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Microculture Plaque Neutralization Test for California Group Arboviruses

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A microculture plaque neutralization test is described for California-group arboviruses that is as precise and quantitative as the standard test conducted in 60-mm petri dishes. It was shown that there was no significant between-panel or between-day variation in determinations and that a single pipette could be used for all serum-dilution levels within a titration without inoculum carry-over effect. The experimental protocol and statistical methods used produce 50% neutralization end points that meet the assumptions of parametric statistics. This permits the power and versatility of the analysis of variance to be exploited in testing for treatment effects in serological and immunological studies with viruses.

The plaque neutralization (PN) test (9) is a highly sensitive method for quantitating viral antibodies (13). Additionally, the test is statistically appealing (3, 5, 6, 10) because it is based on enumerative rather than quantal response data. However, as it is often conducted in 60-mm petri dish cultures (macrocultures), the test is laborious (9) and expensive.

Recognizing these disadvantages, Miura and Scherer (8) and, later, Early et al. (4) developed microculture modifications of the PN test that utilize cultures in wells of leucite trays (8) or of disposable plastic panels (4). These modifications have proven useful and expedient but have the disadvantage of not being interpolative (between-dilution end points) and, owing to plaque convergence, they lack the precision of the macroculture test. However, a later version of the microculture method was adapted for cytomegaloviruses and was shown to be as reliable and quantitative as the macroculture test (2). The success of the cytomegolovirus system was possible because the plaques produced were sufficiently small to allow a large number of plaques per culture without excessive overlap. Accordingly, microcultures are a promising basis for highly quantitative tests where plaque size can be restricted.

The purpose of the present work was to develop a microculture plaque neutralization technique for quantitating antibodies to California-group arboviruses. Objectives were (i) to develop a test with the precision and interpolative potential of the macroculture PN test, and (ii) to develop an experimental and statistical protocol for determining 50% end points that meet the assumptions of parametric statistics (12). The second objective was important because, in a subsequent study (manuscript in preparation), we subjected end point data to analysis of variance for hypothesis testing.

MATERIALS AND METHODS

Viruses. Two subtypes of the California-group of arboviruses were used. LaCrosse (LAC; 14) was supplied by W. H. Thompson (University of Wisconsin, Madison, Wis.) and snowshoe hare (SH; 1) by the Rocky Mountain Laboratory (Hamilton, Mont.). Virus stocks were prepared from a 10% suspension of infected suckling mouse brain homogenized in growth medium consisting of Eagle minimal essential medium with Hanks salts (pH 7.4), 10% heat-inactivated fetal calf serum, and antibiotics. The homogenate was clarified at $6000 \times g$ (4 C) for 60 min, and the supernatant was stored frozen at −70 C.

Cell cultures. Cultures used were Vero cells propagated in minimal essential medium with Earle salts, 10% fetal calf serum, and antibiotics. Plastic panels containing 96 (16 mm) wells (Model 96 CV-TC; Linbro, New Haven, Conn.) were used for microcultures and plastic petri dishes (60 mm) were used for macrocultures. Cells were seeded at a concentration of 2.5×10^5 cells/ml in volumes of 0.5 ml per panel well or 5.0 ml per petri dish. Cultures were then incubated in a humidified incubator (37 C; 5% CO_2) for 48 h, at which time monolayers were confluent.

Antiserum. Rabbit antiserum was prepared from a New Zealand white rabbit (14 weeks old) inoculated once intravenously with $10^{6.2}$ plaque forming units of LAC in 1.0 ml of diluent. Forty days after inoculation, the rabbit was bled and serum was harvested, heat

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inactivated (56 C; 30 min), and stored frozen at −20 C.

Plaque neutralization test. Serum was diluted with growth medium in twofold serial dilutions starting at 1:20. Each serum dilution tube was paired with its own control which contained an equal volume of diluent only. Virus suspensions containing approximately 1,000 plaque forming units per ml were added to serum and control tubes in proportions of 1:1, and the mixtures were incubated at 37 C for 1 h.

