Plaque Production by Arboviruses in Singh's *Aedes albopictus* Cells

C. E. YUNKER* and J. CORY

*National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana 59840*

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We report plaquing tests of 124 virus strains, mostly arboviruses of 21 serological groups, in Singh's line of *Aedes albopictus* cells. Thirty of these plaqued; all were arboviruses of six groups and were known or presumed to be mosquito borne. Failing to plaque were 86 strains of arboviruses, mostly tick borne, two strains of insect pathogens, and six animal viruses not classified as arboviruses. Among mosquito-borne agents, plaquing ability appeared related to serological classification. California group and most A-group viruses failed to plaque, but nearly all members of B and Bunyamwera groups readily plaqued. Within serological group B, 14 of 16 mosquito-borne agents plaqued, but none of 13 tick-borne or vector-unassociated viruses did so. Some implications of these results for recognition and classification of arboviruses are discussed.

In 1969 Suitor (20) reported that Japanese B encephalitis (JBE) virus, a member of Casals' serological group B known to cause cytopathogenic effect (CPE) in Singh's line of *Aedes albopictus* cells, was also capable of producing plaques in monolayers of these cells. However, the broad and practical implications of this demonstration for virus-vector studies remained questionable, possibly because CPE is characteristically inapparent in virus-infected arthropod cells in culture (3) and the strain of virus Suitor used was a unique variant that grows well at the lower temperatures usually required to incubate insect cells. Later, using virus strains not adapted to grow at low temperatures, we confirmed Suitor's observation of plaque production by JBE virus in *A. albopictus* cells and, further, reported similar results with five related Flaviviruses, West Nile, yellow fever, and dengue viruses 1, 2, and 4, and the unrelated rhabdovirus, Indiana vesicular stomatitis (7). In the present study we report results of plaquing attempts with 124 viruses, mostly arthropod borne, in Singh's *A. albopictus* cells.

**MATERIALS AND METHODS**

**Cell line and culture conditions.** The *A. albopictus* cell line of Singh (15), received by us in its 49th passage from Sonja Buckley, Yale Arbovirus Research Unit, New Haven, Conn., was used in its 87th to 135th passages. Since receipt at this laboratory, these cells have been grown as monolayers in a lactalbumin hydrolysate growth medium in Hanks basic salt solution (Grand Island Biological Co.) to which was added the following (per liter): 100 ml of fetal calf serum inactivated at 56°C for 1 h, 1 g of bovine plasma albumin (fraction V) (Armour Co.), 10^4 U of penicillin G (sodium salt), 100 mg of streptomycin sulfate, and 40 mg of neomycin sulfate. The pH was adjusted to 6.8 if necessary, and the medium was sterilized by pressure filtration. Stock cultures were grown as monolayers in 12 ml of medium in 250-ml plastic flasks (Falcon Plastics) in ambient air at 27°C and were subcultivated at intervals of 7 to 10 days.

Normal cell stocks were routinely monitored for the presence of adventitious or latent animal viruses, with negative results, by screening in suckling mice and Vero cell cultures. In addition, neither known virus nor virus-like particles could be detected in this particular strain of Singh's *A. albopictus* cells by electron microscopy thin-section technique (H. Hirumi, personal communication). Mycoplasma could not be demonstrated by testing of selected samples of cells (both plaquing virus infected and uninfected) by conventional methods and also by a commercial testing laboratory. Species identity of the *A. albopictus* cell line was confirmed by the Institute for Medical Research, Camden, N.J.

**Preparation of monolayers for virus inoculation.** Growth medium was decanted from confluent young monolayers of cells grown in four 250-ml flasks and replaced with fresh growth medium. Cells were dissociated with the aid of a 10-ml plastic pipette by vigorously drawing and expelling medium over the monolayers. This was facilitated by using a pipette on which the tip had been bent in a flame. Cell suspensions were pooled in a 100-ml Erlenmeyer flask and stirred by a magnetic stirrer adjusted to medium-slow speed. Cell density was adjusted to 2 × 10^5 to 3 × 10^6/ml with growth medium, and 30-ml Falcon plastic
flasks were each seeded with 5 ml of suspension. Cultures were incubated at 27°C for 2 to 7 days.

