

Title: Ecology of Arboviruses in Thailand

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Objectives:

- a. To determine the ecologic factors which affect the maintenance and dissemination of arboviruses causing human disease in Thailand.
- b. To develop laboratory techniques for support of field investigations.

Description:

Continued field collections of wild vertebrate sera created an urgent need for a rapid but reliable serological antibody screening technique. This need led to the development of a BHK-21 cell metabolic inhibition test for the detection of Japanese encephalitis (JE), Sindbis and chikungunya (Chik) neutralizing antibodies.

Field studies concerned with the maintenance and transmission of JE virus in and around the Red Cross Horse Farm at Bang Phra have been continuing. The mosquito light trapping program has been expanded, and relative physiologic ages of two vector species, Culex tritaeniorhynchus and C. gelidus, were determined by dissection. At the same time, virus isolation attempts were made from mosquito pools in an attempt to correlate relative abundance and physiologic age with infection. Serological monitoring of a new group of susceptible horses on the farm and of wild vertebrates was conducted in an attempt to determine the time of transmission and to identify suspect reservoir species for further field investigations.

Taxonomic studies designed to give a firm base to the ecological studies have also been continuing. Intensive collections of materials in direct support of the virus studies have been carried out in the Bang Phra area. Additional extensive collections have been made in various areas in Thailand to add to distributional and life history data of Thai birds and mammals and to make the SMRL reference collection more complete.

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Recovery of virus from parous Anopheles mosquitoes collected for malaria field studies have been attempted.

A reported epizootic of equine encephalitis in Nakorn Pathom was investigated.

Progress:

BHK-21 Cell Metabolic Inhibition Test. Extensive serologic surveys require the use of a simple, reproducible and specific laboratory antibody assay system. In our experience, the HI test as a screening device for non-human vertebrate sera is complicated by (1) non-specific reactions despite acetone or kaolin extraction of sera (2) removal of specific antiviral macroblogulin by kaolin treatment and (3) misleading intragroup cross-reactions. A neutralization test having the simplicity of the HI test was needed for serologic surveys. The metabolic inhibition test experiments conducted in our laboratory represent an attempt to develop such a neutralization test for three of the arboviruses present in Thailand.

The BHK-21 cell metabolic inhibition (MI) test is similar to MI tests with other cell-virus systems reported by others. An acid (yellow color) indicates cell survival and an alkaline pH (red or purple color) represents virus-induced cell destruction BHK-21 MI tests employing both microtiter and large disposable plastic plates have been used with local strains of Sindbis, Japanese encephalitis (JE), and chikungunya viruses.

Studies with the BKM-21 cell macro (large disposable plate) and microtiter metabolic inhibition test were carried out. A series of experiments were done manipulating several of the variable factors to achieve consistent results and clear color contrast. Experiments with various cell concentrations indicated that 4,000 cells in 0.1 ml growth medium per well for the microplates and 25,000 in 0.5 ml per well for the large plate was optimal. A variety of factors in pH control were examined, including CO₂ vs aerobic incubation, heavy vs extra heavy mineral oil at several volumes per well, mineral oil vs Saran wrap covering of plates, and several concentrations of sodium bicarbonate. Some of the early inconsistencies in cell growth were traced to inadequately cleaned plates. Our present procedure calls for washing plates (0.7% haemosol) followed by thorough rinsing (at least 8 times) in tap water and demineralized water. The plates are then sterilized by ultraviolet light.

The volumes per well of test reagents used are as follows:

<u>Reagents</u>	<u>Microtiter plate</u>	<u>Large plate</u>
Heavy mineral oil	0.08 ml	1.5 ml
Virus (in M 199 + 20% FBS) (100 TCD ₅₀)	0.025 ml	0.1 ml
Serum dilutions in M199+ 20% FBS	0.025 ml	0.05 ml
Cells (live count) in M199+ 5% FBS	4,000	25,000
Volume of cell suspension	0.1 ml	0.5 ml
NaHCO ₃ in medium	0.0007%	0.0015%
Over-feeding with 19S+ 6% FBS	0.5 ml day 4	0.8 ml day 5

The procedure used is as follows :

- (1) Serum dilutions are added to the plates.
- (2) The virus dose is added (100 TD LD₅₀) next.
- (3) Serum-virus mixtures are incubated at 37°C for 2 hours.
- (4) Cell suspensions are added.
- (5) Oil is added (may be done first in micro plates).
- (6) Extra heavy mineral oil is applied to top outside edges of plate.
- (7) Plates are covered with sterile Saran wrap (oil from step 6 should seal Saran wrap to plate).
- (8) Plates are incubated at 37°C in aerobic incubator.
- (9) Saran wrap is removed and cells in each well overfed (do not replace Saran wrap after feeding).
- (10) Plates are reincubated for 2-3 days and read.

Addition of Saran wrap has produced very sharp end-points and virtually eliminated troublesome mycotic contamination.

Comparative microtiter, macro plate and tube neutralization tests with JE virus indicate correlative results by these methods when acute and convalescent phase sera from clinical JEV encephalitis cases were tested (Table 42). Failure of several convalescent phase sera to neutralize in the first test was due to virus "break-through" probably owing to low avidity of the early sera and high sensitivity of the BHK-21 cells to JE virus. Retesting of several of these sera increasing the serum virus incubation period from one to two hours and, in the case of the microtiter test, lowering the virus dose, significantly increased serum titers (Table 42). Similar tests were carried out with Sindbis and chikungunya viruses and reference antisera in microtiter, macro plate and tube neutralization tests. The Sindbis test was carried out before the discovery that longer (two-hour) virus-serum incubation was necessary. Even with the shorter incubation time, the microtiter method was as sensitive as the tube method with two of the three sera tested, whereas the macroplate method appeared to be less sensitive than either (Table 43). The chikungunya tests were carried out with the two hour serum-virus incubation. In this case virus "break through" and rapid cell to cell spread in the established tube monolayers resulted in significantly lower serum neutralizing titers in tubes than in either macro or micro plates, where addition of a cell suspension to the serum virus-mixture did not permit this to happen (Table 43). The microtiter method was the most sensitive of the three techniques compared.

Experiments to determine intragroup neutralization specificity have begun. In two trials there was no cross neutralization between chikungunya and Sindbis viruses. Three group B antisera, Japanese encephalitis hyperimmune rabbit, Tembusu hyperimmune mouse, and dengue 2 immune monkey sera were tested against JE (Nakayama) virus. There was no heterologous neutralization by the sera, which had homologous plaque reduction neutralization titers of 1:900, 1:1280 or >, and 1:1500, respectively. When serum titers from cases of dengue fever and viral encephalitis were compared by hemagglutination-inhibition (HI) and microtiter MI tests, the magnitude of the acute phase-convalescent phase changes were in complete agreement for the encephalitis cases (Table 44). The one encephalitis case with a four-fold group B HI titer rise also had a four-fold MI neutralization rise, doubtless diagnostic of JE encephalitis. In four other encephalitis cases where HI titers were high and relatively fixed and JE encephalitis was suspected, the MI neutralization test failed to show four-fold rises of acute to convalescent phases titers. In these cases either the MI test was not adequately sensitive or these HI titers represent old group B infections not associated with the encephalitis. Perhaps most significant was the failure to demonstrate four-fold acute-convalescent phase cross reacting antibody titer rises to JE virus by MI tests in three cases of secondary dengue infections (Table 44). In these infections a four-fold or greater HI antibody rise occurred to both dengue and JE viruses, with all convalescent phase serum titering to over 1:5,000. These results suggest that it may be possible to exclude JE, serologically, as an etiologic agent where a broadly cross-reactive group-B HI response occurs following infection with other group B viruses.

The BHK-21 cell micro MI test has proved to be reproducible, reasonably accurate, easy to perform, comparatively inexpensive and sparing of field-collected sera which are often in very limited quantity. The limited sensitivity is of less concern where one wishes to exclude all false positive even if a few true positives are also excluded. Studies on the ecology of Japanese encephalitis virus. Studies on the ecology of Japanese encephalitis virus at Bang Phra, an area of year round virus activity, have been continuing. Field activities have focused on two main problems: (1) The capture of large numbers of Culex tritaeniorhynchus and C. gelidus to assess virus infection, relative abundance and physiologic age and (2) the capture of birds and mammals to identify which species were present on the area and to determine which of these species might be involved in virus maintenance.

Bang Phra Mosquito Study. Between February 1966 and February 1967, mosquitoes were obtained by the Department of Medical Entomology from 655 light trap collections made in four areas in the vicinity of the horse farm operated by the Red Cross Society of Thailand at Bang Phra in Cholburi province. The most abundant species captured in the light traps were: Culex fuscocephalus, C. gelidus, C. tritaeniorhynchus, Aedes lineatopennis, A. mediolineatus, A. vexans, Anopheles aconitus, Mansonia annulifera and M. uniformis. A total of 181, 284 mosquitoes belonging to the above species were tested for the presence of virus, and viral isolates were obtained from pools of C. gelidus, C. tritaeniorhynchus, A. lineatopennis, A. mediolineatus and A. vexans. The peaks in abundance of all five species occurred during the rainy season (May-October), while the populations of C. gelidus, C. tritaeniorhynchus, A. vexans and A. lineatopennis were observed to reach two peaks, during the months May-June and again in September-October, the A. mediolineatus population demonstrated a single peak during July and August during which the populations of the other four species were exhibiting an unexplained decline (Figure 12). The aquatic stages of the two Culex species favor semi-permanent frequently polluted bodies of water such as slow-moving streams, paddy-fields and ponds, while the three Aedes species characteristically breed in temporary rain-pools. All five species are known to feed upon large domestic animals, and the greatest numbers were collected at Bang Phra during this period from two light traps located in the vicinity of a cattle barn and a horse stable, respectively. That those mosquitoes were in fact feeding on livestock at Bang Phra was borne out by the results of agar-gel diffusion tests run against the gut contents of engorged mosquitoes from these light trap collections. Details of the agar-gel tests are given under the section on mosquito studies.

The age composition of C. gelidus and C. tritaeniorhynchus populations at Bang Phra were studied during this period through dissection and examination of ovaries of mosquitoes from light trap collections. Parous were distinguished from nulliparous females by the presence of absence of terminal coils on the ovarian tracheoles. A significant rise in the proportion of older (parous) C. gelidus females occurred in March and again in June; it was during those two months that the only isolations of viral agents were obtained from C. gelidus at Bang Phra during this period (Table 45). Unfortunately, the numbers of C. tritaeniorhynchus dissected during this same period were too small to indicate whether the population of that species exhibited similar changes in age composition. Interestingly, all of 12 JE antibody-free horses resident at the Bang Phra horse farm developed JE antibodies between 2 June and 2 July, the time when many parous C. gelidus females were present and when both C. gelidus and C. tritaeniorhynchus were particularly abundant.

Additional mosquito pools were submitted by Bang Phra Red Cross personnel to the Virology Department for recovery of viruses. The total numbers of mosquitoes tested for the presence of virus is given in table 46.

Wild Mammals and Bird Sampling. Through the last 12-month period, birds and mammals were live-trapped in the Bang Phra area. All specimens were bled for serological testing, birds being bled from the external juglar vein and mammals by cardiac puncture or by rupture of the retroorbital sinus. One half of the specimens were sacrificed for the museum skin collection and for virus isolation attempts from organ (spleen, brain and kidney) suspensions. The other animals were marked and released at the point of

capture. Many of these animals were frequently recaptured, and served as field sentinels of virus activity. Sera are being tested for neutralizing activity to JE, chikungunya and Sindbis virus in the metabolic inhibition test. Approximately 0.1 ml. of blood drawn in the field was inoculated into each of two 1 oz. bottles containing LLC-MK₂ cell monolayers. These cells were returned to the laboratory where they were checked for CPE and then overlaid with agar to determine the presence of plaque-forming agents. To date all virus isolation attempts from vertebrates have been negative.

Serological tests have been conducted with over 600 sera representing about 40 species, approximately one third of the total number of specimens collected. Of these, low percentages of sera from 13 species have neutralized JF virus, four species have neutralizing antibody to sindbis virus and two species have neutralizing antibody to chikungunya virus (Table 47). A comparatively small percentage of the total individuals in any reacting species had neutralizing antibody. Sera from five specimens neutralized JE virus at the end of the rainy season (October-November) and were negative when bled and tested two weeks to two months later. This could be accounted for on several bases: (1) the positive MI test reactions may have been spurious (2) two of the second sera had to be diluted 1:10 and all second sera were tested against 300 TCID₅₀ whereas first sera were tested against 70 TCID₅₀, all of which could make the second test less sensitive than the first, and (3) antibody levels may have rapidly declined between the first and second bleedings, and the MI test was not sensitive enough to detect antibody in diluted, low-titered serum. No MI test negative to positive conversions have been found, although many of the sera from serially bled individuals remain to be tested. In no case did a single sera react with all viruses, and only one sera reacted with two viruses, suggesting that these sera did not contain substances capable of non-specifically inactivating arboviruses in general.

