to differences in pH, humidity, temperature and other physico-chemical factors. The short survival time in acidic fruits and vegetables was expected. Japanese workers[h] have shown that an acidic environment (pH $\leqslant$ 5.0) was harmful to *V. cholerae*. These same workers also pointed out that high osmolarity decreased vibrio viability. This would account for the low survival time in foods with a high percentage of sugar. Bengali sweets are famed for their extreme sweetness, and *Rossegolla* is not only made with a great deal of sugar but it is also soaked in sugar-cane syrup.

The results from these studies, in conjunction with those from other countries, point to the conclusion that excessive importation restrictions on goods coming from countries in which cholera is present or endemic are unwarranted.

[h] Miyaki, K., Iwahara, S., Sato, K., Fujimoto, S., & Aibara, K. (1967) *Bull. Wld Hlth Org.*, **37**, 773-778.

Isolation of Dengue Viruses in *Aedes albopictus* Cell Cultures

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The *Aedes albopictus* cell line[b] has been shown to be more sensitive than infant mice and Vero cells to infection with dengue viruses and the infection can easily be detected on the basis of the characteristic cytopathic effect produced by these viruses in the inoculated cultures.[c, d] In view of these findings, a study was undertaken to assess the utility of this cell line for the primary isolation of dengue viruses from sera and mosquitos.

Altogether, 25 human sera collected from cases of dengue-like illness from various places in India during the last 5 years were used in this study. Dengue viruses of types 1, 2, 3 or 4 had been isolated from most of these sera during earlier studies in this laboratory by intracerebral inoculation in infant mice or in vertebrate cell cultures or in both. As it was found that the undiluted sera had anticellular effects leading to rapid cell lysis in *A. albopictus* cultures, the sera were diluted 1 : 10 in 0.75% bovine albumin in phosphate saline (BAPS) buffered to pH 7.2 prior to their inoculation in these cultures.

Each diluted serum was inoculated in batches of 4 culture tubes; 0.1 ml/tube. Two hours after incubation at 30°C, the cultures were washed twice, the growth medium was replaced, and the cultures were reincubated at 30°C in a stationary position. They were observed daily for 10 days for the appearance of cytopathic effects. The cultures showing cytopathic effects were frozen and thawed 3 times and the tissue culture fluid from 4 tubes of the same batch was pooled and centrifuged at 300 *g* for 10 minutes. The supernatant fluid was used either for further passages or as an antigen for identification in complement-fixation (CF) tests.[e] Most specimens required only 1, or sometimes 2, passages before they could be identified.

As the human sera used in this study had been stored at a temperature of −50°C for long periods (between 1 and 5 years), and had been thawed more than once during this period, they were inoculated in 10^{-1} dilutions intracerebrally into infant mice and some of them also in Vero cell cultures simultaneously with the inoculation in *A. albopictus* cell cultures to cross-check the presence of virus in the samples. The inoculated mice were observed for 21 days for signs of sickness and the survivors were challenged intracerebrally with the TR-1751 strain of dengue type 2 virus to test for resistance to the challenge virus.[f] Inoculated Vero cell cultures were observed only for cytopathic agents and the interference technique for detecting the viruses was not used.

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[b] Singh, K. R. P. (1967) *Curr. Sci.*, **36**, 506-508.

[c] Singh, K. R. P. & Paul, S. D. (1968) *Curr. Sci.*, **37**, 65-67

[d] Paul, S. D. & Singh, K. R. P. (1969) *Curr. Sci.*, **38**, 241-242.

[e] Pavri, K. M. & Ghosh, S. N. (1969) *Bull. Wld Hlth Org.*, **40**, 984–986.

[f] Paul, S. D. et al. (1965) *Ind. J. med. Res.*, **53**, 777-789.

It was possible to isolate dengue viruses from 22 out of the 25 samples of human sera tested in *A. albopictus* cultures. The isolates, which were later identified as dengue types 1 or 2, produced a progressive syncytial type of cytopathic effect which involved the whole cell sheet, leading finally to its complete lysis. The dengue types 3 and 4 produced a non-progressive type of cytopathic effect with small foci of syncytial formation and predominantly cytolytic effect but without leading to the complete lysis of the cell sheet even after 14–15 days of infection. However, fairly extensive syncytial formations could be seen in masses of cells which had become detached from the glass wall and were floating freely in the supernatant medium.