**Virus inoculation and assay.** Virus stocks, stored frozen usually as 10% mouse brain suspensions (vol/vol) in 7.5% bovine plasma albumin or 50% normal rabbit serum in 0.15 M phosphate-buffered saline, were mostly from the Rocky Mountain Laboratory collection. These were thawed and prepared as serial 10-fold dilutions in growth medium. Medium was decanted from cell monolayers, 0.2 ml of each virus dilution was introduced into a flask, caps were replaced, and the inoculum was distributed over the monolayer by tilting the flask. Cultures were then incubated at room temperature for 1 h on a rocking platform. Primary overlay medium consisted of 3 parts of growth medium mixed at 44°C with 1 part of molten 2% agarose (Seakem) in Hanks solution. This was brought to 41 to 42°C and introduced in 4-ml amounts into each flask by means of a needleless Cornwall syringe. Caps were replaced, the flasks were returned to a horizontal position, and the medium was allowed to solidify for at least 15 min. Cultures were incubated at 35 or 37°C for 5 to 7 days, whereupon 2 ml of secondary overlay medium was introduced into each. This medium was identical to primary overlay medium except that it contained 1.5% neutral red dye. Plaques were counted after 1 to 5 days of further incubation in the dark at 35 to 37°C, and titers were expressed as plaque-forming units per milliliter. Plaquing in *A. albopictus* cells was confirmed by replicate tests. Identities of certain plaquing viruses were established by plaque-reduction neutralization tests in *A. albopictus* or African green monkey kidney (Vero strain CCL-81) cells (9). In addition, comparative titers for some virus stocks were obtained through parallel inoculation of Vero cells. All virus stocks that failed to plaque in *A. albopictus* cells, except insect viruses, were demonstrated to possess levels of virus adequate to infect Vero cells or newborn mice.

**RESULTS**

Thirty of 124 virus strains tested produced plaques in *A. albopictus* monolayers. Those positive were members of the serological groups A, B, C, Bunyamwera, Kemerovo, and vesicular stomatitis (Table 1). Of the remaining 94 viruses failing to induce plaques in these cells, 39 were members of the first five groups mentioned, 47 were arboviruses of 16 miscellaneous serological groups or were not group associated, six were vertebrate viruses not arthropod borne, and two were mosquito pathogens (Table 2). Two lots of normal mouse brain, each tested twice as serial 10-fold dilutions, also failed to cause plaques or CPE in *A. albopictus* cells.

**Group A arboviruses.** Of nine viruses tested, only one, Chikungunya, formed plaques in *A. albopictus* monolayers (Fig. 1). Two or more attempts to induce plaques in these cells with each of the remaining eight failed; these viruses were Eastern equine encephalomyelitis, Mid-

delburg, O’nyong nyong, Semliki Forest, Sindbis, Una, Uruma, and Western equine encephalomyelitis.

**Group B arboviruses.** Fourteen of 28 viruses tested produced plaques: dengues 1, 2, 3, and 4, Ilheus, Israel turkey meningoencephalitis, JBE (two strains), Kunjin, Murray Valley encephalitis, Ntaya, St. Louis encephalitis, Uganda S, West Nile (three strains), and yellow fever (Fig. 2). Viruses of this group failing to plaque on two or more attempts were Apoi, Bussuquara, Kadam, Langat, Louping Ill, Modoc, Negishi, Omsk hemorrhagic fever, Powassan, Rio Bravo, Royal Farm, tick-borne encephalitis, Tuleniy, and Zika. Two of these, Bussuquara and Zika, unlike the remaining 12, consistently caused a well-defined CPE in agar-overlaid cells.

