ISOLATION OF GETAH VIRUS FROM MOSQUITOS COLLECTED ON HAINAN ISLAND, CHINA, AND RESULTS OF A SEROSURVEY

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Abstract. An isolate of Getah virus was obtained from *Culex* mosquitos collected in Mao'an Village, Baoting County, Hainan Province, China, in 1964. The virus (strain M-1) replicated in laboratory-bred *Aedes aegypti* and *Cx. fatigans* (= *quinquefasciatus*), and was transmitted by laboratory-bred *Ae. albopictus* to healthy newborn albino mice. Skeletal muscles of newborn albino mice experimentally infected with the virus showed degeneration, atrophy, necrosis, and inflammatory changes of muscle fibers. Antibody prevalence in humans and animals ranged from 10.3% by neutralization tests of samples from healthy people in 1979 to 26.4% by CF tests of samples from people with febrile illnesses in 1982. The high prevalence of antibody in pigs, horses, and goats (17.6% to 37.5%) indicated that infection with Getah or a closely related virus is relatively common in domestic animals.

INTRODUCTION

Getah virus, a member of the Semliki Forest antigenic complex (family Togaviridae, genus *Alphavirus*) was first isolated from *Culex (Culex) gelidus* mosquitos collected in Malaysia in 1955 (American Committee on Arthropod-borne viruses, 1985). Since that time Getah virus has been isolated from *Culex, Anopheles,* and *Aedes* species mosquitos in Malaysia, Cambodia, Japan, Australia, Philippines, Taiwan, and eastern Russia (American Committee on Arthropod-borne Viruses, 1985), from Sri Lanka (JSM Peiris, unpublished data), and from a healthy pig in Japan (Matsuyama *et al,* 1967). In 1978 an epizootic of this virus occurred in race horses in Japan (Kamada *et al,* 1980). Fever, rash, edema of the hind legs were the primary symptoms, but none of the animals developed paralysis or died. Nevertheless, this virus is considered an important veterinary pathogen because the illness it causes incapacitates the animal.

During investigations on arboviral diseases in the Wu Zhi Mountain Area (Long 109° 07′ E, Lat 18° 09′ N) of Hainan Island, China, in 1964 a virus isolate was obtained from a pool of *Culex* species mosquitos captured from nature. This article reports the results of the following studies: characterization of the biologic and physicochemical properties of the virus, serologic identification, an experiment in which the virus was transmitted to newborn albino mice by *Ae. albopictus,* and a limited seroepidemiologic investigations on the sera of humans and animals.

MATERIALS AND METHODS

Virus isolation

Hainan Island is off the south coast of China, directly east of northern Vietnam and southeast of Guangzhou on the Chinese mainland. Collection of mosquitos was with a hand-held suction device in an area with various bushes and grasses, banana trees, coconut palms, rubber trees, and a pig sty beside a stream in Mao'an Village (Long 109° 07′ E, Lat 18° 07′ N), Baoting County, Hainan Island. Mosquitos for virus surveillance were collected for 1-2 hours in early evenings in the summer of 1964. Mosquitos were placed in dry ice immediately after capture. In the laboratory they were separated according to place of capture and species and tested in pools of 30-60 mosquitos. For virus testing, mosquitos were washed four times with sterile physiologic saline and macerated in a sterile mortar. Diluent used was 5% sterile egg yolk in phosphate-buffered saline (pH 7.6) containing penicillin 1,000 units/ml and streptomycin 1,000 μg/ml.

After low speed centrifugation, the supernatant fluid was injected into 1- to 3-day-old albino mice (0.01 ml intracranially and 0.1 ml intramuscularly), and the mice observed for 21 days. Litters of fresh mice were inoculated with suspensions of brains of mice that showed signs of illness. Animal inoculation, cell cultivation, plaque formation, and sensitivity to ether and hemagglutination experiments were carried out according to standard procedures (Shope and Sather, 1979).