In all, 22 out of 25 groups of mice inoculated with serum samples showed resistance to the challenge virus. Of the 18 serum samples also inoculated in Vero cells, only 11 produced cytopathic effects in these cells while 15 were cytopathic to *A. albopictus* cells. The 3 serum samples from which no cytopathic agent could be isolated in *A. albopictus* cells also failed to protect mice against subsequent virus challenge. Only first-passage inoculations were observed and no blind passages were made in either infant mice or in the 2 cell cultures.

Dengue type 2 has been previously reported to produce cytopathic effects in Vero cells.[g] In the present studies all the 4 type 1 and 7 out of 11 dengue type 2 viruses were cytopathic to Vero cells. In *A. albopictus* cultures all 15 of the dengue 1 and 2 strains were cytopathic.

Five of the isolates were titrated in *A. albopictus* cultures to assess the extent of viral multiplication. Results showed that even after 1 or 2 passages in these cultures, the dengue type 1 and 2 virus strains could multiply to high enough titres to give 50% cytopathic endpoints at 4.75 dex[h]/ml–≥6.5 dex/ml.

In addition, attempts were made to isolate dengue viruses from mosquitos. At the time of this study, naturally infected mosquitos were not available and laboratory-reared *Aedes aegypti* were therefore infected by intrathoracic inoculations with sera (1 : 10 dilution) representing all 4 serotypes of dengue viruses. After keeping the inoculated mosquitos for 10–13 days at 30°C and 80%–85% relative humidity, they were distributed in pools of 1–5 mosquitos. Each pool was triturated in 1.5 ml of BAPS containing 1000 IU of penicillin, 1.0 mg of streptomycin and 4 μg of amphotericin B per ml; the suspensions were centrifuged at 4°C and 12 100 *g* for 1 hour. Similar suspensions prepared from pools of 10 uninoculated mosquitos per pool were used as controls. The virus isolation and identification procedures used for mosquito suspensions were the same as those described for human sera.

Altogether, 8 suspensions prepared from mosquitos inoculated with dengue type 1, 9 with dengue 2, 3 with dengue 3, 5 with dengue 4 and 5 from uninoculated mosquitos were processed in *A. albopictus* cell cultures. All of the suspensions prepared from pools of infected mosquitos produced a rapidly progressive cytopathic effect, while uninfected suspensions did not show any cytotoxic or cytopathic effect on *A. albopictus* cells.

Most of the isolates were identified in CF tests using tissue-culture fluids as antigen. Though the antigen titres were not very high, the results were clear enough to identify the isolates. The CF titres of antigens from mosquito isolates were higher than those from human sera. The identification results correspond exactly with the results obtained during previous studies.

Attempts were also made to identify the isolates by neutralization tests in *A. albopictus* cell cultures. However, antisera against various dengue types neutralized the different type strains to more or less the same titres, thus excluding the possibility of any diagnostic interpretations.

This study has shown that dengue viruses belonging to all 4 serotypes could be isolated directly from human sera and mosquito suspensions on the basis of characteristic cytopathic effects produced by these viruses in *A. albopictus* cell cultures. All 4 serotypes of dengue viruses were found to multiply in this cell line to fairly high titres so that tissue-culture fluids could be used directly as antigens for the preliminary identification of isolates in CF tests. The 2 types of cytopathic changes in *A. albopictus* cells caused by dengue types 1 and 2 on the one hand, and types 3 and 4 on the other, could also be of help in rapid preliminary differentiation of these 2 groups. It was also observed that preinoculation of human sera in mosquitos facilitated the isolation and identification of dengue viruses, probably by enhancing the infective virus titres.

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[g] Balaya, S. et al. (1969) *Ind. J. med. Res.*, **57**, 767-774.

[h] dex=log units to the base 10 (Haldane, J. B. S. (1940) *Nature (Lond.)*, **187**, 879).