**Group C viruses.** Two of four viruses of this group, Marituba and Murutucu (Fig. 1), caused plaques; negative after two attempts were Apeu and Itaqui viruses.

**Bunyamwera group viruses.** Seven viruses of this group caused plaques: Bunyamwera, Cache Valley, Chittoor, Germiston, Guaroa, Ilesha, and Wyeomyia (Fig. 1). One virus, Kairi, failed to plaque, although it was tested three times. With four members of this group, optimal development of plaques required prolonged incubation periods of up to 12 days postinoculation. Here it was necessary to reduce incubation temperatures to 35°C to prevent deterioration of cells.

**Kemerovo group viruses.** Of nine members of this group tested only Tribeč virus formed plaques. Others tested, each two or more times, were Chenuda (two strains), Great Island, Huacho, Kemerovo, Lipovnik, Sixgun City, Wad Medani, and Yaquina Head (three strains) viruses.

**Vesicular stomatitis group viruses.** Both Indiana and New Jersey serotypes of vesicular stomatitis virus plaqued in *A. albopictus* cells. No other rhabdoviruses were tested.

Plaque-reduction neutralization tests performed in *A. albopictus* or Vero cells confirmed the identity of selected plaqueing viruses from each serological group. These were: Chikungunya, dengue 1, 2, and 4, Israel turkey meningoencephalitis, JBE, West Nile, yellow fever, Murutucu, Chittoor, Tribeč, and Indiana vesicular stomatitis.

Titers (dex plaque-forming units/ml) in *A. albopictus* cells were obtained for all viruses except one (Table 1). The exception was Guaroa, in which plaques were extremely large and diffuse. For comparison, titers in Vero cells were determined for 15 viruses (Table 1). These were comparable in both Vero and *A. albopictus* cells.
### Table 1. Plaque formation in A. albopictus cells by 27 arboviruses of six serological groups

