

Summary of Virus Isolation and Identification JE Project—Chiangmai 1970

Principal Investigators : Ananda Nisalak, M.D.
Debhanom Muangman, M.D.
Dumrong Chiewsilp, CPT, MC, RTA
Pairotana Gunakasem, M.D.
Phanu Sithisomwong, D.D.S. (deceased)
Rapin Sritbhan, M.D.
Robert Edelman, LTC, MC
Suchinda Udomsakdi, M.D.

Assistant Investigators : Anan Boonkanoke
Aree Boriharnvanakett, B.S.
Chumpan Chavachati, B.S.
Jiraporn Supavadee, B.S.
Laddawal Sookcharoen, B.S.
Ming Choohong
Panor Srisongkram, B.S.
Suwana Vithanomsat, B.S.
Sumitda Narupiti, B.S.
Raveevun Leelasatayakul, B.S.
Prapai Thong—Ngarm, B.S.
Somchai Siripatananukulchai, B.S.
Nonglak Khananurak, B.S.
Roypim Tiptanatoranin, B.S.
Chalam Chantrasri, B.S.
Ravithat Putvatana, B.S.
Moragot Tanticharoen, B.S.

SUMMARY: Starting in April, 1970 and extending through March, 1971, the Dept. of Virology processed 5,878 mosquito pools for virus. Table 1 summarizes by month the number of mosquito pools processed, the number of viruses isolated, and the percent of mosquito pools positive for virus. In addition to mosquitoes, 284 serums collected from domestic animals in Chiangmai and four brain specimens obtained at autopsy from patients dying with encephalitis were studied for virus. No virus was recovered from three human brains from Chiangmai; Herpes simplex virus was recovered from one brain from Khon Kaen. An agent was isolated from one pig serum, but this agent could not be reisolated from the original serum specimen.

The mosquito pools, composed of nine species that are known or suspected vectors of arboviruses in Thailand, were inoculated into MK2 monolayer cell cultures and into the brains of 1–2 day old suckling mice. The animal sera and human brain materials described above were similarly processed. The results shown in Table 2 reveal that MK2 cell culture and suckling mouse (S.M.) systems are nearly equal in their capacity to detect and isolate Thai strains of JEV from mosquito pools. The titration of 2 JEV strains shown in Table 3 indicate that 1 LD₅₀ of virus for S.M. is equivalent to 0.7–2.4 plaque forming units in cell culture. Thus both SM & MK2 cells are nearly equally sensitive to JEV contained in mosquito suspensions. Because each system failed on occasion to detect JEV strains that the comparison system successfully detected (Table 2), both systems were used in tandem whenever possible in order to maximize the chance of virus recovery.

The details of the virus isolation protocol are given below. Before August 1970 and after December 1970, when there were fewer numbers of mosquito pools to be processed, all mosquito species except A. albopictus and A. aegypti were placed simultaneously into both isolation systems. The latter two species were titrated

only in MK2 cell culture. During August 1970 to December 1970, when the number of unprocessed mosquito pools rapidly accumulated, we divided the number of mosquito pools into two lots. Each lot was placed into the isolation system considered most sensitive to the expected viruses in that lot. Thus 3 mosquito species (Culex tritaeniorhynchus, Aedes aegypti, Aedes albopictus) were titrated in MK2 cell cultures. Five species were inoculated into suckling mice (Culex gelidus, Culex vishnui complex, Aedes lineatopennis, Aedes mediolineatus, Aedes vexans), and one species (Culex fuscocephala) was inoculated into both isolation systems. In this manner we were able to rapidly process over 3700 mosquito pools with a minimum time of 2 weeks and a maximum time of 3 months separating collection and final processing. Three 1 oz. prescription bottles, containing MK2 cell monolayer cultures were used per mosquito pool. One bottle was overlaid with agar after a 1 1/2–2 hour virus adsorption period; the second bottle was overlaid with agar on day 4, and the third bottle was maintained in fluid phase without agar. The large majority of JEV isolates in MK2 cells were detected as plaquing agents using the delayed agar method. No agent other than JEV was isolated in MK2 cells. The 10 mosquito pools that contained plaquing agents (Table 6) were initially inoculated only into S.M. The fluid phase in the third bottle provided a convenient source of JE virus seed which was often of high titer (greater than $10^{5.0}$ PFU/0.3 ml culture fluid).

One triturated mosquito pool was inoculated into 1 litter of 1–2 day old suckling mice (8 mice per litter) and observed daily for 21 days. Brains from healthy, 21 day old surviving mice were not blind-passed into fresh mice, because in preliminary experiments (described below) we were unable to detect latent JEV in surviving mice. Mice which had survived inoculation with JEV-infected mosquito pools showed no resistance to challenge with JEV given IC (Table 4) or with 100 LD50 Dengue 2, New Guinea C strain, given IC (not shown). In other experiments the brains of mice which survived inoculation with mosquito pools containing JEV did not kill mice on three serial passages in suckling mice or yield plaques in MK2 cell culture.