Panels were divided into 4 quadrants of 24, and all cups in a given quadrant were inoculated with a single tip fixed to a 0.2-ml automatic pipette (Biopette; Schwartz Bio-Research. Orangeburg, N.Y.); before proceeding to the next quadrant, the used tip was replaced with a new one. Inoculation proceeded from the lowest to the highest serum dilution and then in similar order through the respective control tubes. Inoculated panels were incubated in a humidified atmosphere at 37 C for 60 min with shaking at 15-min intervals.

Cultures were then overlayed with 0.75 ml per well of a mixture (37 C) of equal volumes of 2% gum tragacanth (7) and double-concentrated medium (modified from a formulation supplied by N. J. Marchette, University of Hawaii, Honolulu) made up with 20% 10× minimal essential medium (Earle salts), 20% heat-inactivated fetal calf serum, 1% lactalbumin hydrolysate, 0.2% yeastolate, 1.26% sodium bicarbonate, 0.73% glucose, and 0.025% sodium pyruvate. Overlayed cultures were incubated for 4 days and then voided of overlay, washed, and stained with carbolfuchsin.

Fifty percent neutralization end points. Plaques were counted by visual inspection and serum-control ratios were calculated and expressed as the percentage of plaque survival (PS). All percentage values between 10 and 90%, inclusive, were transformed to the corresponding arcsin square root percent values (11). Serum dilutions 1:20, 1:40, ... 1:20,480 were transformed to the coded values of 1, 2, ... 12, respectively, using the transformation $X = \log_2 (X'/10)$, where X is the transformed value and X′ is the reciprocal of the original serum dilution. These transformed values were used for computational procedures.

For each of the two sets of data within a given panel quadrant, a best-fit linear regression line was computed by regression analysis (11). Given the computed values for the constant (a) and slope regression coefficient (b) in $\hat{Y} = a + bX$, where $\hat{Y}$ is the best estimate of the PS at a given X (dilution) value, the 50% end point was computed by setting $\hat{Y}$ equal to the arcsin square root percent value corresponding to 50% (i.e., 45) and then solving the resulting equation for X (11). The calculated X was the best (minimal variance) estimate of the dilution at which 50% of the plaques were neutralized and is expressed as the CED_{50} (coded 50% end point) or the ED_{50} (corresponding uncoded 50% end point relative to the reciprocal of the serum dilution). Only CED_{50} values were used in statistical analyses. Uncoding of CED_{50} to ED_{50} values was achieved by extracting the antilog (base 2) of the CED_{50} and multiplying by 10 (i.e., $X' = 10\ [2^X]$).

RESULTS

Comparison of PN tests in micro- and macrocultures. LaCrosse-specific rabbit antiserum from a single rabbit was quantitated repeatedly for neutralizing activity against the homologous virus in micro- and macrocultures, and the difference between end points by the two methods was tested for significance. The test in macrocultures followed the microculture protocol.

Figure 1 shows a complete microculture PN test in a quadrant of a 96-well panel. Figures 2a