Results of the MI tests suggest infection of rodents, small resident birds and possibly bats in the Bang Phra area. The ecological significance of these observation is unknown. The association of neutralizing properties of the MI test positive sera with their immune globulins has yet to be demonstrated. Tests to these ends are underway.

Isolation of new viruses. During the past year, viral agents were recovered and reisolated from Bang Phra mosquito species which had previously not been associated with viruses in Thailand. Two viruses were recovered from Aedes mediolineatus, two from Ae. lineatopennis and one from Ae. vexans (Table 48). Identification of four of these has been attempted. Two of these, BKM-367/66 from Ae. mediolineatus and BKM-589/66 from Ae. lineatopennis appear to be group B Arboviruses (Table 49) somewhat similar to but not identical with Tembusu, JE and dengue 4 viruses (Table 50) but identical with each other and with BKM-448/66 (Tables 51,52). BKM-457/66, from Ae. vexans, failed to react by HI test with either arbovirus A or B grouping serum.

Taxonomy of Thailand Vertebrates. Work on the taxonomy of birds and mammals continues to progress. A field handbook on the identification of rats of Thailand was prepared and published. The main collecting effort has been at Bang Phra, in support of the Japanese encephalitis virus studies being done there.

Additional areas ecologically dissimilar to the central plains area have also been sampled, particularly a broad-leaved evergreen subtropical forest area along highway 23 between Korat and Kabinburi. In this area Suncus etruscus, Rattus sladeni and an apparently new species (perhaps genus) of yellow horseshoe bat were collected. In a nearby area, Nakorn Nayok, a melanistic variety of Rattus raja was found. In the northwest mountains of Thailand, in the Mae Sariang area, Rattus nitidus was collected by SMRL personnel for the first time.

In the central plains area, Mus musculus was found inhabiting a grain warehouse in Thonburi, and a bird mist netting program on Koh Kret was carried out in an area where previous capturing and releasing of birds had resulted in banding of many individuals.

Attempted virus recovery from Anophelines. Many anopheline species commonly bite man. Their possible role as arbovirus vectors in Thailand is unknown. As a by-product of malaria studies done by the Department of Medical Entomology, salivary glands from parous (hence, having had one or more previous blood meal) female Anopheles mosquitoes of 19 species were pooled by time, place and species and tested for presence of virus by inoculation into suckling mice and LLC-MK₂ cell cultures. To date, all attempts at virus recovery have been unsuccessful, but the numbers of mosquitoes tested has been very small, (1, 147 individual mosquitoes in 219 pools).

Equine Encephalitis in Nakorn Pathom Caused by Japanese Encephalitis Virus. In Nakorn Pathom, Thailand, from August through November, the Department of Livestock Development (Thai Government) estimated 30 equine deaths due to encephalitis among a total population in the area of about 300 horses. Only the last case was seen by SMRL Virology and Veterinary Medicine personnel, who along with Dept. of Livestock Development personnel, obtained acute and convalescent phase sera from that animal. At that time sera were collected from 29 horses and tested for the presence of Japanese Encephalitis (JE) virus antibody. All sera were tested by the micro hemagglutination-inhibition (HI), complement-fixation (CF) and metabolic inhibition (MI) neutralization tests. Results of these tests (Table 53) demonstrate that horses in the Nakorn Pathom area are infected with JE or a closely related virus. The presence of CF antibody suggests fairly recent infection. In one case it was possible to associate JE infection with clinical encephalitis, serologically (Table 54).

Summary:

A reliable BHK 21 cell micro metabolic inhibition test was developed for detecting JE, sindbis and chikungunya antibodies. This test is at least as sensitive as the tube neutralization test. The test appears to be quite specific, and requires very small amounts of reagents.

A large number of mosquitoes were collected in light traps at Bang Phra. All five of the most commonly collected species, Culex gelidus, C. tritaeniorhynchus, C. fuscocephalus, Aedes vexans, Ae. medilineatus and Ae. lineatopennis, were most abundant during the rainy season. C. gelidus the only species for which enough dissections permitted evaluation, had significant rises in the proportion of older (parous) females in March and again in June. It was during these times that viruses were recovered from this species. Between 2 June and 2 July 1966 all of 12 JE antibody-free horses at Bang Phra developed antibody.

During the 1966-1967 collecting period, twelve viral agents were recovered from Bang Phra mosquitoes, nine by us and three by Red Cross personnel. Identification of these viruses has not been complete, but at least three are JE virus. Five of the viruses recovered from Aedes species are unlike any of the arboviruses previously known to be present in Thailand. Three are group B agents and appear to be identical. One is neither group A nor group B and one has not yet been tested for group reaction.

Twenty-nine individuals of 13 vertebrate species from Bang Phra have reacted with JE virus in the metabolic inhibition test. A number adequate for calculation of antibody prevalence rates has not yet been tested, but these data suggest wild vertebrate infection in the area.

Taxonomic studies have resulted in the publication of a field identification book of Thai rats. One new rat and bat species has been added to the SMRL reference collection. The bat may represent a previously unknown species.

Attempts at recovery of viruses from parous Anopheles mosquitoes have been unsuccessful.

About 30 equine cases of encephalitis occurred in Nakorn Pathom in August-November 1966. The last case, the only one seen by us, was confirmed as JE on serologic grounds. This represents the first

proved case of JE in horses in Thailand, although others have been suspected. Of 29 horses, 28 had JE antibody. Of these, two horses had signs of neurological damage, suggesting that wide spread JE infection among horses in this area had occurred.

Publications

1. Russell, P.K. Isolation and identification of dengue viruses in tissue culture. *Proc. Jap. Soc. Trop. Med.*, 7:43, 1966.
2. Halstead, S.B., Yamarat, C., Russell, P.K. Hemorrhagic fever in Thailand and South Vietnam; newer knowledge regarding etiology. *Jap. J. of Exp. Med. and Biol.*, in press.
3. Russell, P.K., Udomsakdi, S., Halstead, S.B. Antibody response in dengue and dengue hemorrhagic fever. *Jap. J. of Exp. Med. and Biol.*, in press.
4. Deller, J.H., Russell, P.K. Fevers of unknown origin in Americans in South Vietnam. *Ann. Int. Med.*, in press.
5. Deller, J.H., Russell, P.K. Chikungunya Disease in adult Americans., in press.
6. Bhamarapavati, N., Boonyaratvej, S., Russell, P.K. Encephalitis and pneumonitis due to chikungunya virus: report of a fatal case. *J. Med. Assoc. Thailand*, 49:627, 1966.
7. Russell, P.K., Chumdermpadetsuk, S., Plyaratn, P. A fatal case of dengue hemorrhagic fever in an American child. *Pediatrics*, in press.
8. Russell, P.K., Nisalak, A., Sukhavachana, P., Vivona, S. A plaque reduction test for dengue neutralizing antibodies. *J. Immunol.*, in press.
9. Russell, P.K., Nisalak, A. Dengue virus identification by plaque reduction neutralization test. *J. Immunol.*, in press.

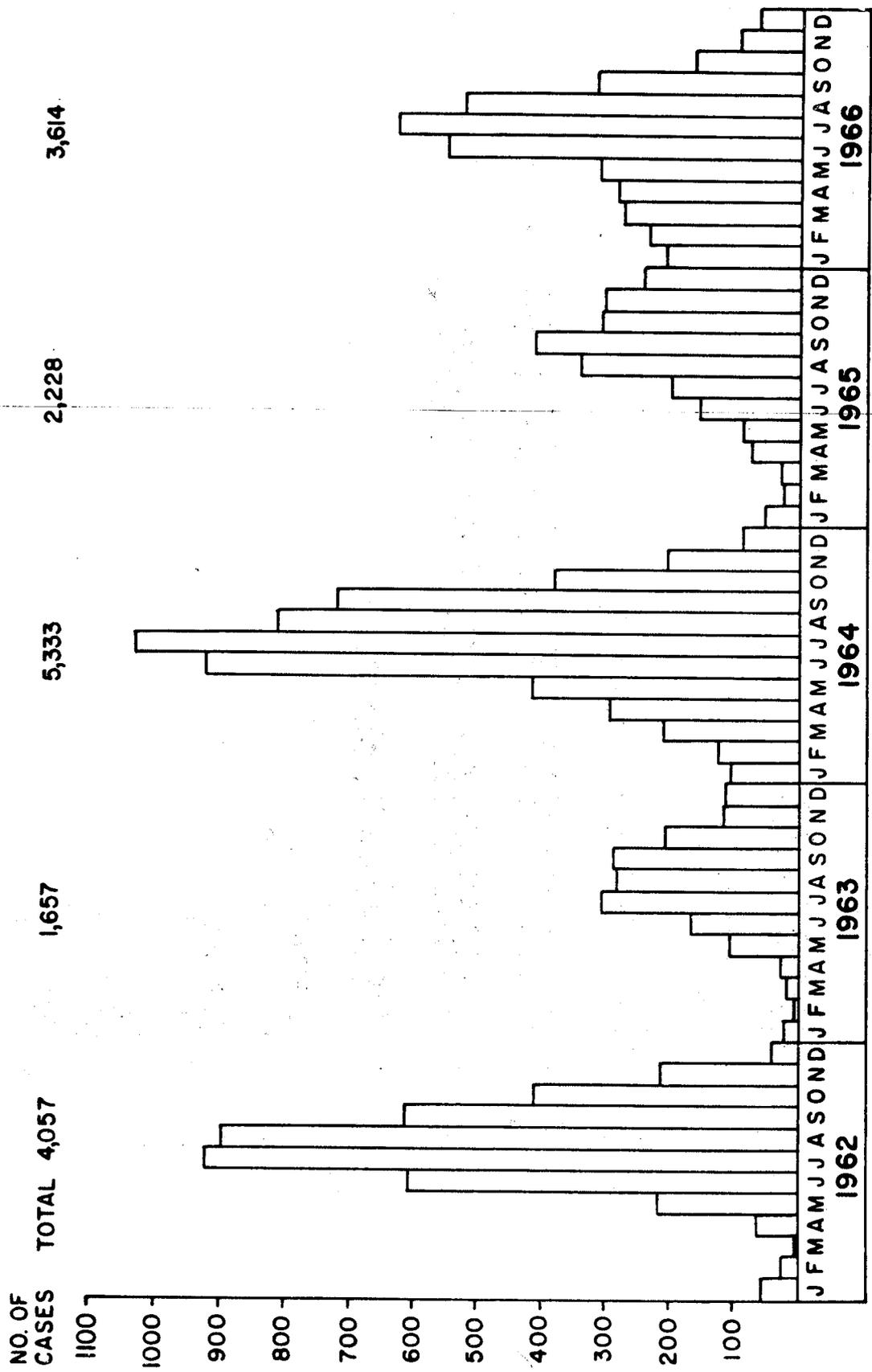


FIG. 1 CASES OF THAI HEMORRHAGIC FEVER ADMITTED TO BANGKOK AND THONBURI HOSPITALS, 1962 - 1966.

