship between PFU's and $MICLD_{50}$'s; the 95% confidence limits are represented by the inner pair of curved lines and the 95% tolerance limits for a point which could be determined by a single plaque assay and a single mouse assay is represented by the outer pair of curved lines.

different, although plaque size was increased by the fourth day. A linear relationship was observed between plaque counts and virus concentration under various treatment conditions, permitting precise virus assays. The results were further extended to include comparison of slopes between $MICLD_{50}$ and PFU. Although the titers for $MICLD_{50}$ were consistently higher, there were no statistical differences in or between slopes of the two assay systems. This observation permitted accumulated data comparisons between the two assay systems to be combined into one linear regression program for development of a model for estimating $MICLD_{50}$ from PFU. The model now provides a technique for observing infectivity in two independent assay systems with certain accuracy at a greatly reduced cost to an experimenter or diagnostic and experimental laboratories where such determinations are routine. To insure that the relationship portrayed in the model continues to hold after additional passage of the virus in tissue culture lines, it will be necessary to perform studies at regular intervals to confirm the relationship between PFU and $MICLD_{50}$. This procedure will serve to spot any changes in mouse pathogenicity, unaccompanied by decrease in PFU.

For the virus system under study, a large reduction in cost has been provided utilizing the model described. The particular model, and the parameter estimates obtained, while not necessarily applicable to other virus strains gives some hope that similar comparative studies may provide a basis for like reduction in cost for routine assays of other viruses. Based on their own experience, the authors recommend such studies where the number of assays is appreciable.

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