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ISOLATION OF JAPANESE ENCEPHALITIS VIRUS FROM MOSQUITOES COLLECTED IN SABAK BERNAM, SELANGOR, MALAYSIA IN 1992

I. VYTHILINGAM,[1] K. ODA,[2] T. K. CHEW,[1] S. MAHADEVAN,[1] B. VIJAYAMALAR,[1] K. MORITA,[3] H. TSUCHIE[4] AND A. IGARASHI[3]

ABSTRACT. Detection and isolation of Japanese encephalitis (JE) virus were attempted from female mosquitoes collected in Kampong Pasir Panjang, Sabak Bernam, Selangor, from May to November 1992. A total of 7,400 mosquitoes consisting of 12 species in 148 pools were processed and inoculated into *Aedes albopictus* clone C6/36 cell cultures. Of these, 26 pools showed the presence of viral antigens in the infected C6/36 cells by specific immunoperoxidase staining using an anti-JE virus polyclonal antibody. Presence of JE virus genome was confirmed in the infected culture fluid for 16 pools by using reverse transcriptase-polymerase chain reaction and JE virus-specific primers. Of these, 3 pools were from *Culex tritaeniorhynchus,* 4 from *Culex vishnui,* 3 from *Culex bitaeniorhynchus,* 2 from *Culex sitiens,* one from *Aedes* species, and 3 from *Culex* species. Isolation of JE virus from *Cx. sitiens, Cx. bitaeniorhynchus,* and *Aedes* sp. (*Aedes butleri* and *Ae. albopictus*) is reported for the first time in Malaysia.

INTRODUCTION

Japanese encephalitis (JE) virus is common over a wide part of Asia. The natural transmission cycle involves *Culex tritaeniorhynchus* Giles and other closely related *Culex* mosquitoes that breed primarily in rice fields and feed on man in the early evenings (Shope 1990).

Japanese encephalitis is endemic and occurs sporadically throughout Malaysia year-round (Fang et al. 1980). A significant number of viral encephalitis cases in northern peninsular Malaysia are caused by JE virus (Cardosa et al. 1991). A few arboviral studies, primarily consisting of the measurement of arbovirus antibody levels in human and animal populations, were carried out in Malaysia in the 1950s (Cruikshank 1951, Paterson et al. 1952, Pond et al. 1954, McCrumb 1955). In the 1970s, Simpson et al. (1970, 1974) isolated JE virus from 3 species of mosquitoes (*Cx. tritaeniorhynchus, Culex gelidus* Theobald, and *Aedes curtipes* Edwards).

No large-scale studies were carried out to determine the vectors of JE when cases of JE were reported. When one positive case of JE was reported from Kampong Pasir Panjang in the district of Sabak Bernam in Selangor, Malaysia, during May 1992, we conducted entomological surveys to determine the vectors of JE and attempted to isolate the virus from field-caught mosquitoes.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was applied to confirm presence of JE virus genome in cell cultures. Detection of antigen in cell culture by ELISA and focus-forming units using BHK 21 cells was also carried out.

MATERIALS AND METHODS

Collection of mosquitoes: Adult mosquitoes were collected using CDC battery-operated light traps baited with CO_2. The traps were operated each night from 1800 to 0700 h. The light traps were hung on trees or poles outdoors. Details of collections, identification, and storage of mosquitoes for isolation studies have been described by Vythilingam et al. (1993).

Preparation of mosquito pools: Each pool of 50 mosquitoes was homogenized with 2 ml of 0.2% bovine plasma albumin in phosphate-buffered saline (PBS), pH 7.2, and was centrifuged at 3,000 rpm for 15 min at 4°C. An aliquot of each supernatant was inoculated into *Aedes albopictus* (Skuse) clone C6/36 cells (Igarashi 1978). After incubation at room temperature for 7 days, infected C6/36 cells were harvested and maintained at −70°C.