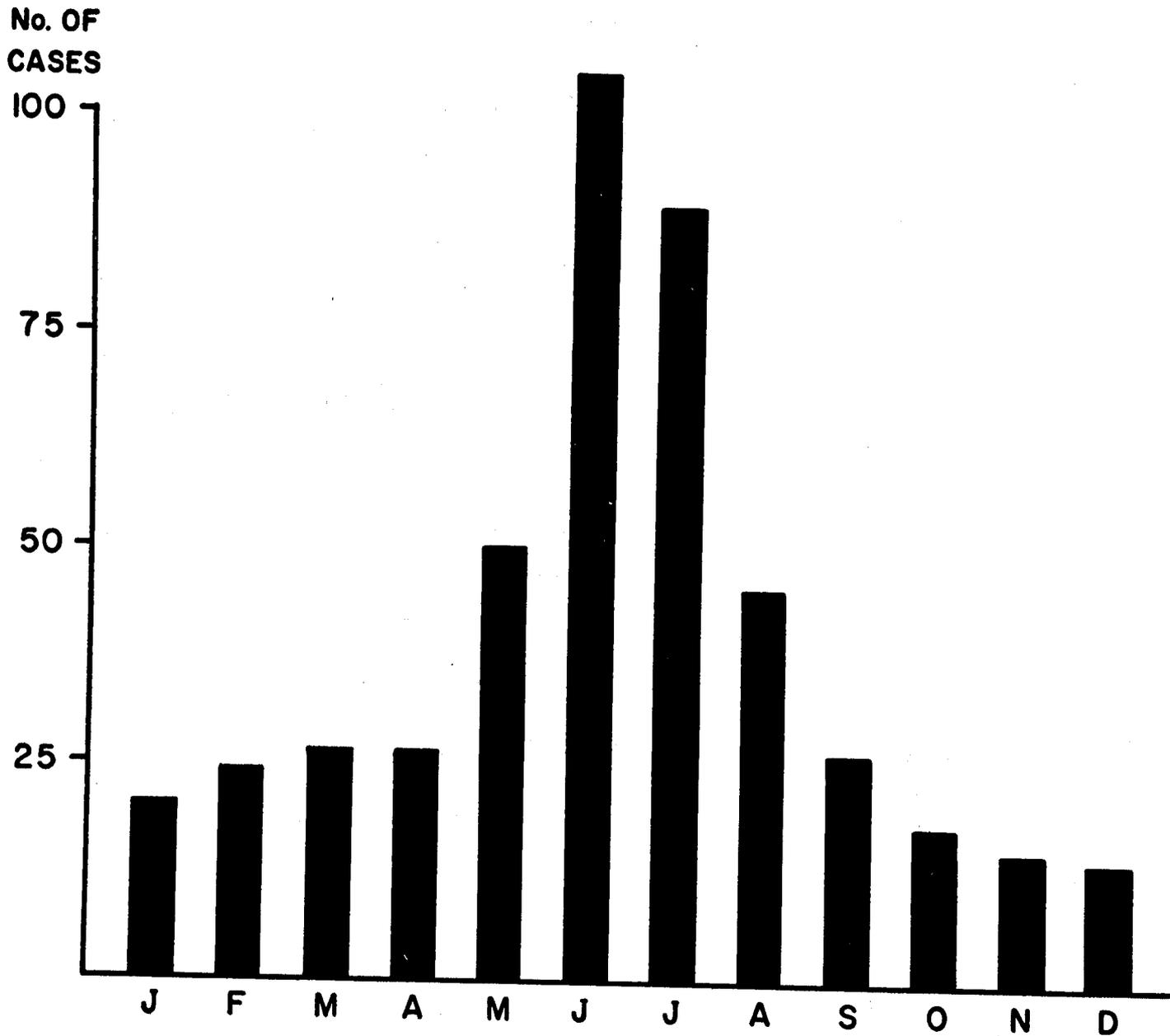


FIG. 2 CASES OF HEMORRHAGIC FEVER ADMITTED TO 3 SAIGON HOSPITALS DURING 1966 BY MONTH.

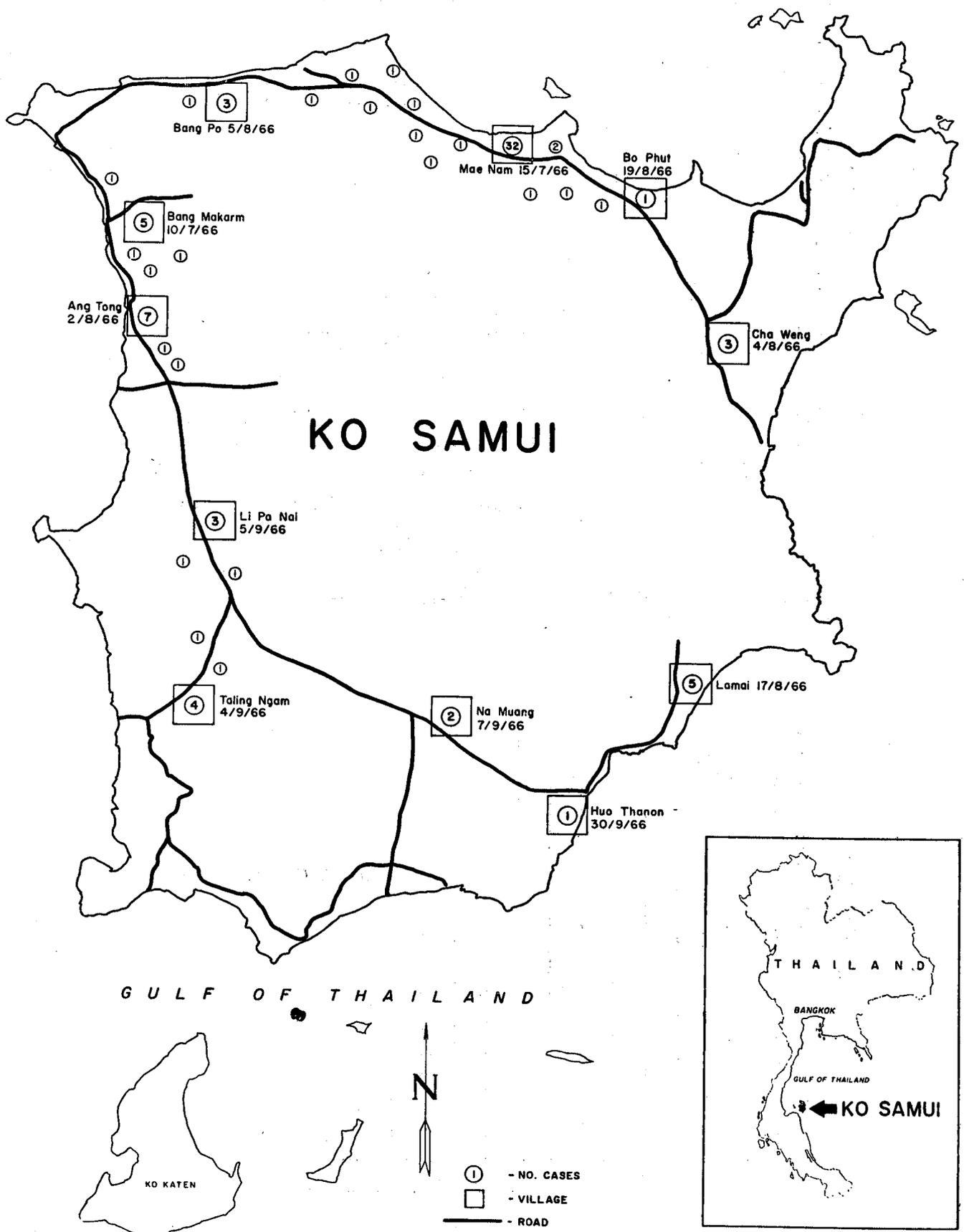


Figure 3. Map of Koh Samui showing location of cases and date of initial case in each village.