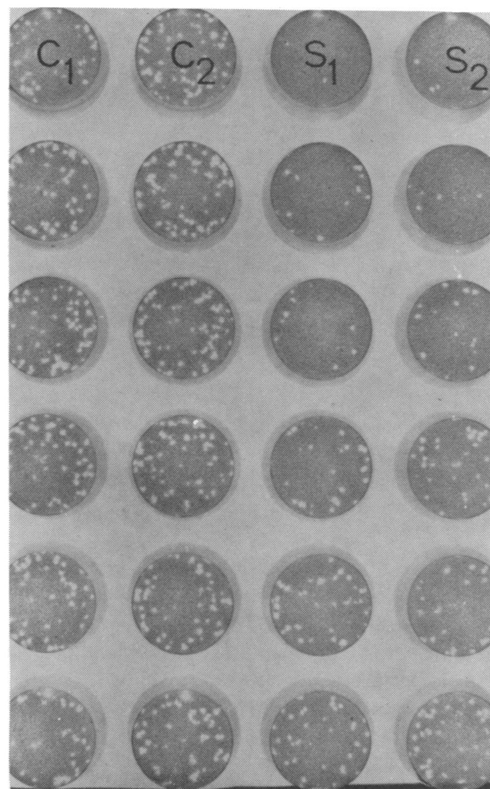

FIG. 1. *Completed plaque neutralization test in one quadrant of a 96-well disposable plastic panel. Microcultures were inoculated with serum-containing reaction mixtures (S) and serum-free control mixtures (C). Serum dilutions (twofold) increase from top to bottom. Ratios of plaque numbers in S_1 to those in C_1 and in S_2 to those in C_2 are calculated and expressed as the percentage of survival. The two ratios are calculated for each of the six dilution levels, so that two independent linear regression lines can be calculated from each of the two sets of observations.*

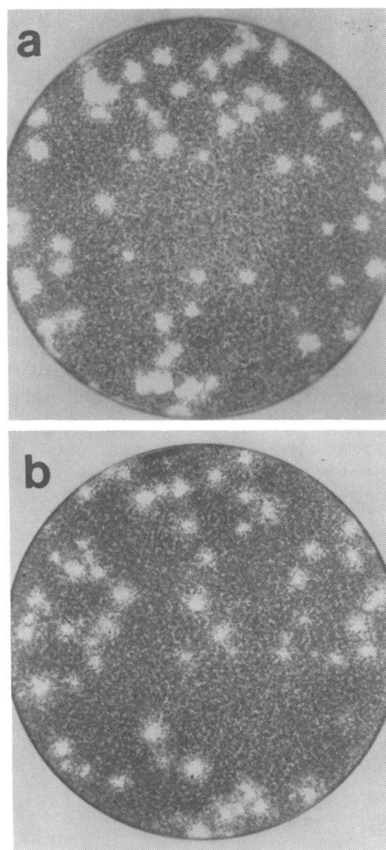

FIG. 2. *Close-up view (×5.3) of plaques in individual microcultures (16 mm). Vero cell monolayers are overlayed with gum tragacanth and stained with carbolfuchsin. (a) LaCrosse plaques; (b) snowshoe hare plaques.*

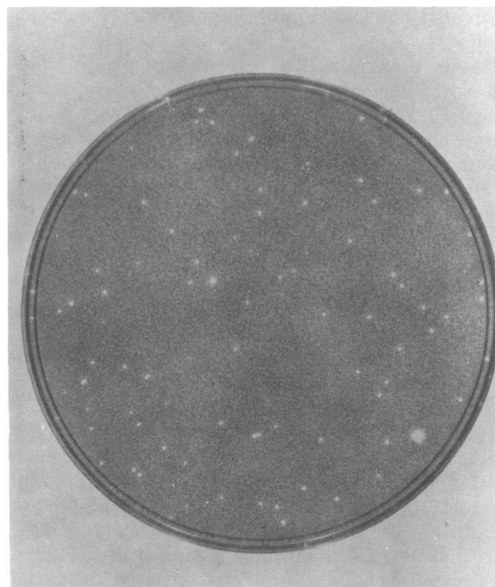

FIG. 3. *LaCrosse plaques in 60-mm petri dish (×1.0). Cells, overlay, and stain are as described for microcultures.*

and 3 show micro- and macrocultures, respectively, containing LAC plaques.

Results of the experiment (Table 1) suggest a very close agreement between assay methods. The small difference between means of 0.019 CED_{50} units was determined to be nonsignificant ($P > 0.25$).

Between-panel variation. Between-panel variation was studied with two California-group subtypes, LAC, which produces plaques with a sharp margin (Fig. 2a), and SH, which produces plaques with a clear center surrounded by a turbid margin (Fig. 2b). Eight end point determinations were made in each of three panels for each of the two viruses, and a single serum dilution series was used for all determinations within a virus.

The data in Table 2 show that differences in determinations between panels are small and

TABLE 1. *Comparison of mean 50% neutralization end point observations in disposable plastic panels versus 60-mm plates*

Vessel	Mean[a]	Difference between means	Student's t test
Panels	8.46 ± 0.06 (3,521)	0.02	0.22 NS[b]
Plates	8.48 ± 0.06 (3,570)		

[a] Mean of 16 CED_{50} observations for panels and 24 CED_{50} observations for plates plus or minus standard error. Values in parentheses are uncoded ED_{50} dilution values.