Detection of viral antigen by peroxidase/anti-peroxidase (PAP): C6/36 cell smears were prepared for each culture on a 12-well microscope slide and fixed in cold acetone for 20 min. Anti-JE mouse serum diluted 1:200 in PBS without calcium chloride and magnesium chloride (PBS[−]) was added and incubated at room temperature for 40 min. Slides were then rinsed with PBS and rabbit anti-mouse IgG serum diluted 1:1,000 in PBS(−) was added and incubated for 40 min at room temperature. Slides were rinsed again with PBS and sheep antirabbit IgG serum diluted 1:500 in PBS(−) (BIORAD) was added

[1] Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia.
[2] IMR-JICA Research Project on Tropical Diseases, Institute for Medical Research, Kuala Lumpur, Malaysia.
[3] Department of Virology, Institute for Tropical Medicine, Nagasaki University, Nagasaki, Japan.
[4] Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

and incubated for 40 min. Slides were then rinsed with PBS and PAP-rabbit serum diluted 1:1,000 in PBS(−) was added and incubated for 40 min. After a final rinse, 0.5 mg/ml 3,3'diaminobenzidine 2 HCl (DAB) (Sigma) in PBS with 0.02% hydrogen peroxide was added and incubated for 5 min. The slides were then rinsed gently in running tap water and examined under the microscope for the presence of viral antigen. For a positive control, a Japanese isolate, JaGArO1, was used; culture medium was used as a negative control. This was the standard procedure carried out on all mosquito homogenates.

Culture fluids positive for the presence of JE virus antigen were further tested using the following methods:

1) Direct reverse transcriptase-polymerase chain reaction (RT-PCR) of culture fluid: The RT-PCR was carried out directly on the culture fluids to demonstrate the presence of JE virus. The primer pairs used were from the Envelope protein region. Sense primer AATGGGCAATCGTGACTTCA (993-1013) and anti-sense primer TCAGTGAAGCCTTGTTTGCA (1251-1271) were from Sumiyoshi et al. (1987). The primers were synthesized by standard methods using an Applied Biosystem DNA synthesizer. Five microliters of infected culture fluid were incubated with an equal volume of detergent mix (1% Nonidet P-40) and 10 units (U) of RNase inhibitor (Takara Co., Japan) in a 500-μl Eppendorf tube for 1 min at room temperature. This was followed by the addition of 90 μl of RT-PCR mixture consisting of (100 pmol of each primer, 0.2 mM deoxynucleoside triphosphate, 10 mM tris [pH 8.9], 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg of bovine serum albumin per ml, 0.1% sodium cholate, 0.1% Triton X-100, 10 U reverse transcriptase [Life Science, Inc.] and 2 U of Tth DNA polymerase—a thermostable DNA polymerase [Toyobo Co., Osaka, Japan]). The reaction mixture was covered by 2 drops of mineral oil. The reactions were allowed to proceed in an Iwakai Thermal cycler programmed to incubate at 53°C for 10 min and then proceed with 35 cycles of denaturation (94°C, 1 min), primer annealing (53°C, 1 min), and primer extension (72°C, 1 min). Further elongation was carried out for 5 min at 72°C (Tanaka et al. 1993).

Seven microliters of PCR product were loaded on a 3% NuSieve 3:1 agarose gel in TAE buffer (40 mM Tris/acetate [pH 8.0], 1 mM EDTA). Gels were stained with ethidium bromide and amplified DNA bands were visualized by UV light illumination to determine the correct size of the DNA product. The JaGArO1 isolate was used as a positive control; culture medium was used for the negative control.

2) Detection of antigen by ELISA: A 96-well plate was coated with capture antibody (prepared from high-titered dengue hemorrhagic fever patient's serum) and incubated overnight at 4°C. Blocking agent was then added and left for 30 min. The plate was then washed with PBS with 0.05% Tween 20 (PBS T). One hundred microliters of the sample were then added to the wells and incubated for 1 h. The plate was then washed with PBS T. Horseradish peroxidase (HRPO) conjugated with antiflavirus IgG was then added and incubated for a further 1 h. The plate was then washed with PBS T and o-phenylenediamine (OPD) in substrate buffer, and hydrogen peroxide was added and incubated in the dark for 1 h. The reaction was stopped by the addition of 75 μl of 4 N sulfuric acid. The optical density was recorded with an ELISA reader at 492 nm. All values below 0.6 were considered negative.