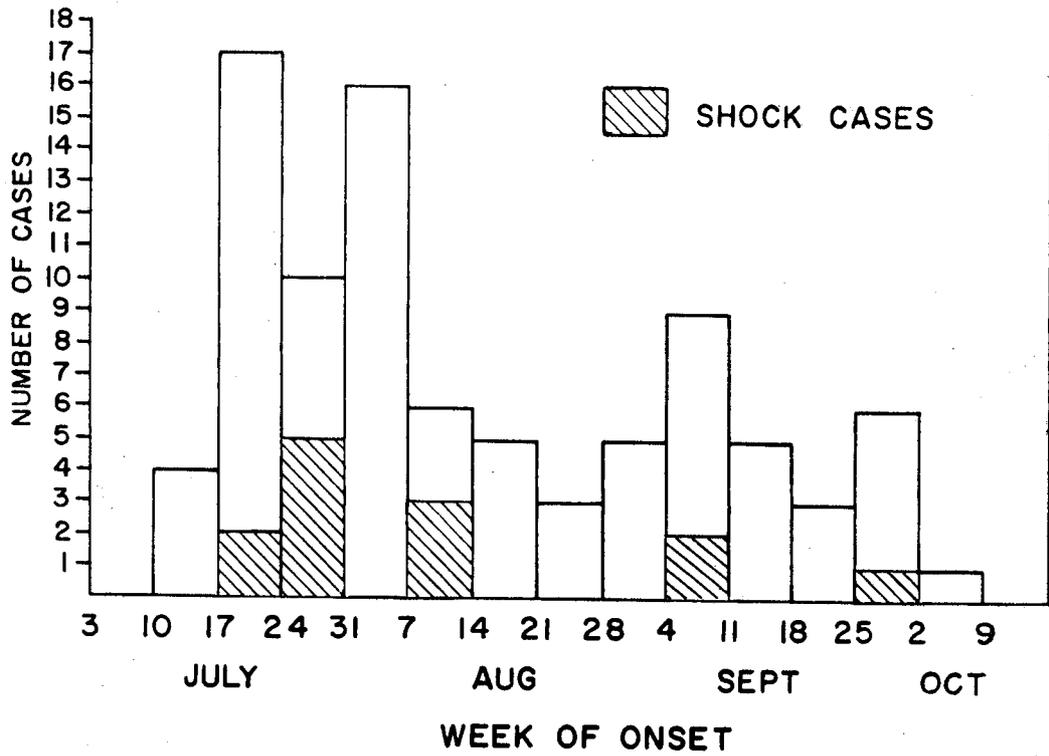


Figure 4. Distribution of 90 dengue cases by week of onset

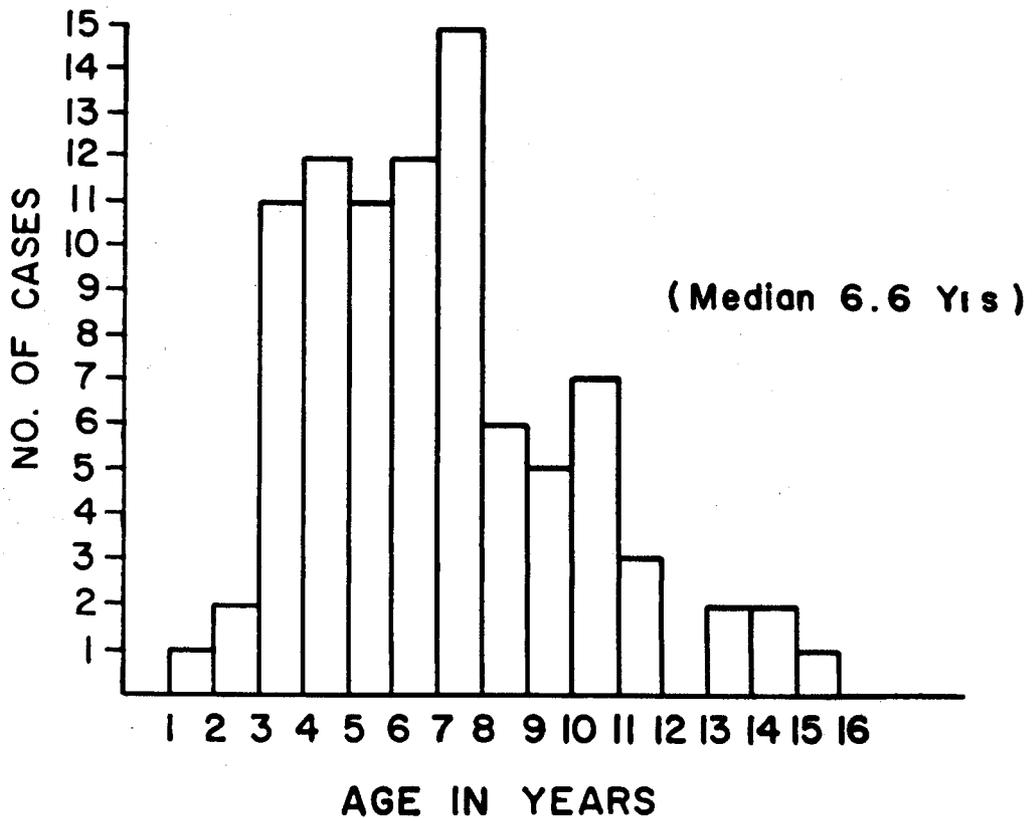


Figure 5. Age distribution of 90 dengue cases

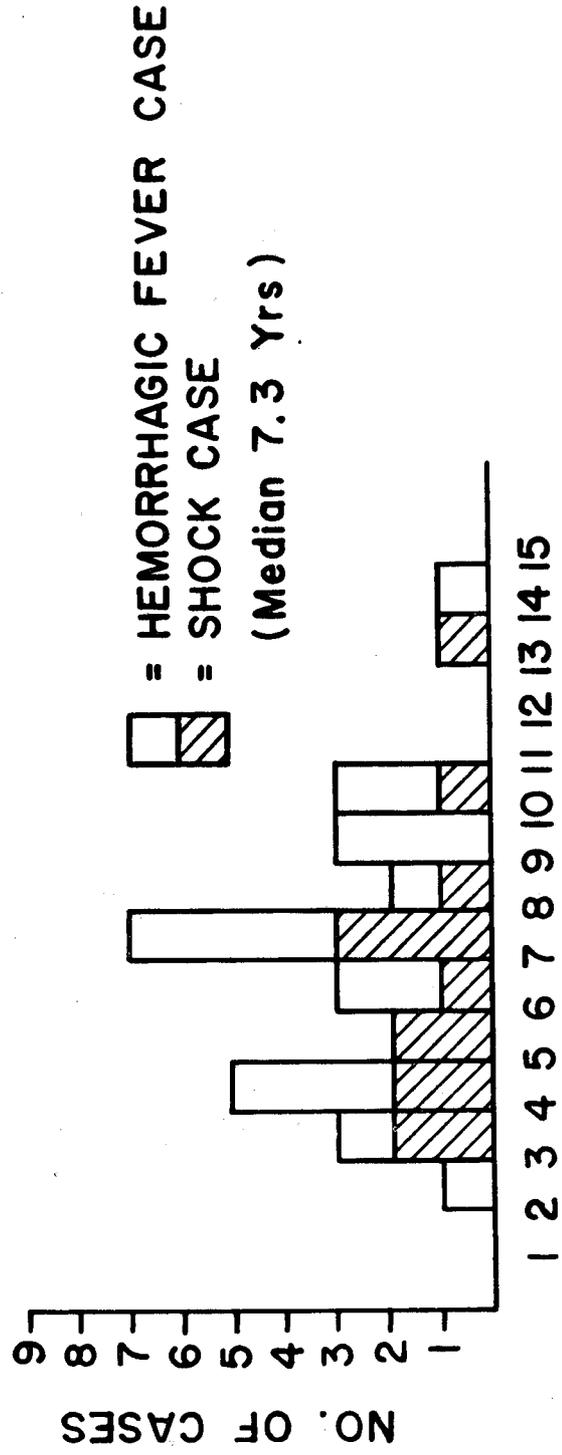


Figure 6. Age distribution of 90 dengue cases by clinical syndrome

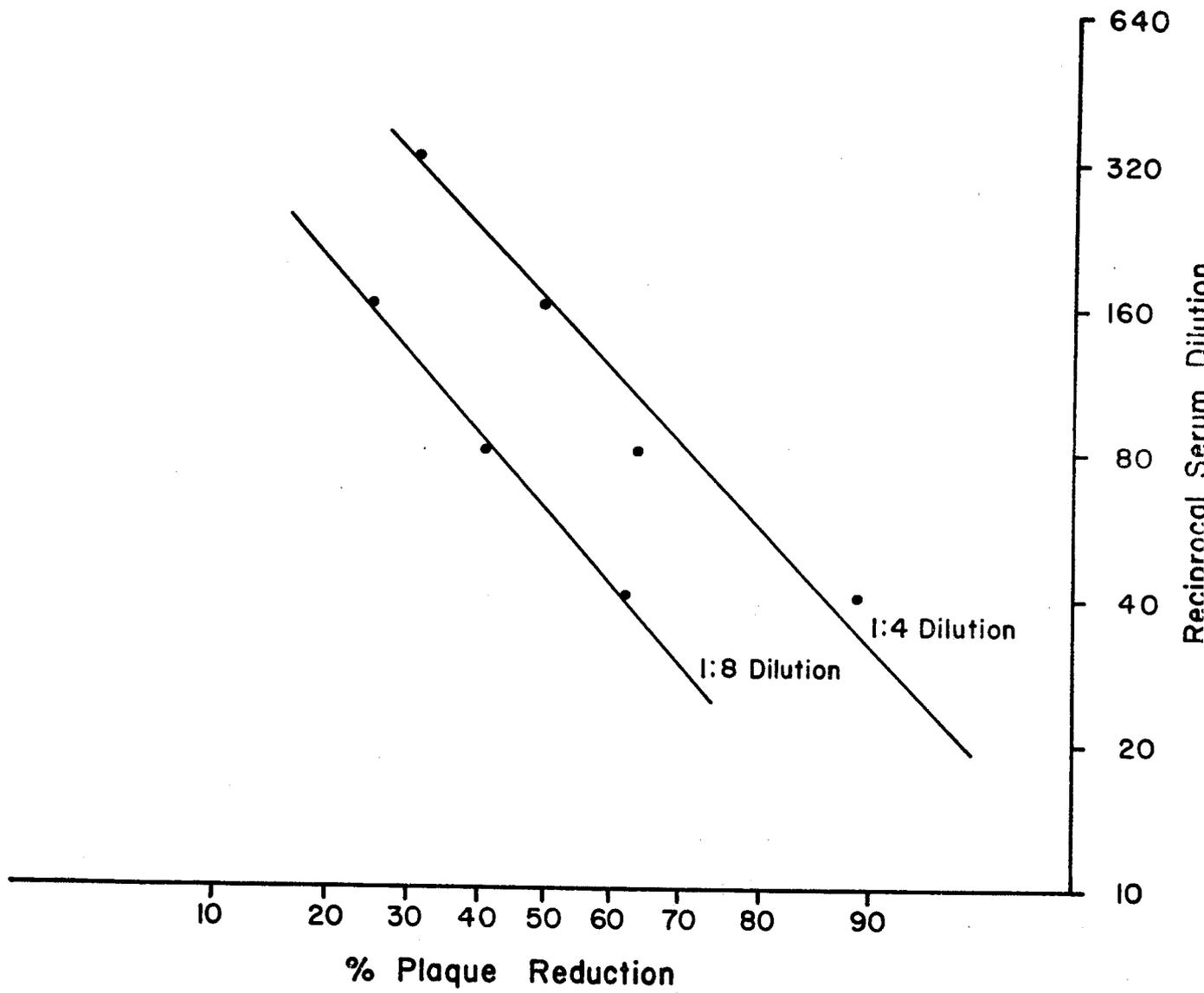


Figure 7. Plaque reduction neutralization of dengue-1 (Hawaii) virus by 2 dilutions of dengue-1 (Hawaii) antiserum

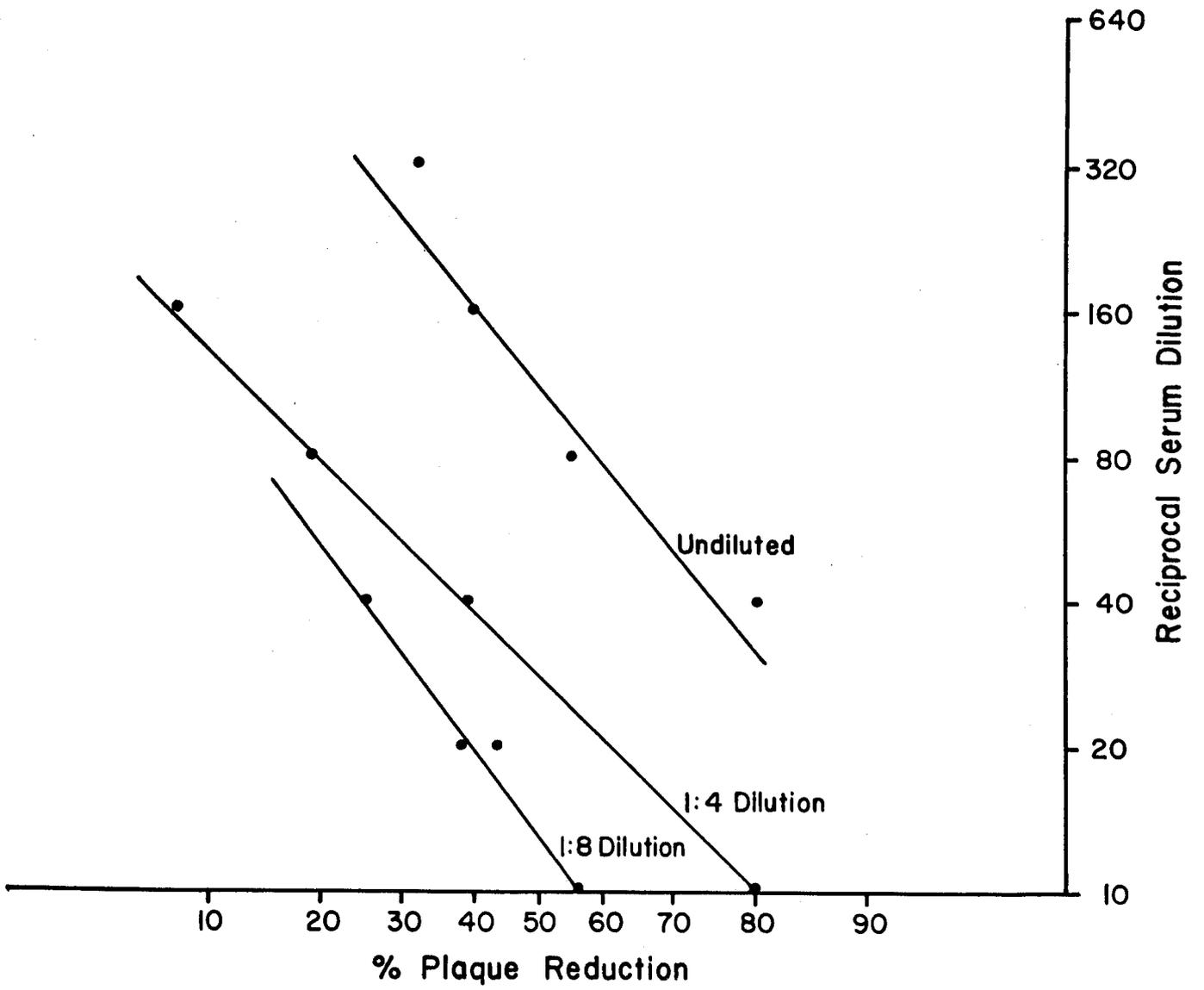


Figure 8. Plaque reduction neutralization of TH-Sman virus by 3 dilutions of TH-Sman antiserum.

