Isolation of Chikungunya Virus Contaminating an 
*Aedes albopictus* Cell Line

*(Accepted 17 December 1974)*

**SUMMARY**

An *Aedes albopictus* cell line was found contaminated with structures morphologically compatible with an alphavirus. Rapid isolation of a cytopathic virus was effected by combining sonication, concentration with Aquacide II, rate zonal sedimentation and subsequent plating of fractions on Vero cells under agar overlay. The virus caused neither death nor disease on inoculation into infant and adult mice. It produced a c.p.e. in Vero and BHK 21 cells, and multiplied in Singh's *Aedes aegypti* cells. The virus was identified serologically as chikungunya by complement-fixation and plaque reduction neutralization test. Virus was not detected in a single attempt by these methods in the American Type Culture Collection *A. albopictus* line. The presence of chikungunya virus in *A. albopictus* cells is not easily recognized and may complicate interpretation of experimental results.

During studies of mosquito iridescent viruses of *Aedes taeniorhynchus* at Purdue University, some aspects of the research involved the use of Singh's *Aedes albopictus* cell line (Singh, 1967) for virus assays. Following experiments requiring 'temperature-stressing' of *A. albopictus* cells, light microscopy revealed the presence of polykaryocytes as well as massive syncytia formation in the uninoculated mosquito cells. Electron microscopy of such cells showed a cytoplasmic, virus-like contaminant, icosahedral in shape, measuring about 60 nm in diam. in thin sections (Fig. 1) and 65 nm in negatively stained preparations. Paracrystalline structures and occasional budding from cell membranes, i.e. findings characteristic of an alphavirus, were observed. Propagation attempts of these virus-like particles in bovine cell cultures were unsuccessful. Clonal selection of cells derived from cultures without overt c.p.e. and free of virus-like particles resulted in the establishment of a 'normal' *Aedes albopictus* cell line.

The starter cell line was kindly supplied by Dr I. Schneider, Walter Reed Army Institute of Research, who, in turn, had obtained it from Dr C. Barry, University of Maryland. The latter had purchased it in 1971 from the American Type Culture Collection. Studies with arboviruses were not being carried out by Dr Barry, Dr Schneider, and Dr Webb at the time the cell line was carried in their laboratories.

Both 'normal' and 'c.p.e.' cell lines were submitted to the Yale Arbovirus Research Unit (YARU) for identification of the virus-like contaminant. For control purposes, an *Aedes albopictus* cell line obtained in 1974 from the American Type Culture Collection ('ATCC' cell line) was examined simultaneously. The three sublines were tested for latent virus contamination as follows: (1) by conventional methods for arbovirus contamination (i.e. inoculation of infant mice, adult mice and plating of cells on Vero and LLC-MK, mono-layers under agar overlay), and (2) by combination of sonication, concentration with Aquacide II, rate zonal sedimentation and subsequent plating of fractions on Vero cells under agar overlay medium containing DEAE (Karabatsos, 1969). Cells were dispersed in 100 ml
of serum-free Mitsuhashi-Maramorosch medium (Mitsuhashi & Maramorosch, 1964) at a concentration of $4 \times 10^8$ cells/ml. The three cell pools, i.e. 'normal', 'c.p.e.' and 'ATCC', were processed identically, but on separate days. Cells were sonicated with a macroprobe (80 W power for cytoplasmic disruption) and clarified by sedimentation at 10000 rev/min for 30 min. The macroprobe was heat sterilized and the working area was decontaminated by u.v. irradiation between procedures. Concentration ($\times 10$) was carried out with Aquacide
Table 1. Results of complement-fixation test comparing ‘c.p.e.’ isolate with chikungunya and other alphaviruses

<table>
<thead>
<tr>
<th>Antibody</th>
<th>BHK 21, infected with c.p.e. isolate</th>
<th>BHK 21 uninoculated</th>
<th>Chikungunya TH 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chikungunya virus ascitic fluid, Ross strain</td>
<td>64/8*</td>
<td>o/o</td>
<td>32/32</td>
</tr>
<tr>
<td>Chikungunya virus serum, TH 35 strain</td>
<td>≥ 128/≥ 8</td>
<td>o/o†</td>
<td>≥ 128/64</td>
</tr>
<tr>
<td>Sindbis virus serum, EgAr 339 strain</td>
<td>16/2</td>
<td>o/o</td>
<td>o/o</td>
</tr>
<tr>
<td>VEE virus serum, TR 1945 strain</td>
<td>8/2</td>
<td>o/o</td>
<td>o/o</td>
</tr>
<tr>
<td>Control ascitic fluid</td>
<td>0/0</td>
<td>o/o</td>
<td>o/o</td>
</tr>
</tbody>
</table>

* Reciprocal of antibody titre/reciprocal of antigen titre; initial dilution of antibody 1:4, of antigen 1:2.
† Traces of reaction at dilution of antigen 1/2, of serum 1/8 or 1/16.
‡ Control ascitic fluid was prepared in mice inoculated with viruses unrelated to alphaviruses.