3) Focus-forming units using BHK 21 cells: The BHK 21 cells were grown on 96-well flat-bottom plates. When a cell monolayer was formed the fluid was removed from the well plates with a suction apparatus. Twenty microliters of samples that had been serially diluted were added to the wells. The plate was rocked every 0.5 h so that absorption could take place. At the end of 2 h, 100 μl of Tragacanth gum was added to the wells and incubated for 30 h. The overlay medium was discarded at the end of the incubation period and the plate was rinsed once with PBS with calcium chloride and magnesium chloride (PBS [+]). The cells were then fixed by adding 50 μl per well of 5% formaldehyde in PBS(−) and incubated for 20 min. After incubation the formaldehyde was discarded and the cells rinsed gently with PBS(−). The cell membrane was then permealized by incubation with 100 μl of 1% Nonidet P-40 (NP 40) at room temperature for 20 min. The NP 40 was then discarded and the cells rinsed with PBS(+). Block Ace (Yukijirushi) was added and incubated for 30 min after which it was washed with PBS(+). Anti-JE mouse serum diluted 1:500 in PBS(−) with 0.02% NaN_3 was then added to the wells and incubated for 1 h at room temperature. After washing with PBS(−) 3 times, 0.5 mg/ml of 3,3'diaminobenzidine 2 HCl (DAB) in PBS(−) and 0.02% hydrogen peroxide were added to the wells and incubated at room temperature for 5 min. The plate was then washed with water and observed under a stereoscope and the number of foci counted.

RESULTS

A total of 148 pools of mosquitoes were processed for virus isolation. Table 1 shows the pools of mosquitoes collected for virus isolation at various times of the year. A large number of pools were collected in September.

Table 1. Pools of mosquitoes collected during the various months and virus isolation attempts.

Month	No. pools tested	No. positive[1]	Percentage of pools positive
May	11	2	18
June	5	1	20
September	85	6	7
October	36	5	14
November	11	2	18
Total	148	16	11

[1] Positive by RT-PCR, ELISA, and focus-forming unit assays.

Twenty-six pools of mosquitoes were found positive by PAP staining, of which only 16 were confirmed to be positive for JE by PCR, ELISA, and plaque-forming units (BHK 21) (Table 2).

Table 3 shows the number of virus isolates per number of pools of mosquitoes and the minimum field infection rates. *Culex vishnui,* Theobald, *Cx. tritaeniorhynchus, Culex bitaeniorhynchus* Giles, *Culex sitiens* Wied., *Aedes* species (*Ae. albopictus* and *Aedes butleri* Theobald) and mixed *Culex* species showed the presence of JE virus genome in the cell culture.

DISCUSSION

Twenty-six pools of mosquitoes showed JE antigen by PAP testing but only 16 pools were con-

Table 2. Japanese encephalitis virus isolations from mosquitoes collected at Kampong Pasir Panjang in 1992.

Pool no.	Species	Antigen PAP	PCR	BHK 21	ELISA
145	*Cx. tritaeniorhynchus*	+[1]	−[1]	0	0.01
321	*Cx. gelidus*				
	Cx. bitaeniorhynchus	+	+[2]	0.45×10^6[3]	0.91[4]
	Cx. quinquefasciatus				
	Cx. sitiens				
322	*Cx. vishnui*	+	+	0.45×10^6	1.17
396	*Ae. albopictus*	+	+	0.36×10^6	1.14
	Ae. butleri				0.01
534	*Cx. tritaeniorhynchus*	+	−	0	
536	*Cx. tritaeniorhynchus*	+	+	0.4×10^6	1.18
540	*Cx. pseudovishnui*	+	−	0	0.04
549	*Cx. gelidus*	+	−	0	0.01
571	*Cx. tritaeniorhynchus*	+	+	0.4×10^6	1.27
579	*Cx. vishnui*	+	−	0	0.01
584	*Cx. tritaeniorhynchus*	+	−	0	0.01
586	*Cx. vishnui*	+	+	3×10^4	0.74
587	*Cx. tritaeniorhynchus*	+	−	0	0.01
598	*Cx. vishnui*	+	+	0.2×10^6	0.73
599	*Cx. vishnui*	+	−	0	0.01
600	*Cx. tritaeniorhynchus*	+	+	1.4×10^6	1.45
601	*Cx. sitiens*	+	+	0.1×10^6	0.99
711	*Cx. tritaeniorhynchus*	+	−	0	0.03
717	*Cx. bitaeniorhynchus*	+	+	0.3×10^6	0.77
720	*Cx. sitiens*	+	+	1.8×10^6	1.58
	Cx. quinquefasciatus				
729	*Cx. bitaeniorhynchus*	+	+	0.1×10^6	1.43
739	*Cx. sitiens*	+	+	0.3×10^6	1.49
745	*Cx. pseudovishnui*	+	+	0.45×10^6	1.43
	Cx. vishnui				
799	*Cx. vishnui*	+	+	0.2×10^6	0.60
803	*Cx. vishnui*	+	−	0	0.00
806	*Cx. bitaeniorhynchus*	+	+	0.3×10^6	0.77