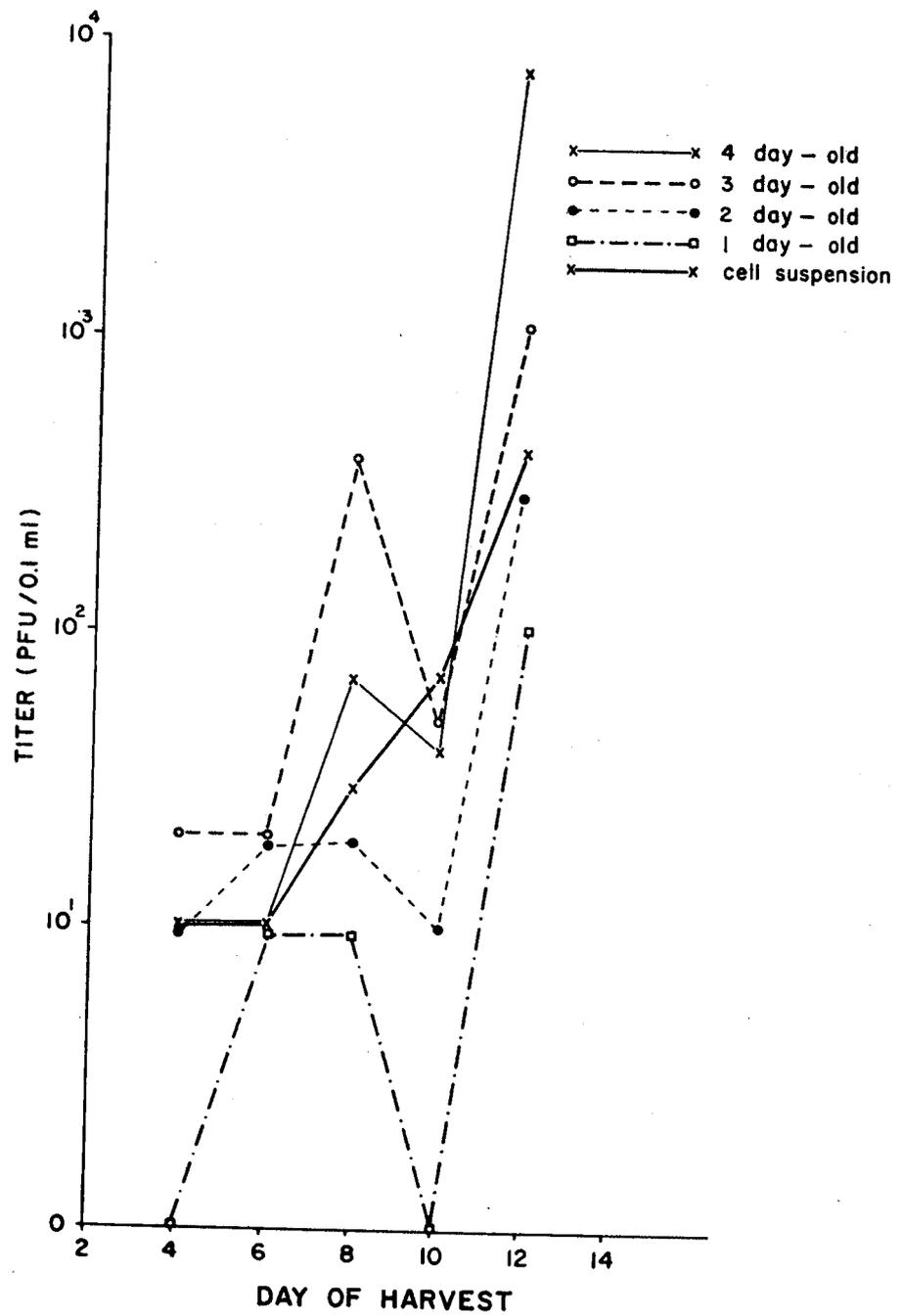


Figure 9. Growth of dengue-2 (BKM-540) virus in LLC-MK₂ cells of various ages

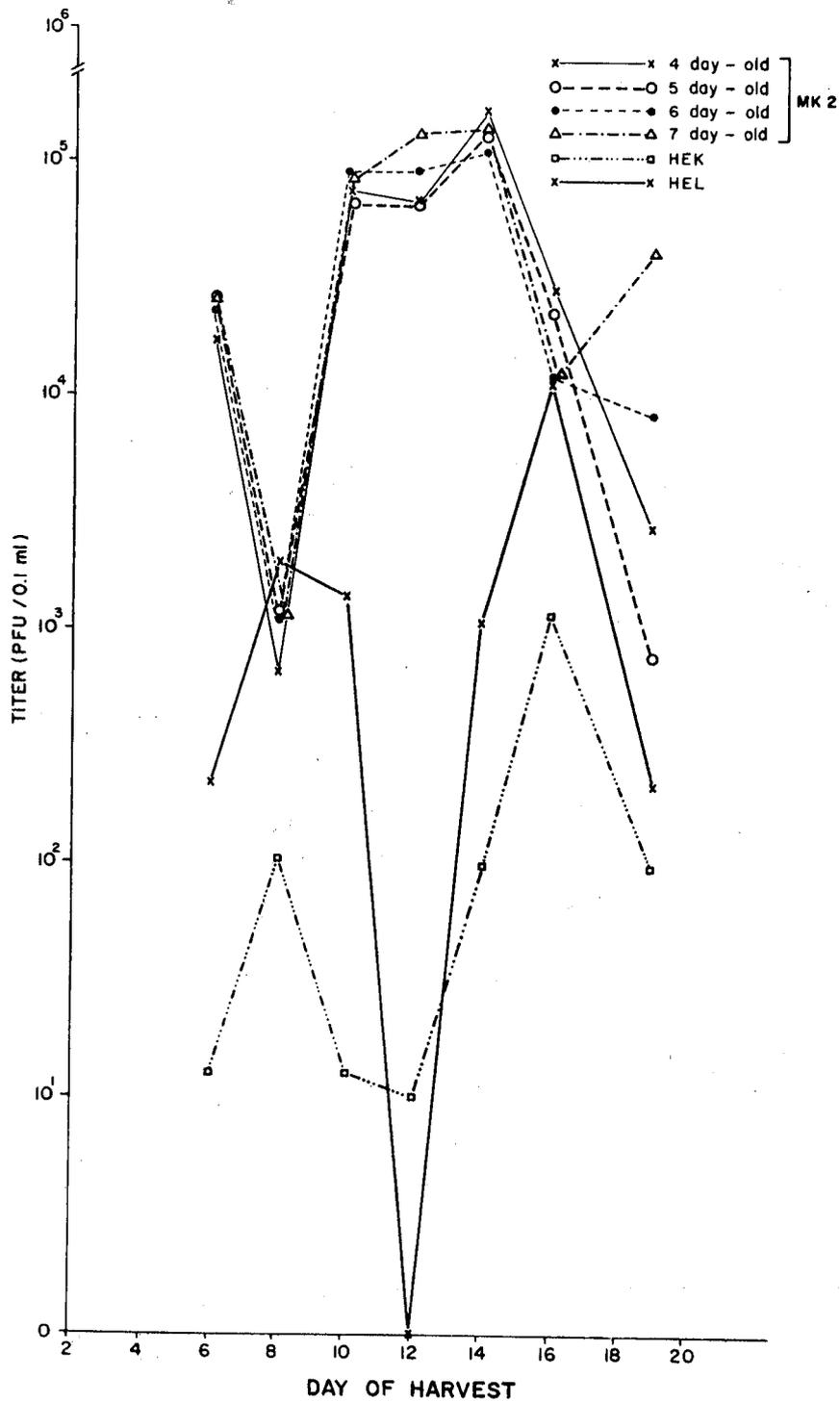


Figure 10. Growth of dengue-2 (BKM-540) virus in LLC-MK₂ cells of various ages and in human embryonic lung and human embryonic kidney cells.

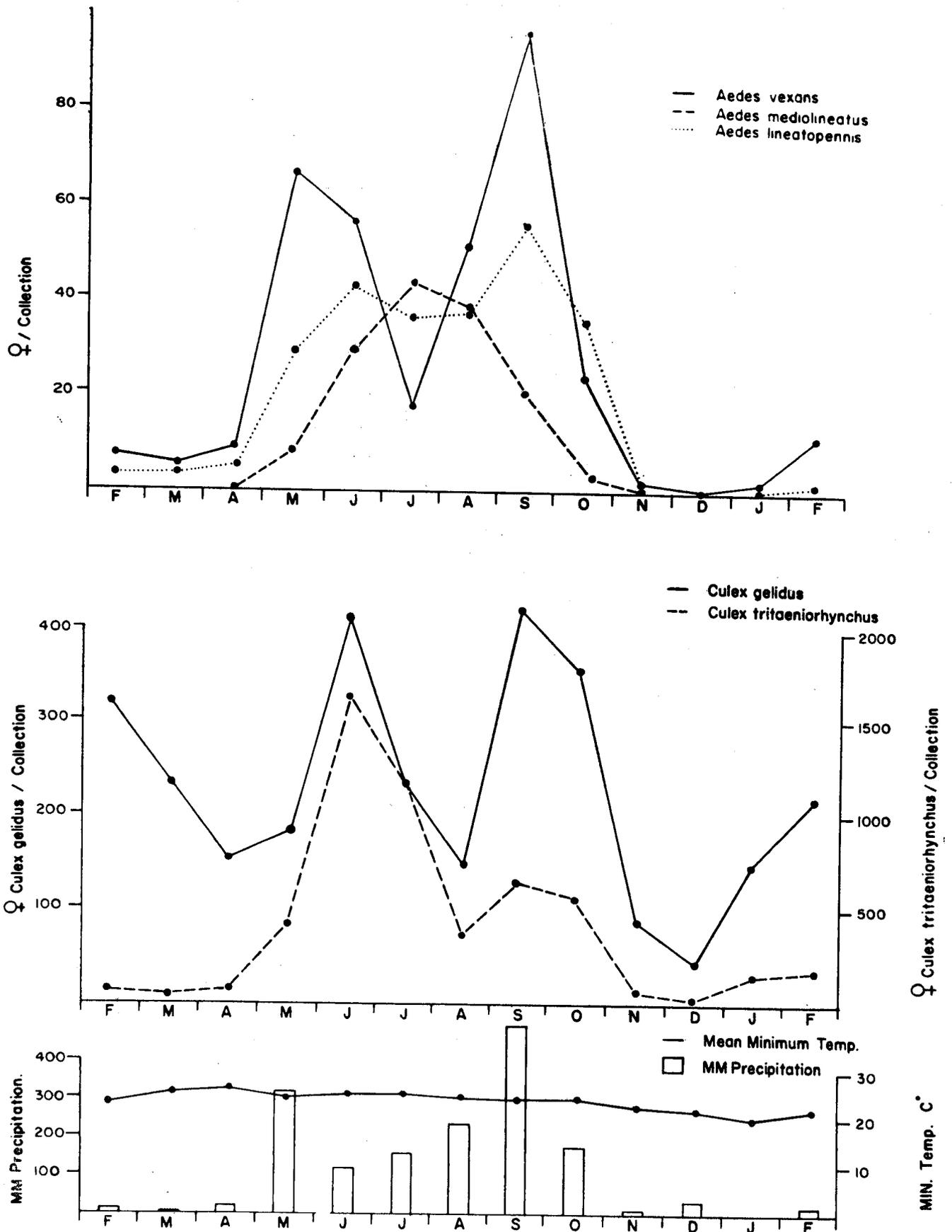


Figure 11. Light trap collections of Culex gelidus, Culex tritaeni-orhynchus, Aedes lineatopennis, Aedes mediolineatus and Aedes vexans at Bang Phra, 1966-67.

Table 1. Age specific hospitalization rates for hemorrhagic fever, Bangkok-Thonburi, 1965-1966.

Age (yr)	1965		1966	
	Cases	Rate/1000*	Cases	Rate/1000*
<1	246	2.9	330	3.9
1	197	2.4	190	2.3
2	260	3.3	275	3.5
3	280	3.7	352	4.6
4	291	3.9	404	5.5
5	222	3.1	431	6.1
6	191	2.9	408	6.2
7	156	2.4	282	4.4
8	115	1.9	260	4.3
9	85	1.6	190	3.5
10	65	1.3	180	3.5
11	57	1.2	149	3.0
12	46	1.0	109	2.4
13	13	0.31	51	1.2
14	13	0.34	30	0.78
15	4	0.003	22	0.018

*based on 1960 census

Table 2. Age distribution of 452 hospitalized cases of hemorrhagic fever in Saigon 1966.

Age (yr)	Cases		Deaths	
	No.	%	No.	%
<1	14	3.1	3	4.2
1	27	6.0	5	7.0
2	38	8.4	2	2.8
3	97	21.6	12	16.9
4	117	25.9	25	35.2
5	49	10.8	10	14.1
6	38	8.4	7	9.9
7	21	4.6	3	4.2
8	15	3.2	2	2.8
9	5	1.1	0	
10	10	2.2	0	
11	8	1.8	0	
12	5	1.1	2	2.8
> 12	8	1.8	0	

Table 3. Distribution of cases by clinical syndrome and etiology.

Syndrome	Etiology		
	Dengue	Unknown	Total
Shock	13	0	13
HF	18	8	26
DF	24	12	36
UF	35	29	64
Totals	90	49	139

Table 4. Distribution of 90 dengue cases by clinical syndrome and serologic class.

Syndrome	Serologic Response		
	Primary	Secondary	Pos., N.E.C.*
Shock	0	9	4
HF	1	9	8
DF	3	18	3
UF	5	25	5
Totals	9	61	20

* Positive, not exactly classifiable (see text)

Table 5. Distribution of dengue Cases by age and sex.

Age (yr)	Male	Female	Total
<1	0	0	0
1	1	0	1
2	1	1	2
3	6	5	11
4	6	6	12
5	2	9	11
6	6	6	12
7	6	9	15
8	2	4	6
9	2	3	5
10	3	4	7
11	1	2	3
12	0	0	0
13	0	2	2
14	1	1	2
15	<u>1</u>	<u>0</u>	<u>1</u>
Total	38	52	90

Table 6a. Dengue viruses from human cases by dengue type and clinical syndrome.

Clinical Syndrome (No. cases)	Numbers of Strains Isolated			
	Type 1	Type 2	Type 3	Untyped
Shock (13)	0	1	1	1
HF (26)	0	0	2	0
DF (36)	1	1	3	2
UF (64)	0	6	0	3
Totals	1	8	6	6

Table 6b. Distribution of 15 dengue virus strains by serotype, area and date of recovery.

<u>Village</u>	<u>Virus Type</u>	<u>No. of Strains</u>	<u>Date (s) of Recovery</u>
Mae Nam	1	1	20 July
	2	5	20, 26 July; 2,8,22 Aug.
	3	1	31 July
Taling Ngam	2	1	11 Aug.
	3	2	4 Aug. 6 Sept.
Ang Tong	3	3	31, Aug; 6, 29 Sept.
Li Pa Noi	2	1	10 Sept.
La Mai	2	1	5 Sept.

Table 7a. Number of dwellings found infested with Aedes aegypti in three Koh Samui villages, 1966—1967

<u>Month</u>	<u>Mae Nam</u>	<u>Ang Thong</u>	<u>Taling Ngam</u>
July, 1966	13/13*	25/25	—
August, 1966	14/15	—	—
February, 1967	76/120	76/76	14/30

* (No infested / no checked)

Table 7b. Sources of A. aegypti and A. albopictus larvae collected on Koh Samui, November 1966.