IIH for 14 h at 4°C. The concentrates were layered on 15 to 35% sucrose gradient, pH 8.0, and spun at 27000 rev/min for 3 h in a Spinco SW 27-1 rotor. Eight fractions (2·1 ml each) were collected from the bottom of cellulose nitrate Spinco tubes by gravity after puncturing with a 26 gauge needle; fractions were stored at −65°C and plaque-assayed on Vero cells thereafter.

The ‘c.p.e.’ subline showed peak infectivity in fraction 4 (4 × 10⁸ p.f.u./ml). Plaque morphology was irregular, both in size and shape, but was clearly recognizable 7 days post-inoculation. Pooled plaque progenies, when harvested with a curved pipette and dispersed into undiluted, inactivated (56°C for 30 min) foetal bovine serum, induced a moderate c.p.e. in BHK 21 cell cultures within 6 days following inoculation. After four serial passages, infected BHK 21 supernatant fluids were used as antigens in a complement-fixation (CF) test (Casals, 1967). Uninoculated cultures were used as controls (Table 1). The fluids were anti-complementary or gave non-specific reactions when used undiluted; at dilutions of 1:2 and higher, they were generally satisfactory. Two chikungunya virus immune reagents (strains Ross and TH 35) reacted to titre with the c.p.e. isolate used as antigen; trace reactions were noted against the BHK 21 control preparation. The reactions given by the Sindbis and Venezuelan equine encephalitis (VEE) virus immune sera were ascribed to group A cross-reaction, the titres being only a small fraction of the homologous titres (1:64 for Sindbis virus; 1:512 for VEE virus). In a plaque reduction neutralization test, using varying dilutions of virus and undiluted immune chikungunya (ATCC VR 64) ascitic fluid distributed by the National Institute of Allergy and Infectious Diseases, the log₁₀ (neutralization index) was 5·4 or greater. The ‘c.p.e.’ isolate was avirulent for mice. It multiplied in Singh’s Aedes aegypti cells showing that the contaminating virus is chikungunya and not o’nyong-nyong virus (Buckley, 1971). This chikungunya strain failed to produce polykaryocytes when tested in a subline of Aedes albopictus brought to Yale University by Dr. Singh in 1970.

A similar isolate was also obtained from the ‘c.p.e.’ subline by conventional plaquing without sonication, concentration or rate zonal sedimentation. However, appearance of plaques following plating of cells was slow (17 days after inoculation) and induction of c.p.e. in BHK 21 cell cultures was also slow (17 days after inoculation with plaque progenies). The ‘normal’ and the ‘ATCC’ sublines failed to yield any contaminating virus under our laboratory procedures.
It has been known since 1968 that *Aedes albopictus* cells may support the growth of chikungunya virus without causing c.p.e. (Banerjee & Singh, 1968) and that the virus may become apathogenic for mice in these cells (Banerjee & Singh, 1969). We are aware of another instance where mouse-pathogenic chikungunya virus was isolated as a contaminant of mosquito cells (B. H. Sweet, personal communication 1974). It is assumed in the present case that chikungunya virus, representing either an extraneous virus or a virus inherent to the cultured larvae themselves (Sanders, 1973), became cytopathic under the conditions of temperature-stressing of the cells.

This investigation was supported by U.S. Public Health Service Research Grants AI 09972, AI 11132, The World Health Organization, U.S. Army Contract DADA 17-72-C-2170, and by The Rockefeller Foundation. The senior author's work was done in partial fulfilment of the requirements for the degree of Master of Public Health at Yale University. We are grateful to Dr C. Barry, Dr I. Schneider, and also Dr H. Hirumi of Boyce Thompson Institute, Yonkers, for information and continued interest and for making unpublished data available to us. We thank Charles Mullen, Elinor Gilson and Mildred Malhoit for technical assistance.

**Yale Arbovirus Research Unit**
Yale University School of Medicine
New Haven, Connecticut 06510, U.S.A.

**Laboratory of Insect Pathology**
Department of Entomology
Purdue University
West Lafayette, Indiana 47907, U.S.A.

**REFERENCES**


(Received 5 November 1974)