| Virus name                  | Strain designation | Serological group | Vector | Virus stocks | Plaques in A. albopictus cells |
|-----------------------------|--------------------|------------------|--------|-------------|--------------------------------|
|                             |                    |                  |        | Passage history | Titer (log₁₀ plaque forming units/ml) | Avg diam (mm) | Read on day |
| Chikungunya*                | 23161              | A                | M      | m174        | 8.4                            | 8.6           | 4.0          | 6           |
| Dengue 1*                   | Hawaii             | B                | M      | m116        | 8.3                            | 8.3           | 3.5          | 7           |
| Dengue 2*                   | NGB                | B                | M      | m45         | 7.1                            | 8.6           | 3.5          | 7           |
| Dengue 3                     | H-87               | B                | M      | m23         | 6.0                            | 6.0           | 3.5          | 6           |
| Dengue 4*                   | H-241              | B                | M      | m9          | 6.6                            | 7.4           | 3.0          | 7           |
| Ilheus                      | 26                 | B                | M      | m28         | 5.5                            | 5.0           | 5.0          | 7           |
| Israel turkey meningo-encephalitis* | None         | B                | U      | m33         | 9.6                            | 10.3          | 5.0          | 6           |
| JBE                         | K-29               | B                | M      | m12         | 7.4                            | 7.6           | 4.5          | 6           |
| JBE*                        | Nakayama           | B                | M      | e41m6       | 10.0                           | 10.0          | 2.5          | 7           |
| Kunjin                      | MRM16              | B                | M      | m4          | 9.0                            | 8.0           | 2.5          | 7           |
| Murray Valley encephalitis | 4286               | B                | M      | m13         | 9.4                            | 9.4           | 5.0          | 9           |
| Ntaya                       | B25833             | B                | M      | m24         | 6.0                            | 6.0           | 3.5          | 9           |
| St. Louis encephalitis      | 798-55             | B                | M      | m34         | 8.0                            | 8.0           | 2.0          | 9           |
| Uganda S                    | B30987             | B                | M      | m22         | 4.3                            | 4.0           | 1.5          | 7           |
| West Nile*                  | 7259-60            | B                | M      | m27         | 9.0                            | 9.0           | 2.0          | 6           |
| West Nile                   | Ar95-60            | B                | U      | m6          | 9.0                            | 9.0           | 2.0          | 6           |
| West Nile                   | Ar108-60           | B                | U      | m7          | 9.0                            | 9.0           | 2.0          | 6           |
| Yellow fever*               | 17D                | B                | M      | x           | 6.0                            | 6.0           | 3.0          | 9           |
| Marituba                    | BeAn15             | C                | M      | m19         | 7.7                            | 7.7           | 5.0          | 7           |
| Murutucu*                   | BeAn974            | C                | M      | m13         | 8.3                            | 8.7           | 5.0          | 6           |
| Bunyamwera                  | 25510              | BUN              | M      | m38         | 10.0                           | 9.3           | 4.0          | 8           |
| Cache Valley                | 6V633              | BUN              | M      | m7          | 6.4                            | 6.4           | 2.5          | 6           |
| Chittoor*                   | AMM2222            | BUN              | M      | m18         | 8.2                            | 9.6           | 7.0          | 11          |
| Germiston                   | AR1050             | BUN              | M      | m12         | 7.6                            | 7.0           | 10.0         | 12          |
| Guaroa                      | 31498              | BUN              | M      | m2          | 9.0                            | 9.0           | 10.0         | 12          |
| Ilesha                      | KO/2               | BUN              | M      | m4          | 8.7                            | 8.7           | 2.5          | 6           |
| Wyeomyia                    | B26 380            | BUN              | M      | m207        | 9.7                            | 9.7           | 2.5          | 6           |
| Tribec*                     | VR 468             | KEM              | T      | m18         | 10.2                           | 8.2           | 2.0          | 6           |
| Vesicular stomatitis        | Indiana*           | VS               | M      | elm5        | 8.5                            | 8.0           | 7.0          | 5           |
| New Jersey                  | Hazelfurst         | VS               | U      | e20m1       | 9.4                            | 9.4           | 14.0         | 6           |

* Vector known or presumed to be: mosquito or other biting diptera (M), tick (T), or undetermined (U). West Nile strains Ar95-60 and 108-60 were isolated from ticks.

- Passage level of stock in mouse brain (m) or embryonated chicken egg (e), or of complex, high passage history (x).

- Identity confirmed by plaque-reduction neutralization test.

- Plaques were large, diffuse, and not countable.

for Chikungunya, dengue 4, Ilheus, Israel turkey meningoencephalitis, JBE, Uganda S, West Nile, Murutucu, Bunyamwera, and Indiana and New Jersey vesicular stomatitis viruses. Dengue 2 and Chittoor viruses gave significantly higher titers in the mosquito cells than in Vero cells, whereas Kunjin and Tribec virus titers were significantly lower in the former cells.

Of 146 plaquing attempts with viruses eventually determined to cause plaques in A. albopictus cell monolayers, 25 (17.1%) were negative. Among these, the rate of success was markedly higher with group B agents (87%) than with those of other groups (79%). Some factors involved in these rates are discussed below.

**Miscellaneous groups and ungrouped viruses.** Negative plaquing results were obtained with 30 arboviruses representing 16 serological groups and with 10 ungrouped arboviruses.
Table 2. Viruses failing to cause plaques in *A. albopictus* cells