[b] NS, Not significant.

TABLE 2. *Means for between-panel variation in 50% neutralization end point observations for a homologous and a related heterologous virus*

Virus[a]	Mean[b]		
	Panel 1	Panel 2	Panel 3
LAC	8.64 (3,989)	8.59 (3,853)	8.45 (3,497)
SH	6.32 (798)	6.64 (997)	6.69 (1,032)

[a] LaCrosse (LAC) is homologous; snowshoe hare (SH) is heterologous.

[b] Mean of eight 50% end point observations per panel. Values in parentheses are uncoded ED_{50} dilution values.

nonsignificant ($P > 0.05$), as determined by analysis of variance.

Between-day variation. Variation in titers obtained on separate days was investigated with LAC virus and homologous serum. Serum and virus stocks were appropriately diluted, and microcultures were separately prepared on each of the 2 days. Eight determinations were made for each of three panels on each of 2 days.

Results show a small, nonsignificant ($P > 0.05$) difference (0.10 CED_{50} units) between means of observations made on the 2 days (Table 3).

Variation from inoculum carry-over. Success of the panel assay depends in part upon the speed with which a panel can be inoculated. If monolayers are not covered with inoculum soon after growth medium has been decanted, cells dry and the assay is unsuccessful (4). To expedite the inoculation procedure, a single Biopette tip was used as described above for all microcultures receiving inoculum from a given serum virus combination. Since inoculation was in the order of ascending serum dilutions, the possibility existed that neutralizing activity could have been carried over from lower to higher serum dilutions. To test for a possible antibody carry-over effect, an experiment was done to compare neutralization end points of assays in which a single tip was used versus assays in which tips were discarded between dilution levels.

The experiment was carried out using macrocultures inoculated with reaction mixtures from a single serum dilution series. Macro- rather than microcultures were used because changing of tips slowed the inoculation procedure such that a successful test could not be conducted in panels. Except for the "change of tips" treatment, the experiment was conducted as described above.

Results (Table 4) indicate that there was no significant ($P > 0.05$) difference between the two treatment groups.

TABLE 3. *Means for between-day variation in microculture plaque neutralization titers for LaCrosse virus antiserum with the homologous virus*

Day	Mean[a]	Difference	t
1	8.56 ± 0.08 (3,774)	0.10	0.37 NS[b]
2	8.46 ± 0.06 (3,521)		

[a] Mean 50% neutralization end points averaged over 16 (day 1) or 24 (day 2) observations. Values in parentheses are uncoded ED_{50} dilution values.

[b] NS, Not significant ($P > 0.05$).

TABLE 4. *Comparison of the effect of discarding pipette versus using the same pipette between dilutions on 50% end point observations*

Condition	Mean[a]	Difference between means	t
Same pipette	8.41 ± 0.09 (3,401)	0.19	1.0 NS[b]
Different pipette	8.60 ± 0.14 (3,880)		

[a] Means of 12 observations per treatment. Values in parentheses are uncoded ED_{50} dilution values.

[b] NS, Not significant ($P > 0.05$).

DISCUSSION

This report describes a microculture PN test for California-group arboviruses that is as precise and quantitative as the standard PN test conducted in 60-mm petri dishes. It was demonstrated that there was no significant between-panel or between-day variation in determinations and that a single pipette could be used with all serum dilution levels within a given titration without a significant inoculum carry-over effect. These findings indicate that the microculture test can be used in place of the macroculture test with no compromise in the quality of data obtained.

We also describe the application of an experimental and statistical procedure that broadens the scope of the test as a quantitative method. The technique produces end points that are precise and interpolative, since they are computed by regression analysis. The advantage of using a range of data points in quantitating viral antibodies has been emphasized before (15, 16). In addition to using all data points, regression analysis has the further advantage of providing a measure of the variance of Y (observed PS values) about the regression line for any given X (serum dilution), hence permitting computation of the appropriate confidence interval (11). Since the midway point on the regression line is the point about which the confidence interval is usually at a minimum (11), the PS level chosen to estimate end points in the present study was 50% (i.e., the serum dilution that neutralized 50% of the plaques).

