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Titration of Dengue Viruses by Immunofluorescence in Microtiter Plates

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A fast, reliable, and inexpensive method was developed for titration of dengue viruses in microtiter plates with an indirect fluorescent-antibody technique. No significant differences were found in median infectious dose endpoints of samples titrated in microtiter plates as compared with titrations in multichambered slides.

Dengue viruses are among the most important arboviruses which infect humans and are among the most difficult to isolate and identify. A variety of techniques are used to assay infectivity of dengue viruses, including plaque formation (1), mosquito inoculations (6), and determination of endpoints in cell cultures by immunofluorescence (7, 9). Each of these techniques has its shortcomings. Plaque formation is difficult and can be relatively insensitive, depending on the cell line used; mosquito inoculation is sensitive but requires the maintenance of a mosquito colony; and determination of immunofluorescent endpoints often requires multichambered tissue culture slides that are expensive and may not be easily obtained around the world. This report describes a fast, reliable, and inexpensive alternative to these methods: titration with microtiter plates.

Our laboratory is presently evaluating dengue candidate vaccine viruses in mosquito vectors. This work entails titrations of a large number of mosquitoes orally infected with dengue virus and of the blood meals used in the experiments. The presence of infectious virus is assayed by detection of fluorescent foci in mosquito cell cultures by an indirect fluorescent-antibody technique. This basic technique was originally used to detect the presence or absence of LaCrosse and eastern equine encephalitis virus in mosquito suspensions (S. W. Hildreth, Ph.D. thesis, Yale University, New Haven, Conn., 1984). A procedure for titration of dengue virus by indirect fluorescent-antibody technique and commonly obtained 96-well, flat-bottom microtiter plates was developed, and results were compared with those of titrations in multichambered slides.

Dengue virus types 1, 2, 3, and 4 were obtained from Kenneth H. Eckels of the Walter Reed Army Institute of Research. Each virus tested consisted of a parental strain and a corresponding clonally derived candidate vaccine strain. All stock viruses were prepared in *Aedes albopictus* C6/36 cells and had titers determined directly or were passaged again in C6/36 cells and used to prepare infectious blood meals on which adult mosquitoes were fed. The suspensions tested were made by grinding individual mosquitoes with 1 ml of diluent, using a mortar and pestle.

Monoclonal antibodies were produced from hybridoma cell lines developed by Henchal et al. (2). Cells were provided by John T. Roehrig, Center for Disease Control, Fort Collins, Colo. Mouse ascitic fluids containing hybridoma-derived monoclonal antibodies were prepared as previously described (3). Antibodies were heat inactivated at 56°C for 30 min and stored at −20°C. Each lot was tested for specificity, and titers were determined by indirect fluorescent-antibody technique to determine the appropriate working dilution.

An established line of the C6/36 clone of *A. albopictus* (Singh) cells (4) was grown in L-15 (Leibovitz) medium containing L-glutamine (catalog no. DM-312; KC Biological, Inc., Lenexa, Kans.), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum. Cells were maintained in the same medium with only 2% fetal bovine serum. Cells were grown at 27°C in a humid incubator without CO_2.

Four replicates each of serial 10-fold dilutions of infectious virus suspension (Table 1) were inoculated into eight-chambered Lab-Tek tissue culture slides (0.10 ml per chamber; Miles Laboratories, Inc., Naperville, Ill.). Each well was seeded with 0.35 ml of a suspension of C6/36 cells containing 2×10^6 cells per ml. The slides were kept in a humid incubator at 27°C for 7 days.

For the microtiter plate assay, we used the above procedure, modified as follows. Eight or 16 replicates of each dilution of infectious virus suspension (Table 1) were inoculated into 96-well, flat-bottom microtiter plates (0.050 ml per well). Each well was seeded with 0.15 ml of a suspension of C6/36 cells containing 10^6 cells per ml. The plates were kept in a humid incubator at 27°C for 7 days.

After incubation, the slides were wet fixed in cold acetone (−20°C) for at least 10 min. To fix the cells in the plastic microtiter plates, we used a modification of the method of Pursell and Cole (8). Briefly, cell culture fluid was aspirated from each well with a 14-gauge, 1.5-inch (ca. 4.8-cm) needle attached to a vacuum system. The bevel of the needle was placed against the side of the well to prevent loss of cells during aspiration. Cold buffered acetone (0.02 M phosphate-buffered saline [PBS; pH 7.5]–acetone [1:3]) was then added to each well and the plates were incubated at room temperature for 30 min. After fixation, the buffered acetone was aspirated from each well. The plates and slides were air dried and stained immediately or stored at −20°C until staining and examination for viral antigen by indirect fluorescent-antibody technique.