<u>Species</u>	<u>Source</u> (Number of Collections)	
	<u>Artificial Containers*</u>	<u>Natural Containers**</u>
<u>Aedes aegypti</u>	10	3
<u>Aedes albopictus</u>	17	42
Both species	8	3

* Artificial containers: water jugs, cans drums, etc.

**Natural containers: coconut shells, husks and bracts.

Table 7c. Summary of collections of adult A. aegypti and A. albopictus from Koh Samui, November 1966.

<u>Species</u>	Indoors		Outdoors	
	<u>Number Times Collected</u>	<u>Number Mosquitoes Collected</u>	<u>Number Times Collected</u>	<u>Number Mosquitoes Collected</u>
<u>Aedes aegypti</u>	25	86	1	2
<u>Aedes albopictus</u>	12	47	9	72

Table 8. Effect of Time (minutes) on Virus Survival (%) for Various Viruses at 37°C and 42°C.

Time (minutes)	Viruses					
	Hawaii (D-1)	TH-Sman (D-1)	#10572 (D-1)	N.G. "C" (D-2)	#10286 (D-2)	H-241 (D-4)
37°C	15	88	84	82	98	88
	30	66	71	—	97	98
	60	46	52	64	64	74
	90	41	42	—	—	68
42°C	15	8	49	65	90	61
	30	0	25	—	88	48
	60	0	0	38	48	18
	90	0	12	—	—	10

*Percent virus survival compared to zero time control, determined by plaque assay

plaque reduction activity of antibody free, fresh, unfrozen sera.

<u>Sera</u>		<u>Virus</u>			
		<u>D-1</u>	<u>D-2</u>	<u>D-3</u>	<u>D-4</u>
Human—A	undil	73*	90	95	65
	1:5	10	18	25	12
	1:10	0	10	0	0
Human—B	1:5	11	20	0	30
	1:10	5	15	0	0
Monkey V90	undil	67	—	69	—
	1:5	21	28	0	28
	1:10	17	30	0	26
Monkey V92	1:5	39	0	9	23
	1:10	0	0	0	0
Rabbit	1:5	—	40	20	0
	1:10	24	16	24	0
Guinea pig	1:5	30	30	38	7
	1:10	19	20	0	0
Mouse	1:10	—	65	—	—

*% reduction compared to controls

Table 10. Prototype and newly isolated dengue virus strains studies.

Dengue Virus Strain	Host* and Passage	Origin	Year Isolated	Associated Disease**	Dengue Serotype
Dengue-1 (Hawaii)	sm-124	Hawaii	1944		1
Dengue-2 (N.G. "C")	sm-26	New Guinea	1944		2
Dengue-3 (H-87)	sm-21	Philippines	1956		3
Dengue-4 (H-241)	sm-24	"	1956		4
TH-36	sm-16	Thailand	1958		2
TH-Sman	sm-13	"	1958		1
2383-62	sm-4	Thailand	1962	U	3
2557-62	sm-5	"	"	D	3
5987-62	sm-6	"	"	HF	3
648-63	sm-7	"	1963	HF	3
2403-63	tc-1, sm-6	"	"	HF	4
2443-63	sm-7	"	"	HF	4
10044	sm-3	"	1964	HF	2
10286	sm-4	"	"	HF	2
10572	sm-7	"	"	HF	1

Table 11. Homologous and heterologous neutralizing antibody titers of monkey antisera to prototype strains of dengue virus.

Viruses	Antisera					
	D-1	D-2	D-3	D-4	TH-36	TH-Sman
Dengue-1	<u>300*</u>	0	90	10	0	100
Dengue-2	0	<u>1400</u>	30	13	1700	0
Dengue-3	0	0	<u>350</u>	0	0	0
Dengue-4	0	0	14	<u>150</u>	0	0
TH-36	0	2500	NT**	NT	2500	NT
TH-Sman	70	0	NT	NT	0	<u>115</u>

* Reciprocal of 50% plaque reduction titer, 0 = <10

** NT = not tested

Table 12. Neutralizing antibody titers of monkey antisera to dengue viruses related to type 1.

Viruses	Antisera									
	D-1	D-2	D-3	D-4	TH-36	TH-Sman	12900	14580	18280	22448
Dengue-1	300*	0	90	10	0	100	520	100	260	420
TH-Sman	70	0	NT	NT	0	115	530	280	> 640	> 640
10572	66	0	25	0	0	80				
12900	50	0	40	0	0	120	670		380	450
13507	35	0	0	0	0	50	410		530	580
13819	25	0	0	0	0	90				
14580	105	20	35	20	15	150		> 640		
18280	40	0	0	0	0	70	420		425	> 640
22448	110	0	15	0	0	140	400		480	490
24001	100	0	40	0	0	160				
24007	160	12	30	0	0	120				
24177	180	0	20	0	10	170				

* Reciprocal of 50% plaque reduction titer 0= \leq 10

Table 13. Neutralizing antibody titers of reference antisera against dengue viruses related to type 2.

Viruses	Antisera						
	D-1	D-2	D-3	D-4	TH-36	TH-Sman	
Dengue-2	0*	1400	30	13	1700	0	
TH-36	0	2500	NT	NT	>2560		
10044	0	>640	60	20			
10286	0	600	50	10			
11340	0	350	10	0	190	0	
16681	0	500	80	0			
20731	0	>640	40	0	>640	0	
20833	0	>320	10	0	>320	0	
21868	0	240	10	0	100	0	
23753	0	270	15	0			

*Reciprocal of 50% plaque reduction titer; 0 = <10

Table 14. Neutralizing antibody titers of reference antisera to dengue viruses related to type 3.

Viruses	Antisera							
	D-1	D-2	D-3	D-4	TH-36	TH-Sman	14670	Pak-18
Dengue-3	0*	0	350	0	0	0	100	40
2383-62	0	0	350	0	0	0		
2557-62	0	0	130	0	0	0		
5987-62	0	0	120	0	0	0		
648-63	0	0	150	0	0	0		
14670	0	0	200	0	0	0	130	
14962	0	0	160	0	0	0		
16005	0	0	180	0	0	0		
Pak-18	0	0	270	0	0	0		55
16876	0	0	190	0	0	0		
17048	0	0	130	0	0	0		
18302	0	0	300	0	0	0		
21153	0	0	200	0	0	0		
24705	0	0	300	0	0	0		
T 502066	0	0	70	0	0	0		

* Reciprocal of 50% plaque reduction titer; 0 = <10

Table 15. Neutralizing antibody titers of dengue-4 antisera to reference virus strains.

Viruses	Antisera		
	Dengue-4 Monkey	Dengue-4 Mouse	No. 14486 Monkey
dengue-1	10*	10	0
dengue-2	15	30	0
dengue-3	0	20	0
dengue-4	150	> 320	130
TH-36	10	10	0
TH-Sman	0	0	0

* Reciprocal of 50% plaque reduction titer; 0 = <10

Table 16. Neutralizing antibody titers of reference antisera to dengue viruses related to type 4.

Viruses	Antisera							
	D-1	D-2	D-3	D-4	TH-36	TH-Sman	No. 14486	
Dengue-4	0*	0	0	> 320	0	0	130	
2403-63	0	0	0	140	0	0	270	
2443-63	0	0	0	100	0	0		
14486	0	0	0	110	0	0	> 320	
16572	0	0	0	100	0	0	100	
16603	0	0	0	280	0	0	200	
16727	0	0	0	80	0	0	160	
23751	0	0	0	> 320	0	0		
24038	0	0	0	> 320	0	0		

*Reciprocal of 50% plaque reduction titer; 0 = <10

Table 17. Identification of dengue viruses of isolate from patients on Koh Samui.

<u>Virus</u>	<u>Passage</u> <u>Level</u>	<u>Antisera</u>				<u>Virus</u> <u>Type</u>
		<u>D-1</u>	<u>D-2</u>	<u>D-3</u>	<u>D-4</u>	
24366	tc-3	80*	0	0	0	1
24367	tc-4	0	50	0	0	2
24453	tc-3	0	40	0	0	2
24464	tc-4	0	25	0	0	2
24546	tc-2	0	0	160	0	3
24552	tc-4	0	160	0	0	2
24705	tc-2	0	0	300	0	3
24718	tc-5	0	160	10	0	2
24742	tc-4	0	25	0	0	2
24801	tc-4	0	300	0	0	2
24956	tc-2	0	0	80	0	3
24967	tc-3	0	100	0	15	2
24969	tc-1	0	0	140	0	3
24970	tc-1	0	0	80	0	3
25076	tc-3	0	40	0	0	2
25163	tc-2	0	0	230	0	3
Homologous viruses		400	450	160	130	—

*Reciprocal of 50% plaque reduction titer, 0 — <10

Table 18. Identification of dengue viruses isolated from mosquitoes from Koh Samui.

<u>Virus</u>	<u>Mosquito</u> <u>Species</u>	<u>Passage</u> <u>Level</u>	<u>Antisera</u>			
			<u>D-1</u>	<u>D-2</u>	<u>D-3</u>	<u>D-4</u>
BKM 536-66	Ae. aegypti	sm-4	0*	450	20	0
BKM 540-66	" "	tc-3	0	50	0	0
BKM 542-66	" "	tc-3	0	100	0	0
BKM 547-66	" "	sm-3	0	120	40	0
BKM 551-66	" "	sm-4	0	1000	40	0
" " "	" "	tc-4	0	40	0	0
BKM 773-66	Ae. albo	tc-4	0	30	0	0
BKM 875-66	" "	tc-2	0	40	0	0

*Reciprocal of 50% plaque reduction titer, 0 = <10

Table 19. Results of plaque reduction neutralization tests with monkey antisera against tissue culture and suckling mouse strains of dengue-2 viruses.

<u>Viruses</u>	<u>Monkey Antisera</u>			
	<u>dengue-2</u> <u>(N.G. "C")</u>	<u>dengue-2</u> <u>(TH-36)</u>	<u>BKM-551</u> <u>tc-4</u>	<u>BKM-551</u> <u>sm-4</u>
Dengue-2 (New Guinea "C" sm-26)	450*	450	65	1000
Dengue-2 (TH-36, sm-16)	160	320	25	160
BKM551, tc-4	40	30	20	40
BKM551, sm-4	2000	NT**	60	160

* Reciprocal of 50% plaque reduction titer

** NT=Not tested

Table 20. Comparison of dengue virus isolation from human serum and blooded mosquitoes by direct and delayed plaque isolation techniques.

<u>Dengue Type</u>	<u>Direct Only</u>	<u>Delayed Only</u>	<u>Both Methods</u>
1	0*	0	1
2	0	4	8
3	0	1	5
Untyped	0	5	9
	<hr/>	<hr/>	<hr/>
Totals	0	10	23

* No. of strains isolated

Table 21. Number of passages required for suckling mouse mortality by 15 dengue agents in acute phase human serum.

<u>Dengue Type</u>	<u>No. of Strains Causing Mortality at Indicated Passage</u>		
	<u>1st</u>	<u>2nd</u>	<u>3rd</u>
1	1	0	0
2	0	2	4
3	1	1	2
Untyped	1	0	3
	<hr/>	<hr/>	<hr/>
Totals	3	3	9

Table 22. Virus isolations from mosquitoes from Koh Samui.

Pool No.	Species	Plaques in LLCMK-2 cells		Suckling Mice		Virus type
		direct	delayed	ill	CVR***	
BKM-497	Ae. aegypti	3	nt*	0	12/12	?
M-527	"	3	nt	0	12/12	?
BKM-536	"	5	nt	11/12**	-	dengue-2
BKM-540	"	6	nt	0	7/12	dengue-2
BKM-542	"	20	nt	0	11/12	dengue-2
BKM-545	"	8	nt	0	0	?
BKM-546	"	30 ±	nt	2	8/10	?
BKM-547	"	30 ±	nt	6/16	nt	dengue-2
BKM-551	"	30 ±	nt	4/16	nt	dengue-2
BKM-773	Ae. albo.	0	31	0	11/12	dengue-2
BKM-842	"	0	35	0	0	?
BKM-844	"	18	nt	0	12/12	?
BKM-875	"	12	nt	0	10/12	dengue-2

* nt = not tested

** No. ill or dead/No. inoculated

*** CVR-resistant to 100 LD₅₀ dengue-2 challenge

Table 23. Viremia in monkeys (Macaca irus) following subcutaneous injection of dengue viruses.

