

## JEV Plaque—Reduction Neutralization Test (PRNT): A Statistical Evaluation

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### OBJECTIVES:

- 1) To evaluate the efficiency, precision and reproducibility of the JEV—plaque reduction neutralization test (PRNT) and to determine the requirements for a valid PRNT;
- 2) To compare the standard JEV strain (Nakayama) with a Chiengmai JEV strain by PRNT evaluation;
- 3) To evaluate a microculture PRNT test in terms of efficiency, precision, and reproducibility;
- 4) To statistically evaluate the mechanism of the neutralization reaction of JEV and the four dengue serotypes.

DESCRIPTION OF ANTIBODY ASSAYS: We employed the standard macroculture, plaque reduction neutralization test method (macro—PRNT) using MK2 cell cultures in 1 oz prescription bottles and 0.25 ml aliquots of antiserum per bottle. Further details of this method are described in a separate section of this annual report entitled, "Kinetic Neutralization Study of JE Viruses". For the micro—culture plaque reduction neutralization test (micro—PRNT), we used MK2 cells in 15 mm flat—bottom wells and 0.025 ml aliquots of antiserum per well, according to methods previously published (see Pairatana, Yuill, and Russell, Transactions of the Royal Society of Tropical Medicine and Hygiene 63: 446, 1969). The virus strains and antisera assayed are described separately under each of the four experimental headings of this study.

DESCRIPTION OF STATISTICAL ANALYSIS: The arithmetic mean of the control bottles or micro—wells was used to calculate percent reduction for each individual bottle or well in a test. Serum dilutions were transformed to logarithms and percent plaque reduction to corresponding probit values. Comparisons between replicates and between sera were calculated by the method for a parallel—line graded response bioassay. Comparison between the PRNT results on four dengue serotypes and JEV using the same antisera were made by using the technique of analysis of covariance.

### PROGRESS: 1. Macro—PRNT test.

Four acute—convalescent serum pairs from four encephalitis patients were selected to evaluate the PRNT against JEV, Nakayama strain, in the 21st mouse passage level. Of the two serum pairs described below, one pair had a 25—fold neutralizing antibody titer rise with a high convalescent titer of  $>1:1200$ , while the second pair had a high fixed antibody titer of approximately 1:640. Fixed antibody titer is defined as less than a 4—fold PRNT antibody titer rise in the convalescent specimen as compared with the acute. Three or four replicate determinations on each serum pair were run independently at the same time by the same person. Excellent agreement ( $\pm 1\%$ ) was obtained by two different persons in counting plaques.

Two replicates of serum pairs A & B are summarized in Tables 1 and 2. In these tables, the "standard" serum is the acute phase specimen, and the "unknown" serum is the convalescent specimen. As shown in Table 1, statistically identical results were obtained on 2 independent replicates of serum pair A, with both replicates showing equal potency of standard and unknown sera; that is, the confidence limits ( $95\% \text{ CI}_{95\%}$ ) for both replicates clearly overlapped 100%. Similar statistical agreement between confidence

limits of the two replicates of serum pair B was obtained (Table 2). Unlike the fixed titers of serum pair A, there was approximately a 25-fold antibody titer rise in serum pair B (i.e. relative potency of approximately 4%). These data are graphically illustrated in Figures 1 and 2.

A valid PRNT, with good precision and reproducibility, as seen in Tables 1 & 2, was uniformly obtained only when the following statistical requirements were met:

- a) Control plaque counts between 30-130;
- b) Plaque reduction responses bracket 50%;
- c) Three (preferably four) responses between 15-85% plaque reduction.

In addition, three plaque bottles per serum dilution were found to be adequate, and using 2-fold dilutions produced statistically more powerful results than did using 4-fold dilutions.

Over 50% of the data collected did not meet one or more of the above conditions and thus should not be interpreted. These invalid data include 2 additional serum pairs, each with 3-4 replicates, as well as 2 additional replicates of serum pairs A & B. Statistically invalid tests resulted from our inability to reproducibly obtain the required numbers of control plaques and to successfully bracket the 50% plaque reduction value, even though 4 or 5 dilutions of antiserum were used. The large amount of data that had to be discarded, even when the tests were performed under these very standardized conditions, unfortunately makes this serological test too costly and inefficient for large-scale survey use if quantitative antibody titer values are required.