The chambered slides were stained with a 1:100 (working) dilution of the specific dengue antibodies and incubated for 40 min at 37°C in a humidified chamber. After incubation, the slides were washed two times in PBS for 10 min each, rinsed in distilled water, and air dried. Then a 1:100 dilution

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TABLE 1. Comparisons of virus titers of dengue viruses in microtiter plates and multichambered slides[a]

Virus sample	Virus titer[b]	
	Microtiter plates	Chambered slides
Working stock virus		
Den-1 parent (Western Pacific 1974)	6.55 ± 0.15	6.00 ± 0.25
Den-1 vaccine (45AZ5)	6.93 ± 0.12	6.50 ± 0.24
Den-2 parent (PR-159)	6.55 ± 0.15	6.50 ± 0.31
Den-2 vaccine (PR-159/S-1)	8.36 ± 0.14	8.75 ± 0.22
Den-3 parent (CH 53489)	7.05 ± 0.11	6.75 ± 0.22
Den-3 vaccine (clone 24/28)	6.49 ± 0.15	6.25 ± 0.33
Den-4 parent (H-241)	6.43 ± 0.17	6.25 ± 0.22
Den-4 vaccine (H-241, Lot 1)	4.68 ± 0.12	5.00 ± 0.25
Blood meal preparations		
Expt 84002		
Den-1 parent (Western Pacific 1974)	7.68 ± 0.23	7.75 ± 0.22
Den-1 vaccine (45AZ5)	7.68 ± 0.19	8.00 ± 0.25
Expt 84004		
Den-1 parent (Western Pacific 1974)	6.80 ± 0.17	6.75 ± 0.22
Den-1 vaccine (45AZ5)	7.23 ± 0.15	7.50 ± 0.31
Mosquito suspensions[c]		
Den-1 vaccine (45AZ5) with *Aedes aegypti* (day 0, 27°C)		
1	5.30 ± 0.20	5.00 ± 0.25
2	4.80 ± 0.00[d]	3.50 ± 0.00[d]
3	3.80 ± 0.00	3.50 ± 0.00
4	5.13 ± 0.19	4.50 ± 0.31
Den-4 vaccine (H-241, Lot 1) with *Aedes albopictus* (day 21, 33°C)		
1	0	0
2	0	0
3	4.44 ± 0.19	4.50 ± 0.23
4	0	0
Den-1 parent (Western Pacific 1974) with *Aedes aegypti* (day 21, 33°C)		
1	4.36 ± 0.12	4.75 ± 0.22
2	4.05 ± 0.11	4.25 ± 0.22
3	5.05 ± 0.23	4.50 ± 0.31
4	3.80 ± 0.17	4.00 ± 0.25

[a] No statistically significant difference was found ($P < 0.05$) between the two methods.
[b] Titers are expressed as $\log_{10}$ (50% tissue culture infective dose) per milliliter ± standard error.
[c] Day of extrinsic incubation and ambient temperature are given in parentheses after the specific epithet of each test group of four (designated 1 through 4) mosquitoes.
[d] Estimates of standard error cannot be calculated by the Karber method.

of a commercial, fluorescein-conjugated, goat immunoglobulin G (IgG) anti-mouse IgG was added to each slide and incubated again for 40 min at 37°C in a humidified chamber. Cells were counterstained by adding Evans blue (0.0005%) to the conjugate. The slides were then washed as above and cover slips were mounted with PBS-glycerol (1:1).

The microtiter plates were stained by the same procedure as described above. Washes were done with an eight-channeled pipettor or an enzyme-linked immunosorbent assay plate washer. After washing, 0.10 to 0.20 ml of PBS-glycerol (1:3) was added to each well and incubated at room temperature for 30 min. The PBS-glycerol mixture was aspirated out of each well, leaving a thin film to cover the cells. The cover was sealed to the microtiter plate with tape.

The plates were inverted and examined with an epifluorescence microscope (Olympus model BH-2) equipped with a 20× long working distance objective, an HBO 100-W high-pressure mercury burner, and an IF-490 exciter filter. Negative and positive controls were included in all tests. Virus titers were calculated by the method of Karber (5).

Virus titers obtained by the microtiter plate system were comparable with those obtained by the multichambered slide system (Table 1). There were no statistically significant differences ($P < 0.05$) between the titers for particular samples by either method. It should also be noted that the standard errors of titers obtained by the microtiter system were generally markedly lower than those obtained in the chambered-slide system. This difference is due to the use of 8 or 16 replicates of each sample in microtiter plates as compared with only 4 in multichambered slides.

When we used the long working distance objective, examination of the microtiter plates for fluorescence presented no