<u>Dengue Type</u>	<u>Virus strain and Passage</u>	<u>Inoculum (pfu)</u>	<u>Days Viremia Detected</u>
1	14580 BS-C-1 p-4	10 ³	7,8,9,10
1	18280 BS-C-1 p-4	10 ³	6,7,8,9,10
1	22448 BS-C-1 p-4	10 ⁴	7,8,9
1	12000 BS-C-1 p-4	10 ⁴	4,5,6,7,8,9,10
1	Hawaii sm-123	10 ⁴	7,8,9,10
1	TH-Sman sm-14	10 ^{3.5}	5,6,7,8
1	13507 sm-8	10 ⁴	7,8,9,10
3	AP-16 BS-C-1 p-3	10 ^{2.3}	7
4	11220 BS-C-1 p-4	10 ^{2.1}	4,5,6,7,8
4	14486 BS-C-1 p-3	10 ²	5,6
1	12900 BS-C-1 p-4	10 ^{4.5}	1,2,3,4,5,6,7,8,9
4	2443-63 sm-4 LLCMK2 p-2	10 ^{2.3}	5,7

Table 24. Viremia in gibbons (Hylobates lar) following subcutaneous injection with dengue-2 virus*.

<u>Gibbon No.</u>	<u>Days Viremia Detected</u>	<u>Maximum Titer (pfu/ml.)</u>
S-21	4,5,6,7,8	4.5x10 ²
S-29	6,7,8,9	2.5x10 ²
S-30	3,4,5,6	1.5x10 ²
S-34	3,4,5,6,7	2.5x10 ²
S-38	2,3,4,5	4.1x10 ²
S-41**	6	15
S-42**	6,7,8	30

* 100 pfu of No. 10044 (3rd BS-C-1 passage)

**S-41 and S-42 had a dengue-1 infection 5 months previously

Table 25. Appearance of 19S and 7S dengue HI antibodies following a primary dengue—2 infection and a secondary dengue—1 infection one year later.

Day After Inoculation	Monkey A—21				Monkey A—37			
	1st Infection*		2nd Infection**		1st Infection*		2nd Infection**	
	19S	7S	19S	7S	19S	7S	19S	7S
2	0***	0	0	160	0	0	0	80
4	0	0	0	160	0	0	0	80
6	0	0	0	160	0	0	0	80
8	0	0	0	160	0	0	0	80
10	10	0	0	80	0	0	0	80
12	80	20	0	640	0	0	0	320
14	160	80	0	1280	0	0	0	1280
21	80	160	0	1280	0	0	0	1280
28	80	160	0	1280	0	0	0	1280
35	20	320	0	1280	0	0	0	1280
42	0	160	0	1280	0	0	0	1280
60	0	320	0	1280	0	0	0	1280

* Dengue—2 virus, 120 pfu

** Dengue—1 virus, 1000 pfu

***Reciprocal HI titer in highest titer fraction in 19S zone or 7S zone, 0 = <10

Table 26. Specificity of 19S and 7S dengue neutralizing antibody 14 days after dengue-2 infection.

<u>Antibody Fraction</u>		<u>Prototype Dengue Viruses</u>					
		<u>D-1</u>	<u>D-2</u>	<u>D-3</u>	<u>D-4</u>	<u>TH-36</u>	<u>TH-Sman</u>
Monkey A-21	19S	0*	45	0	0	30	0
	7S	0	180	0	0	100	0
Monkey A-39	19S	10	15	0	0	30	0
	7S	20	1,200	90	0	1,800	30

* Reciprocal of 50% plaque reduction titer

Table 27. Neutralizing antibody response to dengue-1 infection one year following dengue-2 infection.

Monkey No.	Days After Inoculation	Viruses			
		D-1	D-2	D-3	D-4
Monkey A-21	0	15*	1800	30	2
	21	> 2560	> 2560	45	200
Monkey A-37	0	0	750	0	0
	21	> 2560	> 2560	320	300

* reciprocal of 50% plaque reduction titer

Table 28. Homologous neutralizing antibody titers following dengue-1 infection one year after dengue-2 infection.

	Days after Inoculation					
	2	4	6	8	10	14
Monkey A-21	40*	60	40	40	200	> 2560
Monkey A-37	20	20	60	40	40	> 2560

* Reciprocal of 50% plaque reduction titer

Table 29. Density gradient centrifugation of serum from a primary dengue infection*

Day of Illness	Fraction No.									
	1	2	3	4	5	6	7	8	9	10
5	0**	0	0	0	0	0	0	0	0	0
6	0	0	20	20	0	0	0	0	0	0
7	0	0	20	20	0	0	0	0	0	0
8	0	0	20	40	0	0	0	0	0	0
10	0	20	40	80	10	20	80	0	0	0
19	0	0	40	20	0	80	160	20	0	0
30	0	0	0	20	0	40	80	40	0	0

* Case No. HFI 735, mild hemorrhagic fever due to dengue 3 virus.

**Reciprocal of HI titer, 0 = 10

Table 30. Density gradient centrifugation of serum from a secondary dengue infection*

day of Illness	Fraction No.									
	1	2	3	4	5	6	7	8	9	10
4	0**	0	0	0	0	40	160	20	0	0
6	0	0	0	0	20	320	1280	160	40	0
8	0	0	20	0	80	640	1280	320	80	40
19	0	0	0	0	40	320	1280	160	40	0
31	0	0	0	0	20	640	1280	160	40	40

*Case No HFI 720, dengue shock syndrome due to dengue 2 virus.

**Reciprocal of HI titer, 0 = 10

Table 31. Dengue HI titers of sera and serum pools from patients with dengue shock syndrome

Case No.	Serum No.	Day After Onset*	HI Antibody Titer			
			D-1	D-2	D-3	D-4
HFI-773	25327	6**	320	640	2560	2560
	25328	7**	640	2560	> 20480	2560
	25335	8	2560	2560	> 20480	> 20480
	25338	9	2560	2560	> 20480	> 20480
	pool		1280	1280	> 20480	> 20480
HFI-747	23548	3**	5120	10240	> 20480	> 20480
	23565	7	> 20480	> 20480	> 20480	> 20480
	23567	9	> 20480	> 20480	> 20480	> 20480
	23617	13	> 20480	> 20480	> 20480	> 20480
	pool		> 20480	> 20480	> 20480	> 20480
HFI-749	23735	5**	> 20480	> 20480	> 20480	> 20480
	23745	10	> 20480	10240	> 20480	> 20480
	23818	20	> 20480	> 20480	> 20480	> 20480
	23843	30	1280	1280	1280	5120
	pool		10240	10240	10240	20480
HFI-744	22516	5**	640	640	5120	640
	22575	6	5120	5120	20480	2560
	22581	8	2560	1280	20480	1280
	22629	20	1280	1280	20480	2560
	pool		5120	1280	20480	2560

* Day after onset of illness
 ** Days on which shock was observed

Table 31. (cont'd)

Case No.	Serum No.	Day After Onset*	HI Antibody Titer			
			D-1	D-2	D-3	D-4
HFI-737	22277	5**	320	640	1280	640
	22307	10	> 20480	> 20480	> 20480	> 20480
	22324	20	10240	10240	> 20480	5120
	22361	30	10240	10240	> 20480	5120
	pool		> 20480	> 20480	> 20480	5120
HFI-782	25857	4**	5120	1280	5120	2560
	25858	5	10240	1280	10240	10240
	25878	6	> 20480	5120	> 20480	> 20480
	pool		> 20480	5120	10240	10240

* Day after onset of illness
 ** Days on which shock was observed

Table 32. Relationship of immunoglobulin concentration to HI antibody titers in fractions from DEAE-cellulose Chromatography of serum pools from patients with dengue shock syndrome.

<u>Case No.</u>	<u>Fraction No.</u>	<u>Immunoglobulin Conc.</u>			<u>HI titer*</u>
		<u>Ig-G</u>	<u>Ig-A</u>	<u>Ig-M</u>	
HFI-773	I	560**	0	0	2560
	II	15	20	0	40
	III	40	0	98	160
HFI-747	I	440	0	0	10240
	II	30	66	0	160
	III	30	tr.***	86	160
HFI-749	I	520	0	0	10240
	II	20	70	0	80
	III	20	tr.***	110	80
HFI-744	I	280	0	0	10240
	II	tr.***	30	0	40
	III	tr.***	0	46	20
HFI-737	I	450	0	0	10240
	II	tr.***	32	0	20
	III	tr.***	0	30	> 20
HFI-782	I	280	0	0	5120
	II	tr.***	31	0	80
	III	tr.***	0	15	40

*Reciprocal of titer vs 8 units of the dengue antigen which gave highest titer.

**Mg%

***Trace

Table 33. Sucrose density gradient ultracentrifugation of serum pool from case HFI 773

	<u>Fraction No.</u>									
	1	2	3	4	5	6	7	8	9	10
Untreated	0*	0	0	0	80	320	1280	640	40	40
2-ME Treated	0	0	0	0	80	320	1280	640	40	40

*Reciprocal of HI titer vs dengue 3 antigen, 0 = <20

Table 34. HI and CF titers of whole sera and 19S and 7S fractions of early convalescent sera from arbovirus infections.

Convalescent Sera	Dengue-2	HI Titers**		JEV	CF Titers***		JEV
		Dengue-2	Chik		Dengue-2	Chik	
24043 (D)*	whole serum	20480	0	5120	128		
	19S	640			0		
	7S	5120			32		
22241 (D)	whole serum	160	0	80	8		
	19S	20			0		
	7S	80			4		
22631 (D)	whole serum	1280	0	640	16	0	16
	19S	320	0	320	0	0	0
	7S	1280	0	1280	16	0	32
25321 (C)	whole serum	0	320	0	0	8	0
	19S	0	320	0	0	0	0
	7S	0	320	0	0	8	0
25524 (JE)	whole serum	0	0	320	0	0	8
	19S	0	0	320	0	0	0
	7S	0	0	320	0	0	8

*(D) = Dengue infection, (C) = Chikungunya infection, (JE) = Japanese encephalitis infection

**Reciprocal of titer vs 8 units of indicated antigen, 0 = <20

***Reciprocal of titers vs 4 units of indicated antigen, 0 = <4

Table 35. Inhibition by specific 19S antibody of complement fixation by whole antisera and arbovirus antigens

Type of 19S Antibody Tested for Inhibition	Antidengue Serum vs Dengue Antigen	Aeti-Chik Serum vs Chik. Antigen	Anti-JE Serum vs JE Antigen
None (control)	32	8	8
Anti-dengue*	0	8	8
Anti-Chik**	32	0	8
Anti-JE***	32	8	0

* 19S fraction of serum 22631

** " " " " 25321

*** " " " " 25524

Table 36. Serum antibody titers at time of death (5th day of disease).

Viruses	Neutralizing Antibody Titers*	Hemagglutination-Inhibiting Antibody Titers**
Dengue-1	1:350	1:2560
Dengue-2	1:640	1:5120
Dengue-3	1:600	
Dengue-4	1:240	
Jap. Enceph.	<1:10	1:5120

* 50% plaque reduction titers

** Vs. 8 units of indicated antigen

Table 37. Dengue hemagglutination-inhibition titers* of sucrose density gradient fractions of serum at time of death.

Fraction No.	1	2	3	4	5	6	7	8	9	10
Untreated	0	0	0	40	0	40	320	160	20	0
Mercaptoethanol Treated	0	0	0	0	0	40	320	160	20	0

* Reciprocal of titer vs. 8 units of dengue-2 antigen, 0 = <10

Table 38. Results of diagnostic studies on 112 patients with F.U.O. at 93rd Evacuation Hospital, 1 April 66-31 August 66.

<u>Diagnosis</u>	<u>No. of Cases</u>
Malaria	10 (incl. 2 scrub typhus)
Leptospirosis	1
Scrub Typhus	11 (incl. 2 malaria)
Dengue	31
Chikungunya	10
Other Diagnosis	7
<hr/>	
Total With Diagnosis	68 (60%)
Undiagnosed	44 (40%)

Table 39. Results of diagnostic studies on patients with FUO at 93rd Evacuation Hospital, 1 Sept 66 to 15 Feb 67.

Total patients admitted to study	295
Withdrawn (administrative & operational necessity)	86
	<hr/>
Total Studied	209

<u>Final Diagnosis on 209 patients</u>	<u>No. cases</u>
Malaria	53 (incl 2 scrub typhus)
Leptospirosis	18
Scrub Typhus	13 (incl 2 malaria)
Dengue	10
Japanese Encephalitis	5
Other Diagnosis	46
	<hr/>
Total With Diagnosis	143 (69%)
Undiagnosed	66 (31%)

Table 40. Clinical diagnoses on 46 patients listed above as "other diagnosis".

Mumps	1	Acute bronchitis	1
Pyelonephritis	1	Melioidosis	1
Prostatitis	6	Inf. mononucleosis	6
Lymphogranuloma	1	Serum sickness	3
Strept. pharyngitis	5	Hemolytic disease	1
Amoebiasis	8	Cellulitis/abcess	4
Pneumonia	8		

Table 41. Distribution of cases by month and diagnosis, 93rd Evac. Hosp. F.U.O Study.