| Virus name* | Strain designation | Serological group* | Vector* | Passage history* | No. of attempts |
|-------------|------------------|-------------------|---------|-----------------|----------------|
| EEE         | 85               | A                 | M       | m9              | 4              |
| Middleburg  | Ar749            | A                 | M       | m12             | 2              |
| O’nyong nyong| MP30             | A                 | M       | m12             | 3              |
| Semliki Forest*| (ATC)         | A                 | M       | m8              | 6              |
| Sindbis     | Ar339            | A                 | M       | m10             | 4              |
| Una         | BT1495-3         | A                 | M       | m9              | 2              |
| Uruma       | Mayaro           | A                 | U       | m9              | 3              |
| WEE         | 999-51           | A                 | M       | c1m6            | 3              |
| Apoi        | Apoi             | B                 | U       | m6              | 2              |
| Bussuquara* | BeAn4073         | B                 | M       | m13             | 4              |
| Kadam       | MP8640           | B                 | T       | m4              | 2              |
| Langat      | TP21             | B                 | T       | m9              | 2              |
| Louping Ill | 334-64           | B                 | T       | m1              | 4              |
| Modoc       | M544             | B                 | !       | m5              | 2              |
| Negishi     | —                | B                 | U       | m11             | 2              |
| Omak        | Guriev           | B                 | T       | ?+m1            | 2              |
| Powassan    | M794             | B                 | T       | m6              | 1              |
| Powassan    | M791A            | B                 | T       | m12             | 1              |
| Rio Bravo   | M64              | B                 | !       | m9              | 2              |
| Rio Bravo   | Burns bat        | B                 | !       | m15             | 2              |
| Royal Farm  | EgArt371         | B                 | T       | m6              | 2              |
| CETBE       | Hypr             | B                 | T       | ?m2             | 2              |
| Tuleniy     | 3-Arch           | B                 | T       | m3              | 2              |
| Zika*       | B24982           | B                 | M       | m147            | 4              |
| Apeu        | BeAn848          | C                 | M       | m5              | 2              |
| Itaqui      | BeAn12752        | C                 | M       | m5              | 2              |
| Anopheles A | 166              | ANA               | M       | ?+m2            | 1              |
| Bakau       | M2325            | BAK               | M       | m13             | 3              |
| Bluetongue  | Cal BT8          | BLU               | M       | e90m54          | 2              |
| Kairi       | TRVL8900         | BUN               | M       | m15             | 3              |
| CE          | BFS283           | CAL               | M       | ±m15            | 2              |
| La Crosse   | PR105826A        | CAL               | U       | m9              | 3              |
| Melao       | TRVL9357         | CAL               | M       | m2              | 2              |
| Snowshoe hare| MC3150          | CAL               | U       | m16             | 2              |
| Tahyna      | 92               | CAL               | M       | m21             | 2              |
| Trivittatus | 993              | CAL               | M       | m15             | 2              |
| Trivittatus | 7941             | CAL               | M       | m3              | 1              |
| Hazara      | JC280            | CON               | T       | m9              | 1              |
| Dugbe       | Ar1792           | GAN               | T       | m16             | 1              |
| Ganjam      | G619             | GAN               | T       | m8              | 3              |
| Farallon    | Ar846            | HUG               | T       | m5              | 1              |
| Hughes      | Original         | HUG               | T       | m11             | 1              |
| Punta Salinas| Ar888           | HUG               | T       | m3              | 1              |
| Raza        | 5/18/64          | HUG               | T       | m9              | 2              |
| Sapphire II | 14               | HUG               | T       | m3              | 1              |
| Chenuda     | Ar1170           | KEM               | T       | ?+m4            | 1              |
| Chenuda     | Ar1152           | KEM               | T       | m19             | 1              |
| Great Island| Main 45          | KEM               | T       | m3              | 1              |
| Huacho      | 883             | KEM               | T       | m6              | 3              |
| Kemeroovo   | R10             | KEM               | T       | m7              | 4              |
| Lipovnik    | 91              | KEM               | T       | ccl+m3          | 3              |
| Mono Lake   | Ar861           | KEM               | T       | m4              | 1              |
| Sixgun City | 52451           | KEM               | T       | v4m2            | 1              |
| Wad Medani  | Ar492           | KEM               | T       | m5              | 2              |
| Yaquina Head| 62              | KEM               | T       | m5              | 1              |
| Yaquina Head| 15              | KEM               | T       | m5v9            | 3              |
| Yaquina Head| 90              | KEM               | T       | v13             | 1              |
| Lanjan      | TP94            | LIN               | T       | m14             | 1              |
| Silverwater | 131             | LIN               | T       | m8              | 1              |
Table 2—Continued