## 2. Comparison of the antigens of two JEV strains.

The PRNT was used to determine the antigenic similarity of two JEV strains—40783, isolated from a human brain from Chiangmai, and the Nakayama strain. Strain 40783 was in 5th mouse passage and the Nakayama strain in 21st mouse passage. Both viruses were incubated with four dilutions of JEV Nakayama antiserum, and the dose-response curves for both viruses are plotted in Figure 3. Statistically valid results were obtained. The antigenic similarity of the two strains was suggested by the two curves being linear and parallel (i.e. Same slope). Antiserum to strain 40783 should be similarly titered against both virus strains to further substantiate antigenic identity.

## 3. Evaluation of the Micro-PRNT.

The micro-PRNT test was performed by one technician running three virus-antiserum mixtures in duplicate. Two low-titered anti-JEV monkey serums and one high-titered anti-JEV rabbit serum were titered against JEV Nakayama in 15th mouse passage. Four or six 15 mm tissue culture wells were used.

Although plaque numbers were small, good agreement ( $\pm 2$  plaques) was obtained at each dilution, resulting in error terms of similar magnitude to the macroculture PRNT. In each of the six tests (on the three antisera), four or five dilutions had plaque reductions between 15% and 85% and bracketing 50% reduction. There was no apparent increase in precision by using six, rather than four, culture wells per dilution. Statistical analysis further revealed that the data were fully valid for four of the six tests. In two tests, there was slight departure from a linear dose-response curve.

The replicates of the two low-titered anti-JEV serums agreed well. The slopes were similar, as were the  $ED_{50}$ 's; 50.0 and 59.1 for one antiserum; 72.8 and 88.8 for the other. Both the slopes and the  $ED_{50}$ 's (3,200 and 13,800) were significantly different between the replicates of the high-titered rabbit antiserum, even though each test produced statistically valid results. Whether this is a function of the test system (e.g., dilution errors) or decrease in statistical efficiency for such high 50% end point titers is not clear and deserves further investigation.

4. Evaluation of Several Group B Arbovirus Neutralization Responses.

The purpose of this experiment was to determine if the presumed group B arbovirus causing a recent infection might be differentiated from other group B viruses by showing a uniquely different dose-response curve.

Convalescent sera from two Japanese encephalitis patients from Chiangmai and one Thai hemorrhagic fever patient from Bangkok were analyzed, using the analysis of covariance technique. Macro PRN tests were run against dengue serotypes 1 thru 4 and JEV concurrently; each serum showed widely cross-reactive antibody titer patterns. The analysis of covariance technique allows the best fitting dose-response regression lines to be estimated for the five arbovirus serotypes, utilizing all the data together. A fairly wide range of slopes were obtained on each serum. More sera need to be tested before the results can be interpreted.

Table 1.

JE plaque-reduction neutralization test (PRNT) (A) serum pair 44605-44606 replicate analysis

Serum No.	Serum Dilution (Reciprocal)	Average* % Plaque Reduction	
		Replicate II	Replicate III
44605 "Standard" (S)	160	83	83
	320	78	75
	640	57	57
	1,280	27	31
44606 "Unknown" (U)	80	96	98
	160	91	88
	320	73	73
	640	37	46

\* Average of 3 Bottles Per Dilution

Parameter Estimate	Replicate II	Replicate III
Slope	-2.0707	-1.9650
ED <sub>50</sub> "Unknown"	1/584	1/682
ED <sub>50</sub> "Standard"	1/636	1/666
Relative Potency (RP)	109%	98%
95% CL <sub>RP</sub> (%)	86-138	58-165

Table 2.

JE plaque—reduction neutralization test (PRNT) (B) serum pair 44480—44481 replicate analysis

Serum No.	Serum Dilution (Reciprocal)	Average* % Plaque Reduction	
		Replicate I	Replicate II
44470 "Standard" (S)	10	84	87
	20	77	68
	40	67	64
	80	47	36
44481 "Unknown" (U)	320	88	81
	640	72	74
	1,280	60	48
	2,560	47	25

\* Average of 3 Bottles Per Dilution

Parameter Estimate	Replicate I	Replicate II
Slope	-1.2817	-1.6560
ED <sub>50</sub> "Unknown"	1/2,819	1/1,208
ED <sub>50</sub> "Standard"	1/73	1/49
Relative Potency (RP)	3.3%	4.1%
95% CL <sub>RP</sub> (%)	2.2-5.0	3.0-5.6

FIGURE I.