Serologic identification of the isolate

Hemagglutination-inhibition (HI), complement-fixation (CF), and neutralization (N) tests were done according to standard procedures (Shope and Sather, 1979; Hunt and Calisher, 1979) Reference viruses and antibodies to them were available from the Centers for Disease Control. Alphaviruses for N tests were: Getah (MM-2021), Sagiyama (Original), Bebaru (MM-2354), chikungunya (S-27), Sindbis (EgAr-339), and Mayaro (TRVL-4675). HI, CF and virus dilution N tests of were done with a field isolate that had been passed three times in suckling mouse brain.

Antibodies against eastern equine encephalitis, western equine encephalitis, Venezuelan equine encephalitis, Japanese encephalitis (Beijing 1 strain from the China Central Institutes of Health), and yellow fever (17D vaccine strain) viruses were prepared in rabbits or guinea pigs. Immune ascitic fluids against other alphaviruses and against prototype West Nile, Japanese encephalitis, Murray Valley encephalitis, dengue types 1-4, Langat, and Bunyamwera viruses were prepared in mice (Brandt *et al,* 1967).

Mouse brain and mouse muscle antigens were prepared with sucrose-acetone; cell culture antigens were prepared by the same method from supernatant fluids of infected cells (Clarke and Casals, 1964).

Observation on the replication of the virus isolate in mosquitos

Aedes albopictus and *Cx. fatigans* used in these experiments were laboratory-bred Chenzhou strains which had been shown to have no pathogens for newborn mice or for cell cultures. Intrathoracic inoculation of the virus isolate into mosquitos was carried out according to the method of Rosen and Gubler (1974).

Experimental transmission

Mosquitos which had been enclosed for about 10 days and starved for 2 days were allowed to feed on infected (viremia titer $10^{4.5 \text{ to } 5.5}$ $\log_{10}$ LD_{50}) mice for about 4 hours. After being chilled to immobility, mosquitos which had fed were selected for breeding until about 10 days after laying eggs. They were subsequently allowed to feed on healthy newborn albino mice for 2-8 hours, and these mice were observed daily for 21 days for signs of illness. Brains were taken from sick mice for preparation of brain antigen or for inoculation into susceptible cell cultures to prepare cell culture antigen.

Assay of antibody in human and animal sera

Serum specimens from Hainan Island were collected from 128 healthy 15- to 50-year old persons living for more than two years in Baoting County in 1979 and from 58 people in Haikou City (Long 110° 03′ E, Lat 20° 01′ N), in 1982; from 91 patients with fever of unknown cause (June 1980 and May 1982); from 39 healthy pigs about 1 year of age (1979 and 1982); from 16 horses and mules transported to Hainan Island more than two years before (1979); and from 16 black goats purchased from Wenchang County (Long 110° 07′ E, Lat 19° 06′ N), Hainan Island (1982).

Preliminary N tests were qualitative virus dilution assays. An 0.1-ml aliquot containing 20-100 $TCID_{50}$ virus was added to an equal volume of serum diluted 1 : 5 in Hanks' medium containing 10% heat-inactivated fetal bovine serum. If this serum dilution neutralized more than 50% of the inoculum, the serum was retested at 1 : 10 for titer by virus dilution N. All virus dilution N tests were done using primary golden hamster kidney monolayer cell cultures. Serum dilution-plaque reduction N tests were done using Vero cells.

RESULTS

Virus isolation

A total of about 10,000 mosquitos collected in 1964 from the Wu Zhi Mountain Area, Hainan Island, were tested for virus. A virus strain (M-1) was isolated from a pool of 50 *Culex* species mos-

quitos collected on July 14, 1964. On the twelfth day after inoculation one of 10 inoculated newborn albino mice developed illness, manifested as prostration followed by paralysis of the hind limbs. The brain was taken for passage, and these and all further mice in which the virus was passaged became ill and died 3 to 7 days after inoculation. Cultures of brain suspensions showed no growth of bacteria.