[1] + = positive; − = negative.
[2] Correct size of PCR product.
[3] Focus-forming units/pool.
[4] Optical densities (496 nm) equal to or greater than 0.6 were considered positive.

Table 3. Number of mosquito pools processed for virus isolation, the number of JE virus isolates, and the minimum field infection rates at Kampong Pasir Panjang.

Species	No. pools tested	No. JE virus isolates	MFIR[1]
Cx. bitaeniorhynchus	11	3	5.5
Cx. gelidus	11	0	
Cx. pseudovishnui	19	0	
Cx. sinensis	3	0	
Cx. sitiens	8	2	5.0
Cx. tritaeniorhynchus	26	3	2.3
Cx. vishnui	33	4	2.4
Culex spp. mixed	9	3	6.7
Ae. butleri	16	0	
Aedes spp. mixed	1	1	
Anopheles spp.	3	0	
Armigeres spp.	2	0	
Mansonia uniformis	3	0	
Other species	3	0	
Total	148	16	

[1] Minimum field infection rate/1,000 specimens tested.

firmed by the other 3 methods. This could have been due to low titer and subsequent loss due to storage and handling. Of the 3 methods used to confirm the presence of JE virus, it was found that RT-PCR was a rapid method though more expensive than the other 2.

Japanese encephalitis virus had been previously isolated from *Cx. gelidus, Cx. tritaeniorhynchus,* and *Ae. curtipes* in Sarawak by Simpson et al. (1970). The isolation of JE virus from *Cx. tritaeniorhynchus* has also been documented from Malaysia by many other workers (McCrumb 1955).

Japanese encephalitis virus isolations from *Cx. bitaeniorhynchus,* although reported for the first time in Malaysia, have been reported earlier from India and Indonesia (Banerjee et al. 1979, Ratna Tan et al. 1993). Laboratory studies have shown that this mosquito can act as an efficient vector (Banerjee et al. 1978). The ability of *Cx. bitaeniorhynchus* to bite man has been recorded in India as well as in Japan (Sasa and Sabin 1950, Ramachandra and Rajagopalan 1957).

Although *Cx. bitaeniorhynchus* formed only 2.7% and 3.3% of the light trap and human bait collections, respectively, JE virus was isolated from 3 pools of *Cx. bitaeniorhynchus* with the highest minimum field infection rate.

Isolation of JE virus from *Cx. sitiens* is a recent finding (Vythilingam et al. 1994). Furthermore, the isolation from a pool consisting of a mixture of *Ae. albopictus* and *Ae. butleri* is being reported for the first time in Malaysia. Japanese encephalitis virus has been isolated from field-collected *Ae. albopictus* and this species can transmit JE (Huang 1957, Wu and Wu 1957). Isolation attempts were carried out from 16 pools of *Ae. butleri* but no virus was isolated. The role of this mosquito as vector of JE is uncertain and it may only be an incidental host. This mosquito is common in the coastal area and is a vicious biter of humans throughout the day.

Although large numbers of *Cx. tritaeniorhynchus* and *Cx. vishnui* were processed for virus isolation, the minimum field infection rates were 2.3 and 2.4, respectively.

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