Month	No. Cases	Malaria	Lepto	Scrub	Dengue	JE	Other	Undx
Sept	33	12	4	2	2	1	7	5
Oct	36	8	2	2	1	3	8	12
Nov	47	13	4	3	4	1	8	14
Dec	35	7	6		2		7	13
Jan	41	2	2	4	1		11	21
Feb	19	11		2			5	1
Totals	211	53	18	13	10	5	46	66

Table 42. Comparative neutralization antibody assays of human sera from patients with Japanese encephalitis.

Patient	Tube Method	Macro	Micro
1. Acute	7.5/300*	<5/300	15/1000
Convalescent	<20/900	<20/300	<20/1000
2. Acute	7.5/300	10/200** <5/300	5/1000
Convalescent	30/300	20/200** <20/200	20/1000
3. Acute	<5/300	<5/200** <5/300	30/70** <5/1000
Convalescent	20/300	40/200** <20/300	80/70** <20/1000
4. Acute	18/300	5/200** 10/800	7.5/70** <5/1000
Convalescent	20/300	40/200** <20/300	80/70** <20/1000
5. Acute	7.5/300	<5/200** <5/300	<5/70** <5/1000
Convalescent	<20/300	60/200** <20/300	40/70** <20/1000
6. Acute	<20/300	<5/200** <20/800	<20/1000
Convalescent	60/300	120/200** 60/300	30/100

* MI titer (recip)/virus dose (LD₅₀)

** Retested where possible, increasing serum-virus incubation from 1 to 2 hours.

Table 43. Comparison of homologous serum neutralization titers against chikungunya and Sindbis virus measured by tube, macroplate metabolic inhibition and micro titer metabolic inhibition neutralization tests. Serum-virus mixtures incubated at 37°C for one (Sindbis) or two (Chikungunya) hours.

Chikungunya antiserum-virus trial.

Serum	Neutralization Method		
	Tube	Macro MI	Micro MI
Rabbit	10/200*	160/100*	> 500/70*
Rabbit	10/200	240/100	≥ 500/70
Mouse	30/200	≥ 500/100	≥ 500/70
Human Conv.	> 5/200	10/100	120/70

Sindbis antiserum-virus trial

Mouse**	7.5/200	> 5/500	15/500
Mouse IAF***	10/200	> 5/500	15/500
Mouse	7.5/200	> 5/500	5/500

* Reciprocal of serum titer over virus dose (LD₅₀) used.

** Antiserum to Thailand strain BKM-599.

***IAF = immune ascitic fluid.

Table 44. Agreement of hemagglutination-inhibition (HI) test titers with BHK 21 cell-Japanese encephalitis (JE) virus metabolic-inhibition test titers of human sera from a dengue epidemic (patients 1-3) and from scattered cases of viral encephalitis.

Patient No.	HI Test		Micro JE MI	Type Illness
	Dengue	JE		
1	80*	20*	<5*	Undifferentiated Fever
	10,240	20,480	<5	
2	1,280	1,280	20	Dengue-like Fever
	5,120	5,120	60	
3	640	2,560	20	Dengue-like Fever
	5,120	10,240	30	
4	80	2,560	15	Viral Encephalitis
	160	5,120	30	
5	0	0	0	Viral Encephalitis
	0	0	0	
6	80	1,280	30	Viral Encephalitis
	320	2,500	60	
7	40	640	30	Viral Encephalitis
	20	320	15	
8	0	320	30	Viral Encephalitis
	0	640	30	
9	80	2,560	15	Viral Encephalitis
	640	10,240	60	

* Reciprocal of Titer

Table 45. Proportion of parous Culex gelidus collected at Bang Phra, 1966-67.

Month	Number Dissected	Number Parous	Proportion Parous	95% C.L.
March	63	42	.66	.54-.78
April	198	72	3.6	.30-.42
May	99	41	.41	.31-.51
June	157	104	.66	.58-.74
July	153	69	.45	.37-.53
August	177	67	.38	.30-.46
September	168	68	.40	.32-.48
October	167	79	.47	.39-.55
November	93	36	.38	.28-.48
December	148	59	.40	.32-.48
January	91	37	.40	.30-.50
February	65	25	.38	.26-.50

Table 46. Virus isolation attempts from Bang Phra mosquitoes, 1966-1967.

<u>Species</u>	<u>Suckling Mice</u>		<u>Tissue Culture</u>	
	No. Indiv.	<u>Pools*</u>	No. Indiv.	<u>Pools</u>
<i>Culex gelidus</i>	82,169	2/714	51,875	2/367
<i>C. tritaeniorhynchus</i>	75,841	1/602	65,259	0/467
<i>C. fuscocephalus</i>	15,558	0/217	10,566	0/139
<i>Aedes Vexans</i>	5,844	1/75	5,651	1/65
<i>Ae. lineatopennis</i>	4,045	2/51	3,650	2/46
<i>Ae. mediotineatus</i>	3,054	2/51	2,977	1/48
<i>Anopheles aconitus</i>	167	0/5	127	0/4
<i>Mansonia uniformis</i>	660	0/19	454	0/12
<i>M. annulifera</i>	184	0/7	144	0/6

* Pools containing virus/total--pools tested

Table 47. Results to date of metabolic inhibition neutralization test screening of reactive wild bird and mammal species from Bang Phra against 30-300 TCLD₅₀ of virus. Only those species reacting are listed.

<u>Sera* from</u>	<u>Virus</u>		
	<u>JE</u>	<u>Sindbis</u>	<u>Chik**</u>
<u>Mammals</u>			
<i>Cyanopterus brachyotis</i>	2/56***	0/56	0/56
<i>Rattus rattus</i>	3/87	2/87	0/87
<i>Rattus norvegicus</i>	1/1	0/1	0/1
<i>Mus cervicolor</i>	2/17	2/17	0/17
<i>Bandicota indica</i>	1/7	0/7	0/7
<u>Birds (all resident species)</u>			
<i>Pycnonotus blanfordi</i>	5/65	0/65	1/65
<i>Pycnonotus goiavier</i>	5/58	0/58	0/58
<i>Passer flaveolus</i>	3/63	1/63	0/63
<i>Rhipidura javanica</i>	3/17	0/17	0/17
<i>Copsychis saularis</i>	1/35	0/35	0/35
<i>Sturnus tristis</i>	1/9	0/9	0/9
<i>Sturnus nigricollis</i>	1/2	0/2	0/2
<i>Rosettus amplexicaudatus</i>	1/1	0/1	0/1
<i>Phragmaticola aedon</i>	0/9	1/9	0/9

* Sera diluted 1:4 - 1:10

** Chikungunya

*** No. of sera neutralizing virus over total sera tested

Table 48. Recovery of arboviruses from Aedes mosquitoes from Bang Phra in 1966.

<u>Designation</u>	<u>Date Collected</u>	<u>Species</u>	<u>No. in Pool</u>	<u>Reisol. In</u>
BKM-367/66	14 Jul	<u>Ae. mediolineatus</u>	39	Mice, TC*
BKM-448/66	6 Jul	<u>Ae. mediolineatus</u>	85	Mice
BKM-457/66	7 Jul	<u>Ae. vexans</u>	11	Mice, TC
BKM-589/66	30 Jun	<u>Ae. lineatopennis</u>	11	TC
BKM-660/66	20 Jun	<u>Ae. lineatopennis</u>	36	TC

* TC = LLCMK₂ cell cultures. Primary isolations of call agents were in suckling mice

Table 49. Arbovirus group reaction of two unidentified Bang Phra viruses.

<u>Antigens</u>	<u>Antisera</u>	
	<u>Group A</u>	<u>Group B</u>
BKM-367/66	<20	640
BKM-589/66	<20	640

Table 50. Hemagglutination-inhibition titers (reciprocals) of mouse anti unknown virus serum against several arbovirus group B antigens.

<u>Antigen (Units)</u>	<u>Antisera</u>	
	<u>BKM-367/66</u>	<u>BKM-589/66</u>
West Nile (8)	80	80
Japanese Encephalitis (8)	320	640
Tembusu (8)	640	640
Dengue 1 (16)	40	40
Dengue 2 (4)	160	640
Dengue 4 (4)	640	1,280
BKM-367/66	<u>1,280</u>	<u>2,560</u>
BKM-589/66	> 2,560	> 2,560

Table 51. Hemagglutination-inhibition reactions (reciprocals) between two Bang Phra unknown viruses and Tembusu and West Nile viruses.

<u>Antigen (Units)</u>	<u>Antisera</u>		
	<u>BKM-367/66</u>	<u>BKM-589/66</u>	<u>Tembusu</u>
BKM-367/66 (8)	<u>640</u>		<u>160</u>
BKM-589/66		<u>1,280</u>	
Tembusu (8)	640	640	<u>≥ 5,120</u>
West Nile (8)		160	

Table 52. Plaque reduction neutralization tests of three Bang Phra unknown viruses by homologous and other arbovirus group B antisera.

<u>Antiserum (Source)</u>	<u>BKM-367/66</u>	<u>BKM-589/66</u>	<u>BKM-448/66</u>
BKM-367/66 (Mouse)	> 10,240*	> 10.240	
BKM-589/66 (Mouse)		> 10.240	640
Dengue 1 (Monkey)	<10/300**	<10/300	
Dengue 2 (Monkey)	<10/1400		
Dengue 3 (Monkey)	<10/350		
Dengue 4 (Monkey)	<10/150		
Tembusu (Mouse)	120/> 1,280	<10/> 1,280	
Japanese Encephalitis (Rabbit)	<10/900	<10/900	<10/900

* Reciprocal of 50% plaque reducing titer.

** Heterologous titer (reciprocal)/Homologous titer (reciprocal).

Table 53. Results of a serologic survey of horses in Nakorn Pathom.

Horse No.	Age	Serologic Tests					
		Hemagglutination-Inhibition		JE ²	JE	MI	
		Chk ¹	Dengue-1		CF ³	Neuf ⁴	
1	2 yrs	40	0	80	0	5	
2	9 mos	0	0	20	0	<5	
3	5 yrs	40	0	160	0	5	
4	3 yrs	20	40	320	8	5	
5	2 yrs	20	0	40	0	<5	
6	1 1/2 yrs	0	20	320	0	5	
7	1 1/2 yrs	0	0	80	0	10	
8	2 yrs	20	0	320	4	10	
9	2 yrs	40	0	40	0	<5	
10	1 yr	0	0	40	0	<5	
11	1 yr	20	20	320	8	7.5	
12	1 yr	0	0	60	4	7.5	
13	3 yrs	0	0	80	0	7.5	

Table 53. (Cont'd)

Horse No.	Age	Serologic Tests					
		Hemagglutination-Inhibition		JE ²	JE ³	MI	Neut ⁴
		Chik ¹	Dengue-1				
14 ⁵	13 yrs	20	20	2,560	8	80	
15	7 mos	0	0	0	0	<5	
16 ⁶	15 yrs	40	20	1,280	8	40	
17	6 yrs	40	0	120	0	10	
18	9 yrs	320	20	1,280	8	60	
19	6 yrs	80	40	1,280	8	60	
20	5 yrs	40	40	640	8	60	
21	6 yrs	40	40	320	4	10	
22	10 yrs	120	0	1,280	8	60	
23	9 yrs	40	40	640	8	40	
24	9 yrs	80	80	1,280	8	120	
25	6 yrs	0	0	640	8	160	
26	12 yrs	0	0	320	4	30	

Table 53. (Cont'd)

<u>Horse No.</u>	<u>Age</u>	<u>Hemagglutination—Inhibition</u>		<u>JE²</u>	<u>JE</u>	<u>MI</u>
		<u>Chik¹</u>	<u>Dengue—1</u>			
27	6 yrs	0	40	640	8	20
28 ⁵	10 yrs	40	40	1,280	16	120
29	3 yrs	20	0	320	0	10

1. Chikungunya Virus
2. Japanese Encephalitis Virus
3. Complement—fixation starting at serum dilutions of 1:4
4. Metabolic—Inhibition Neutralization vs 300 TC LD₅₀ of JE virus
5. Weakness of hand quarters at time of bleeding.
6. Aborted in September in 5th month of gestation.

Table 54. Serological response (reciprocal titers) to 3 arbovirus antigens in a horse with clinical encephalitis

<u>Sampling Date</u>	<u>Hemagglutination—Inhibition</u>		<u>JEV</u>	<u>JEV Complement Fixation</u>	<u>JEV Neut. Metabolic Inhibition</u>
	<u>Chik</u>	<u>Dengue—1</u>			
10 Nov 66	20	<20	40	0	<5
17 Nov 66	40	<20	640	4—8	60