JE-PRNT (A) : CURVES OF REPLICATES II AND III

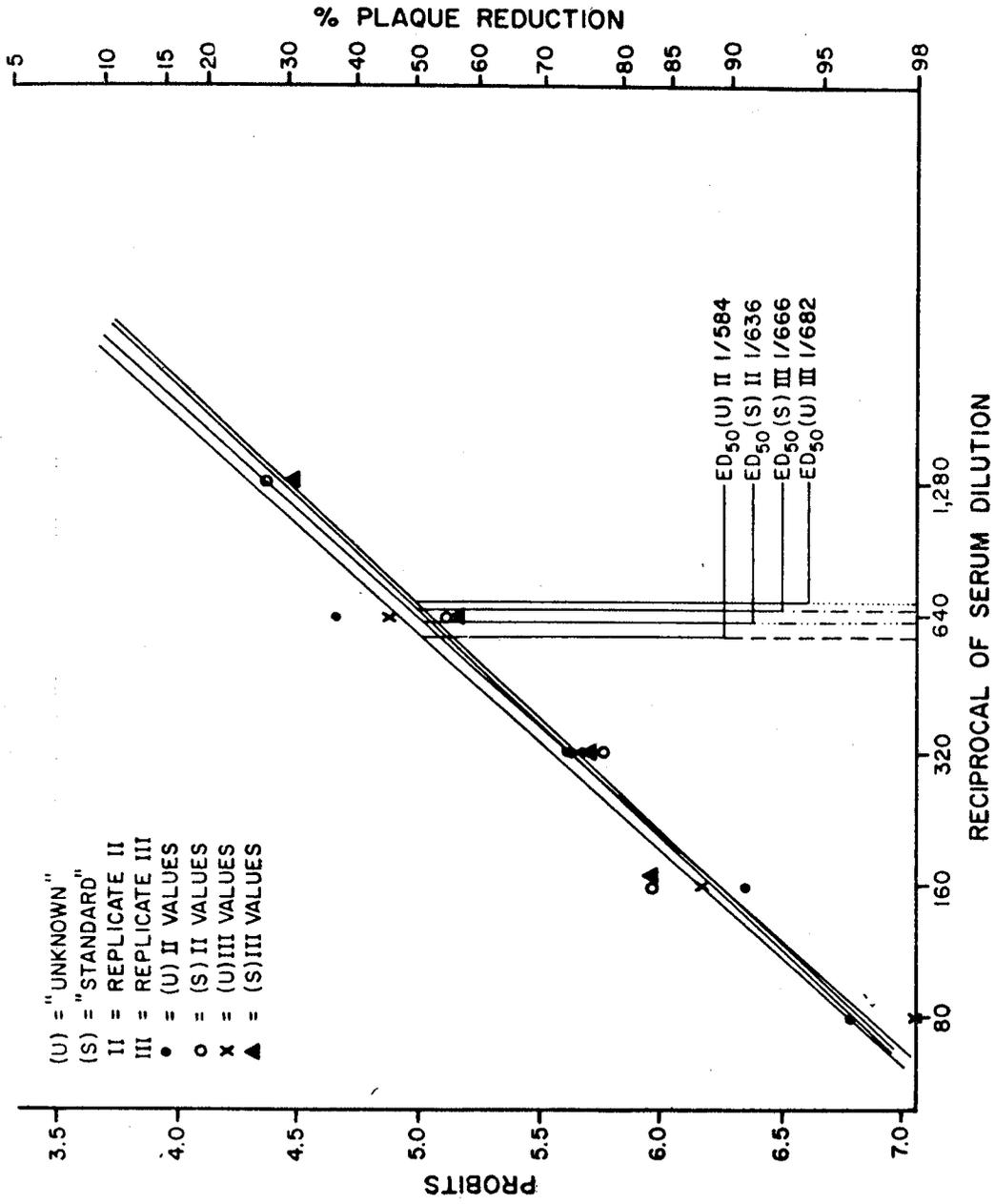


FIGURE 2.