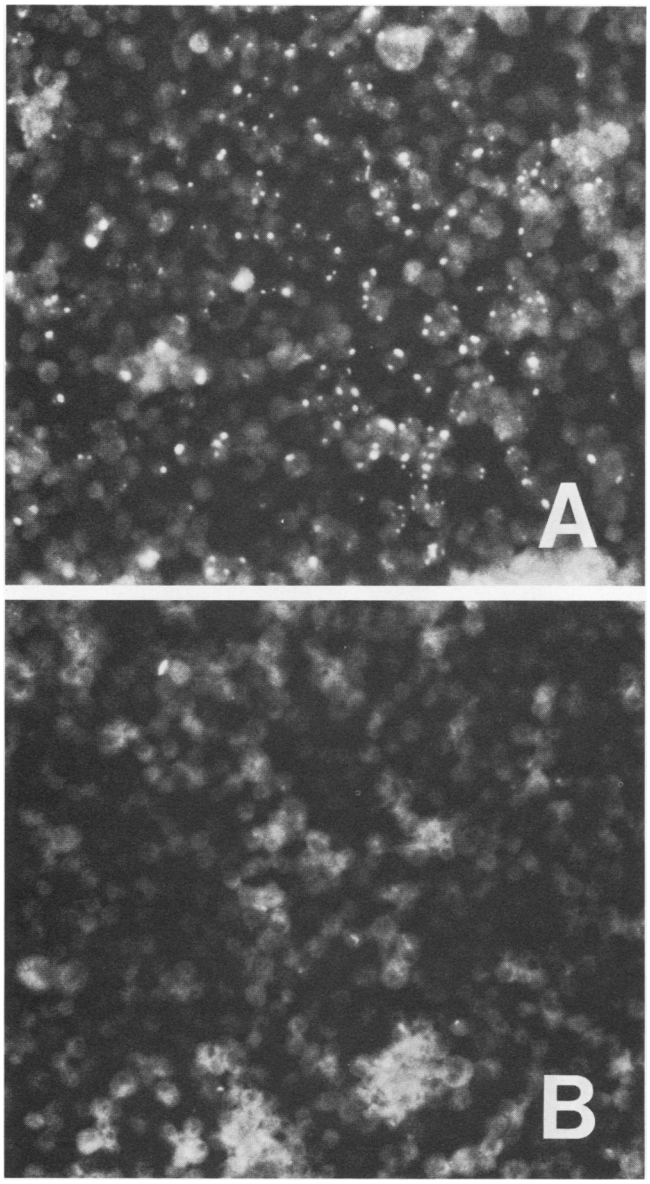

FIG. 1. (A) Fluorescing C6/36 *A. albopictus* cell cultures infected with dengue-1 parent virus (Western Pacific 1974); and (B) uninfected control C6/36 *A. albopictus* cell cultures (magnification, ×62.5).

real problem. Images viewed through the plastic bottom of each well were less distinct than with slides, but specific fluorescence could easily be distinguished from that in control wells (Fig. 1). The majority of the titrations were done in Costar (Cambridge, Mass.) 96-well tissue culture clusters with flat-bottom wells (catalog no. 3596); however, there appeared to be no difference in tests performed with similar plates from other manufacturers.

The microtitration system had three basic advantages over the multichambered slide system. (i) The cost of supplies was higher for titrations with slides. Titration of a single mosquito suspension required two slides at a current cost of $2.50 each. In a microtiter plate, titers could be determined for three mosquito suspensions, with twice as many replicates per suspension, at a current cost of $1.80 per plate. (ii) The biggest savings were in laboratory personnel man-hours. It was considerably quicker and easier to work with three mosquito titrations as a single unit than with six individual slides when adding samples, antibodies, or conjugate. There was no need for gasket removal, which at times can be a difficult process. In addition, washings during the staining procedure were greatly facilitated by the use of the enzyme-linked immunosorbent assay plate washer. (iii) Valuable reagents were conserved; the wells of the microtiter plate greatly reduced the amount of immune reagents needed to stain the cells.

Titration of dengue viruses by immunofluorescence in microtiter plates is a fast, reliable, and inexpensive technique and should be considered as an alternative to the use of multichambered slides.

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LITERATURE CITED

1. **Eckels, K. H., W. E. Brandt, V. R. Harrison, J. M. McCown, and P. K. Russell.** 1976. Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. Infect. Immun. **14**:1221–1227.
2. **Henchal, E. A., M. K. Gentry, J. M. McCown, and W. E. Brandt.** 1982. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am. J. Trop. Med. Hyg. **31**:830–836.
3. **Hudson, L., and F. C. Hay.** 1980. Hybridoma cells and monoclonal antibody, p. 303–327. *In* Practical immunology, 2nd ed. Blackwell Scientific Publications, Boston.
4. **Igarashi, A.** 1978. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. J. Gen. Virol. **40**:531–544.
5. **Karber, G.** 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reiheversuche. Arch. Exp. Pathol. Pharmakol. **162**:480–483.
6. **Kuberski, T. T., and L. Rosen.** 1977. A simple technique for the detection of dengue antigen in mosquitoes by immunofluorescence. Am. J. Trop. Med. Hyg. **16**:339–347.
7. **Miller, B. R., B. J Beaty, T. H. G. Aitken, K. H. Eckels, and P. K. Russell.** 1982. Dengue-2 vaccine: oral infection, transmission, and lack of evidence for reversion in the mosquito, *Aedes aegypti*. Am. J. Trop. Med. Hyg. **31**:1232–1237.
8. **Pursell, A. R., and J. R. Cole, Jr.** 1976. Procedure for fluorescent-antibody staining of virus-infected cell cultures in plastic plates. J. Clin. Microbiol. **3**:537–540.
9. **Tesh, R. B.** 1979. A method for the isolation and identification of dengue viruses, using mosquito cell cultures. Am. J. Trop. Med. Hyg. **28**:1053–1059.