| Virus name | Strain designation | Serological group | Vector | Passage history | No. of attempts |
|------------|--------------------|-------------------|--------|----------------|----------------|
| Midway     | Green Kure         | NYM               | T      | m13v1          | 1              |
| Nyamanini  | A1304              | NYM               | T      | m16            | 1              |
| Palyam     | G5287              | PAL               | M      | m2             | 1              |
| Sandfly fever | Naples         | PHIL              | M      | m55            | 1              |
| Johnston Atoll | LB39579       | QRF               | T?+m7  | m4             | 1              |
| Quaranfil  | Ar1113             | QYB               | T      | m7             | 1              |
| Qalyub     | Ar370              | QYB               | T      | m3             | 1              |
| Tacaribe   | TRVL11573          | TAC               | !      | m6             | 2              |
| Turlock    | MP847-32           | TUR               | M      | e1m8           | 2              |
| Uukuniemi  | S23                | UUK               | T      | m10            | 1              |
| Uukuniemi  | 56298-38           | UUK               | T      | m3v6           | 1              |
| Grand Arbaud | Argas27           | UUK               | T      | m6             | 1              |
| Jos        | Iban17854          | UNG               | T      | m5             | 1              |
| Lone Star  | TMAI381            | UNG               | T      | m4             | 1              |
| Matsu care | 21343              | UNG               | T      | m12            | 1              |
| Mut        | EqAn4906           | UNG               | T      | m9             | 1              |
| Sakkalin   | Lief-71c           | UNG               | T      | m8             | 1              |
| Sakkalin   | 56300-86           | UNG               | T      | m7             | 1              |
| Sapphire I | IXH5a              | UNG               | T      | m9             | 1              |
| Sawgrass   | PR96406            | UNG               | T      | m9             | 1              |
| Thogoto    | IIIA               | UNG               | T      | m9             | 1              |
| Upolo      | CS581              | UNG               | T      | m8             | 1              |
| Wanowrie   | G700               | UNG               | T      | m7             | 1              |
| Anoph eles B | 178                | UNG               | M      | ?+m2           | 2              |
| CTF        | Florio             | UNG               | T      | x+66m          | 1              |
| CTF        | SS18               | UNG               | T      | m7             | 1              |
| EMC        | EMC75c             | n.a.              | !      | m4v1           | 2              |
| HVH(II)    | (wild)             | n.a.              | !      | m2             | 2              |
| Mosquito iridescent | R        | n.a.              | !      | lar            | 5              |
| Mosquito iridescent | T        | n.a.              | !      | lar            | 2              |
| Poliovirus murius | GD7      | n.a.              | !      | m52v1m1        | 2              |
| Myxoma     | Moses              | n.a.              | !      | r?             | 2              |
| Pseudorabies | Aujeszky       | n.a.              | !      | r43m1          | 2              |
| Vaccinia   | Lilly              | n.a.              | !      | ?+v1           | 2              |

* CE, California encephalitis; CETBE, Central European tick-borne encephalitis; CTF, Colorado tick fever; EEE, Eastern equine encephalomyelitis; EMC, encephalomyocarditis; HVH(II), Herpesvirus hominis (type II); WEE, Western equine encephalomyelitis.

* ANA, Anoph eles A; BAK, Bakau; BLU, Bluetongue, BUN, Bunyamwera; CAL, California; CON, Congo; GAN, Ganjam; HUG, Hughes; KEM, Kemero vo; LJN, Lanjan; NYM, Nyamanini; PAL, Palyam; PHIL, Phlebotomus; QRF, Quaran fil; QYB, Qalyub; TAC, Tacaribe; TUR, Turlock; UUK, Uukuniemi; UNG, ungrouped; n.a., not applicable.