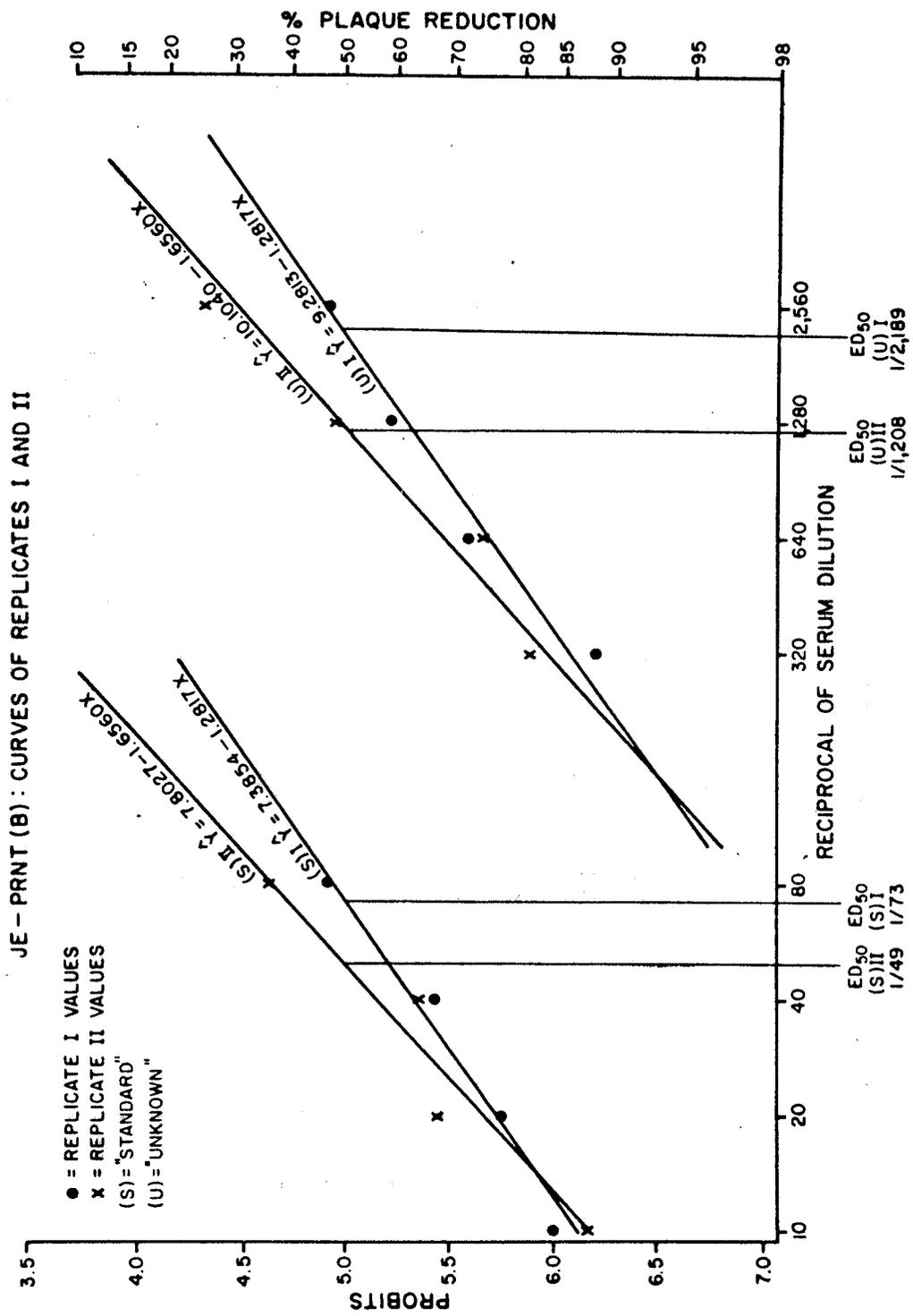


FIGURE 3.

PLAQUE REDUCTION NEUTRALIZATION TEST (PRNT)  
 JEV (NAK) ANTISERUM VS. JEV (NAK) AND JEV 40783 (CHIENGMAI)