Biological characteristics of strain M-1

A 10% suspension of brains of mice infected with strain M-1 was inoculated into each of three groups of newborn albino mice by intracranial (ic), subcutaneous (sc), or intraperitoneal routes, and induced illness and death in all mice. After ic inoculation of strain M-1 into 2- to 3-week-old mice (body weight, 7-12 g), illness and death occurred in only about 20%. None of >12-g mice, about 260-g guinea pigs, or about 750-g rabbits became ill after ic inoculation with strain M-1. The LD_{50} titers in newborn mice inoculated with 20 µl of virus ic and sc were $10^{6.5}$ and $10^{6.7}$ per ml, respectively. The LD_{50} titers of strain M-1 in whole blood and in the brain of symptomatic mice of the same litter were $10^{5.5}$ and in muscle, $10^{6.5}$. After strain M-1 was treated with ether, the LD_{50} titer in mice was reduced by $10^{3.7}$.

The following virus titers were found: in serially propagated Vero cells $10^{7.5}$ LD_{50}/ml, in primary gold hamster kidney monolayer cell cultures $10^{6.0}$ LD_{50}/ml, and in primary albino mouse kidney $10^{4.5}$ LD_{50}/ml. Cytopathic changes appeared rapidly and were complete by 48 hours after inoculation. These changes were manifested as an increase in intracellular granules, cell shrinkage, a mild degree of cell aggregation, cell disruption, and by cells becoming detached from the surface of the container in which they were grown. C6/36 *(Ae. albopictus)* cells irregularly showed confluent cytopathic changes. Primary human embryonic kidney cells did not appear to be affected.

In chick embryonic fibroblast cells, plaques formed within 35 hours. These gradually increased in size and in clarity, reaching 2-4 mm in diameter by 72 hours; the plaque-forming unit titer in these cells was about 10^7/ml. In serially propagated newborn golden hamster kidney (BHK-21) cells plaques reached 3-5 mm in diameter at 72 hours, with distinct a outline; the plaque-forming unit titer in these cells was $10^{6.4}$/ml. Five plaques isolated in BHK-21 cells were picked and inoculated into newborn mice. The incubation period, manifestations and LD_{50} titer of ic infection and those of sc infection were the same. Immune ascitic fluids were prepared separately from these five plaques and used to test antigen of strain M-1 by HI. Antibody titers were 1 : 40-1 : 80, suggesting that there were no gross differences between these subpopulations.

Hemagglutination titers of mouse brain antigen were <10, titer of muscle antigen was 320. Titers of antigen prepared from infected C6/36 cells were as high as 640. The optimal pH for hemagglutination with muscle antigen was 6.2 and with C6/36 cell culture antigen 5.75 or 6.0. The optimal temperature was 4°C; however, satisfactory results were also obtained at room temperature.

Pathologic changes in tissues of mice infected with strain M-1

By light microscopy, degeneration, atrophy, necrosis, and inflammatory changes could be seen in the skeletal muscle fibers of diseased mice. Some of these mice had encephalitic changes of various degrees, mostly seen in the anterior part of the brain. Lymphocytes in the cerebral cortex exhibited extensive necrosis and shrunken and fragmented nuclei. By electron microscopic examination, many incomplete virions were seen in the myelin sheath, and many mature virus particles were seen in muscle cells. Virus particles were 50-60 nm in diameter, round, and contained an envelope with surface projections. Most virus particles were found to aggregate in the distended cisterns of the sarcoplasmic reticulum or were seen in crystalline arrays in the severely degenerated muscle fibers; some were present in the extracellular spaces. Extracellular virus particles, arranged like strings of beads, were seen between the undersurface of the sarcoplasmic membrane and the basal membrane.

Replication and transmission of strain M-1 in mosquitos

Laboratory-bred *Ae. albopictus* and *Cx fatigans* were inoculated intrathoracically with about 10^3 LD_{50} of strain M-1 and the LD_{50} titer of these mosquitos was determined by titration at daily intervals for 20 days. Results showed that strain

Table 1

Cross-neutralization of M-1 virus with six other alphaviruses.