* Known or presumed to be: born by mosquito or other biting diptera (M), tick borne (T), borne by undetermined vector (U), not vector borne (!).

* Passage level of stock in wet chick (c), chicken embryo cells (cc), embryonated chicken egg (e), ground mosquito larvae (lar), mouse brain (m), rabbit tissue (r), Vero (African green monkey kidney) cells (v), or of complex, high passage (x) or unknown history (?).

* Well-defined CPE produced by this virus.

(Table 2). In addition, six viruses that are not arthropod borne, as well as two insect viruses, failed to induce plaques in A. albopictus cells (Table 2).

**DISCUSSION**

Since Suitor's original (20) demonstration of virus plaquing in insect cells, only two published reports describing use of this phenome non have appeared (7, 10). We believe that the rarity of these reports reflects the technical difficulties involved in preparing and maintaining satisfactory monolayers of insect cells. These cells, in comparison with many of vertebrate origin, grow more slowly and are less tolerant of manipulative and qualitative variables. Monolayers must be confluent and firmly attached to the vessels. In our experience,
Fig. 1. Plaque production in Singh's Aedes albopictus cells by some arboviruses of various serological groups. (1) Uninfected culture with overlay, day 6; (2) Chikungunya virus, day 6; (3) Murutucu virus, day 6; (4) Bunyamwera virus, day 6; (5) Ilesha virus, day 6; (6) Chittoor virus, day 11; (7) Wyeomyia virus, day 6; (8) Tribe virus, day 6. Scale, 0.7 x.

Inadequate confluence and poor attachment of cells are most often attributable to qualitative differences among lots of fetal calf serum. Such variations can generally be overcome by prolonged heat inactivation of the serum (1 h at 56 C). In addition, at various times we have been able to ascribe plaquing difficulties to advanced age of stock or assay cultures, excessive temperatures (>42 C) of primary overlay, and toxicity of some lots of agarose. Incubation temperatures of infected cells may also affect the development of plaques with certain agents. Many arboviruses, especially those of group B, plaqued best when overlaid cultures were incubated at 37 C (in fact, this temperature was mandatory for plaquing dengue 4 virus), but Bunyamwera group agents and Tribe virus yielded best plaques when cultures were incubated at 35 C. Also, as in vertebrate cells, high multiplicities of virus have often prevented plaque formation in A. albopictus cells. Thus, in screening for a plaquing virus, care must be
Fig. 2. Plaque production in Singh's *Aedes albopictus* cells by some group B arboviruses. (1) Uninfected culture with overlay, day 7; (2) dengue 2 virus, day 8; (3) dengue 3 virus, day 8; (4) dengue 4 virus, day 8; (5) Ilheus virus, day 7; (6) Israel turkey meningoencephalitis virus, day 6; (7) Kunjin virus, day 7; (8) Murray Valley encephalitis virus, day 9. Scale, 0.7 x.

Our results show that the plaquing technique in *A. albopictus* cells is applicable to a variety of arboviruses, nearly all of which are mosquito borne. Among these mosquito-borne agents, plaquing ability in these cells appears to be related to the serological group to which the virus belongs. California group viruses and most members of group A tested failed to plaque, but nearly all B group and Bunyamwera group viruses (the latter are related to those of the California group) readily did so. The possibility that such contrasting patterns may be used as classifying characteristics is noted.