Ideally, data derived from a PN test, in addition to being precise and quantitative, should be amenable to valid statistical analysis. For example, a question commonly encountered in viral serology is whether the difference between titer values in a cross-neutralization test is significant, or, in an antibody response study,

whether the shape of a response curve is linear, quadratic, or cubic, etc. Adequate statistical analysis of end point data enables the investigators to avoid making empirical judgements in answering these questions.

The test described provides end points that meet the assumptions of one of the most powerful statistical tools available to the biologist (12), the analysis of variance. The assumptions of normality, additivity, and homogeneity of variance were met by the transformations applied to dilution and percentage values (12). The assumption of independence of observations was met by pairing every serum observation (i.e., every serum dilution tube and inoculated microculture) with an unneutralized control. Since each serum value was independent, the resulting error terms were random and independently distributed. In a statistical analysis of end point data, this provides a valid error term for testing significance of treatment effects.

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LITERATURE CITED

1. Burgdorfer, W., V. F. Newhouse, and L. A. Thomas. 1961. Isolation of California encephalitis virus from the blood of a snowshoe hare (*Lepus americanus*) in western Montana. Amer. J. Hyg. **73**:344-349.
2. Chiba, S., R. L. Striker, Jr., and M. Benyesh-Melnick. 1972. Microculture plaque assay for human and simian cytomegaloviruses. Appl. Microbiol. **23**:780-783.
3. Copper, P. D. 1967. The plaque assay of animal viruses, p. 243-311. *In* K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. III. Academic Press Inc., New York.
4. Early, E., P. H. Peralta, and K. M. Johnson. 1967. A plaque neutralization method for arboviruses. Proc. Soc. Exp. Biol. Med. **125**:741-747.
5. Lorenz, R. J. 1962. Zur Statistik des plaque testes. Arch. Gesamte Virusforsch. **12**:108-137.
6. Lorenz, R. J., and B. Zoeth. 1966. An estimation of the overlap bias in plaque assay. Virology **28**:379-385.
7. Mircampsy, H., and F. Rapp. 1968. A new overlay for plaquing animal viruses. Proc. Soc. Exp. Biol. Med. **129**:13-17.
8. Miura, T., and E. F. Scherer. 1962. Comparison of chicken embryonic cell cultures and mice for detecting neutralizing antibody to Japanese encephalitis virus. Use of microcultures for virus- and serum-dilution neutralization tests. Amer. J. Hyg. **76**:197-208.
9. Porterfield, J. A. 1964. The plaque inhibition test, p. 341-362. *In* J. F. Ackroyd (ed.), Immunological methods. Blackwell Scientific Publications, Oxford.
10. Reid, D. B. W. 1968. Statistical methods in virology, p. 104-124. *In* A. J. Rhodes and C. E. van Rooyen (ed.), Textbook of virology, 5th ed. Williams and Wilkins, Baltimore.
11. Snedecor, G. W., and W. G. Cochran. 1967. Statistical methods, 6th ed., p. 135. Iowa State University Press, Ames, Iowa.
12. Sokal, R. R., and F. J. Rohlf. 1970. Biometry. W. H. Freeman, San Francisco.
13. Svehag, S. E., and B. Mandel. 1964. The formation and properties of poliovirus neutralizing antibodies. I. 19s and 7s antibody formation: differences in kinetics and antigen dose requirement for induction. J. Exp. Med. **119**:1-19.
14. Thompson, W. H., B. Kalfayan, and R. O. Anslow. 1965. Isolation of a California encephalitis-group virus from a fatal human illness. Amer. J. Epidemiol. **81**:245-253.
15. Westaway, E. G. 1965. The neutralization of arboviruses. I. Neutralization in homologous virus-serum mixture with two group-B arboviruses. Virology **26**:517-527.
16. Westaway, E. G. 1965. The neutralization of arboviruses. II. Neutralization in heterologous virus-serum mixtures with four group-B arboviruses. Virology **26**:528-537.