Virus	Strain	Titer of antibody to virus: M-1	GET	SAG	BEB	CHIK	SIN	MAY
	M-1	160	1,280	320	<10	<40	<20	10
Getah	MM-2021	80	1,280	160	<10	<40	20	<10
Sagiyama	Original	20	80	≥5,120	<10	<40	20	<10
Bebaru	MM-2354	<10	<10	<40	80	<40	<10	<10
Chikungunya	S-27	10	10	40	<10	≥1,280	20	<10
Sindbis	EgAr-339	10	<10	<40	<10	<40	320	<10
Mayaro	TRVL-4675	10	<10	<40	<10	<40	20	80

Table 2

Neutralizing antibody against M-1 virus in human and animal sera, Hainan Island, China.

Species	Location[a] and year	No. tested	No. with antibody by test[b] (%)		
Human	BT 1979	128	14	(N)	(10.9)
	H 1980/1982	91	24	(CF)	(26.4)
	HK 1982	58	2	(CF)	(3.4)
Pig	BT 1979	17	3	(N)	(17.6)
	BT 1982	22	5	(CF)	(22.7)
Equines	BT 1979	16	4	(N)	(25.0)
Goat	WC 1982	16	6	(CF)	(37.5)

[a] BT = Baoting County, H = Hainan Island-wide collection of sera from febrile patients, HK = Haikou City, WC = Wenchang County.
[b] N = neutralization, CF = complement-fixation.

M-1 reached maximum titers in *Ae. albopictus* and *Cx. fatigans* about five days after inoculation, with LD_{50} titers of $10^{5.0}$ and $10^{5.4}$, respectively.

Four of 5 mice fed upon by infected laboratory-bred *Ae. albopictus* mosquitos became ill 3-6 days later. Seven control mice from the same litter were observed to be healthy after 15 days. Antigen prepared from the brains of sick mice was identified as M-1 antigen by HI; mice inoculated with the brains of these mice developed antibody to strain M-1.

Serologic identification of strain M-1

A cross HI test was performed between M-1 and various flaviviruses, but results were all negative, except for homologous controls. When other HI tests indicated that strain M-1 was an alphavirus, subsequent antigenic comparisons with other viruses of this genus were done. Results indicated that strain M-1 is essentially identical to Getah virus (Table 1).

Antibody survey in humans and animals

Antibody against strain M-1 was determined in serum samples collected from healthy humans and from animals 1979-1982; test results are summarized in Table 2. Unfortunately, assays used were not uniform due to personnel changes at that time. Twenty-six percent of serum specimens from patients with fever of unknown cause on Hainan Island in June 1980 and May 1982 had CF antibody to strain M-1, in contrast with 10.9% of serum specimens from healthy people in Baoting County tested by N in 1979 and 3.4% from healthy people in Haikou City tested by CF in 1982. Nearly 18%

of pigs tested by N and 22.7% of pigs tested by CF, 25% of horses and mules tested by N, and 37.5% of goats tested by CF also had antibody.

DISCUSSION

In its ability to induce pathologic changes in muscles of newborn albino mice, the M-1 strain of Getah virus is similar to Ross River virus (Murphy *et al,* 1973), another member of the Semliki Forest antigenic complex.

Although Getah virus has been isolated from many countries in Asia and from Australia, to our knowledge this is the first report of an isolate of Getah virus from China. The distribution of this virus is the rest of China remains to be determined.

Seroepidemiologic investigations showed that Getah or a closely related virus infects humans and animals on Hainan Island. Antibody, essentially equally distributed by age group in 15- to 50-year old local people, suggests that the virus is endemic in Hainan Province. That the prevalence rate of antibody to strain M-1 was markedly higher in people with fever than in healthy people suggests a possible etiologic role for this virus in febrile illnesses on Hainan Island. Clinical, epidemiologic, and virologic studies of this virus on Hainan Island, in nearby areas of mainland China, and in other countries of Asia are needed.

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