Within serological group B the ability of a virus to plaque correlates well with its vector relationship. Except for two, all mosquito-borne or suspected mosquito-borne group B agents plaqued in the mosquito cells. The exceptions,
Bussuquara and Zika, did, in fact, produce a well-defined CPE in the cell monolayer after agar overlay, indicating that a plaquing potential may exist. Conversely, no tick-borne group B virus could be made to plaque despite repeated attempts. Also failing to plaque were group B viruses (Modoc and two strains of Rio Bravo) for which arthropod transmission is questioned on the basis of evidence supporting direct transmission from infected to uninfected hosts (1, 8, 11).

These results parallel those from studies of virus growth in fluid overlay cultures of Singh's A. albopictus cell lines, wherein group B viruses would propagate in the mosquito cells only if mosquito borne (3-5, 16, 17, 21). Obviously, plaquing ability may be used to infer vector relationships or corroborate suspected ones for group B isolates having undetermined modes of transmission. For example, Israel turkey meningoencephalitis virus, as yet unassociated with any arthropod vector and experimentally only poorly transmissible by Aedes aegypti (13), behaves as do mosquito-borne group B viruses in A. albopictus agar overlay cultures. Similarly, the two West Nile virus strains isolated by Schmidt and Said (14) from field-collected ticks (Ar95-60 and Ar108-60) are indistinguishable in this plaquing system from a strain isolated from the classical vector, mosquitoes (Table 1). This tends to support an alternative hypothesis proposed by these authors that "the actual source of the virus was blood from infected birds, and not from the tissues of ticks which were maintaining the virus biologically."

Additional group B viruses whose vector associations (or lack thereof) are suggested in part by results of this study are Apoi and Negishi. The former, known only from rodents in Japan, failed to multiply in Culex tritaeniorynchus mosquitoes that had fed on virus, and for this reason its status as an arbovirus has been questioned (19). Negishi virus, recovered only from two persons dying of encephalitis and antigenically a member of the Russian spring-summer encephalitis complex of group B arboviruses, also lacks demonstrated vector associations. Failure of these two agents to plaque in A. albopictus cells provides in vitro evidence that they are not mosquito borne.

Of 53 tick-borne agents tested, only one, Tribeč, caused plaques in the Aedes cells. This virus, along with Kemerovo, Lipovnik, and Colorado tick fever viruses, are placed in the "relatively solvent-resistant arbovirus" taxonomic group recently designated as Orbiviruses (2). Unlike mosquito-borne arboviruses of groups A and B, a number of tick-borne Orbiviruses, including the above-mentioned four, are capable of in vitro growth in arthropod cells that are unrelated to natural vectors of the arboviruses (4, 5, 22). For this reason we made repeated attempts to plaque Colorado tick fever, Kemerovo, and Lipovnik viruses, as well as certain well-known arboviruses (Semliki Forest, Sindbis, and California encephalitis) found to multiply in the A. albopictus cell line (3, 12, 16).

Uniformly negative results may indicate that virus replication, in these instances, is limited to a small proportion of the mosquito cells.

A potential application of the A. albopictus plaquing system is suggested by these results. The sensitivity and utility of Singh's A. albopictus cells for the isolation of various dengue virus types in epidemic situations was demonstrated by Singh and Paul (18) and Chappell et al. (6). However, identification of the isolates by plaque-reduction neutralization test requires their passage into LLC-MK2 cells (6). We show that the four major types of dengue viruses will produce clear plaques in the mosquito cells. Thus, if wild strains of dengue viruses will plaque as readily as the strains tested here, these cells may be used as a single system for the rapid isolation and identification of these viruses in routine screening or large-scale operations.

We conclude that the plaquing technique offers a means for direct assay of growth of a number of arboviruses in mosquito cells, that it is especially applicable to the study of viruses for which CPE in these cells has not been demonstrated, and that, with regard to the well-known (lipid-solvent susceptible) arboviruses as opposed to the Orbiviruses, it provides a useful device for determination of virus-vector relationships or a potential aid to arbovirus classification.

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ADDENDUM IN PROOF

Additional tests of Lipovnik virus have shown that it is capable of producing plaques, inconsistently, in A. albopictus cells.
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