The 13 JEV strains isolated in 1970 with their host mosquito species, *in vitro* passage history, PRN identification data, and reisolation results are all listed in Table 5. Each strain produced identical and morphologically characteristic plaques in MK2 cells. The 13 strains grew to high titer ($\geq 10^9$ PFU) in S.M. brain inoculated with once-passaged material. Thus the failure to isolate 2 strains of JEV in S.M. (Table 2) was probably not due to their non-virulence in mice. As previously shown (Table 3) we calculate that 0.7 to 2.4 PFU of JEV inoculated intracerebrally will infect and kill 50% of our outbred strain of mice. Two inbred strains of mice, Balb/c, and C57–black, were equally susceptible to IC inoculation. A neutralization kinetic test, described in detail elsewhere in this annual report, has revealed no major antigenic differences between three Thai strains (isolated from Bang Phra, Udorn, and Chiangmai) and the standard Nakayama strain. The results of all *in vitro* studies therefore suggest that JEV strains of Thailand have closely similar biologic and antigenic characteristics.

Isolates not identified as JEV are listed in Table 6. We have observed that suckling mice are more efficient than the direct and delayed plaque system in MK2 cells for the isolation of virus other than JEV (Table 6). Except for three isolates tentatively identified as Tembusu, these agents are as yet unidentified and 19 of the 29 do not form plaques in MK2 cell monolayers.

Identification data obtained to date on the Tembusu isolates are listed in Table 7. Twenty-six agents have not been identified as to virus type. None of these 26 unidentified viruses reacted with hyperimmune group B mouse ascitic fluid in a screening complement fixation test. A complement fixation screening test using anti-group A mouse hyperimmune ascitic fluid against twenty-six unidentified agents, has produced inconclusive results. Eight of the 26 agents are resistant to ether and desoxycholate, and one (BKM–1165–70) is resistant to ether but not desoxycholate (Table 8). Two plaquing agents (#705 and #2063) are not neutralized by typing antiserum to JEV, dengue 1–4, Tembusu, Batai, Wesselsbron, Chikungunya, and Sindbis. We are therefore dealing with a multiplicity of mosquito isolates never before described for Thailand. With the exception of A. albopictus and A. aegypti, at least one new agent has been isolated from each of the nine mosquito species processed for virus (Table 6).

Table 1.
 A Summary of Monthly Reports on Virus Isolations from Wild-caught Mosquitoes,
 Chiangmai Province, Thailand, Using Suckling Mouse and MK-2 Culture
 Systems (April 1, 1970 - March 31, 1971)

Month ⁽¹⁾	Total Mosq. Pools Processed	Total Viruses Isolated	Percent of Mosq. Pools Positive
April 1970	180	0	0
May "	239	1	0.4
June "	504	9	1.8
July "	559	19	3.4
August "	756	1	0.1
September "	736	3	0.4
October "	702	2	0.3
November "	544	6	1.1
December "	904	1	0.1
January 1971	353	0	0
February "	209	0	0
March "	192	*	*
Total	5878	42	0.63 (Average)

(1) Mosquitoes were collected in the field about one month earlier.

* Final results pending.

Table 2.
Comparison of Suckling Mouse and MK-2 Cell Culture Methods in Terms of
Their Ability to Isolate JE-Virus from Mosquito Pools*

	<u>Arbovirus Isolation Method</u>		<u>Total Number of Isolates</u>
	Suckling Mice (0.02 ml/inoculum)	MK-2 Cell Culture (0.3 ml/inoculum)	
1.	Positive	Negative	1
2.	Negative	Positive	2
3.	Positive	Positive	5
4.	Positive	(Not Done)	3
5.	(Not Done)	Positive	1
6.	Positive	Fungal Contamination	1
		Total JEV Isolates =	13

* Triturated pools inoculated simultaneously into both isolation systems.

Table 3.

Comparison of the Sensitivity of Suckling Mouse and MK-2 Cell Culture Virus Isolation Systems

Virus Dilution	Mosquito Suspensions			
	JE Virus "Lab" Strain ¹ (JE # 40783)		JE Virus "Field" Strain ² (JE # BKM-11137-70)	
	Mice ³	MK-2 ⁴	Mice ³	MK-2 ⁴
Undil.	0/16	TNTC ⁷	—	—
10 ⁻¹	0/16	TNTC	0/16	45,52
10 ⁻²	0/16	TNTC	3/16	17,36
10 ⁻³	1/16	79,90	14/16	2,5
10 ⁻⁴	12/16	10,13	16/16	0,1
10 ⁻⁵	16/16	2,2	16/16	0,0
Control	16/16	0,0	16/16	0,0
	<u>1 LD₅₀ = 2.4 PFU</u>		<u>1 LD₅₀ = 0.7 PFU</u>	

1. Isolated from human brain and passed 3 times in S.M.
2. Isolated from mosquitoes and passed once in MK₂ cell culture.
3. Total number of suckling mice surviving IC inoculation over the total number of mice inoculated.
4. PFU/0.3 ml inoculum.
5. 1 LD₅₀ = 10^{-3.6} dil, 0.02 ml IC/mouse
6. 1 LD₅₀ = 10^{-2.8} dil.. 0.02 ml IC/mouse
7. Plaques too numerous to count.

Table 3.
Comparison of the Sensitivity of Suckling Mouse and MK-2 Cell Culture Virus Isolation Systems

Virus Dilution	Mosquito Suspensions			
	JE Virus "Lab" Strain ¹ (JE # 40783)		JE Virus "Field" Strain ² (JE # BKM-11137-70)	
	Mice ³	MK-2 ⁴	Mice ³	MK-2 ⁴
Undil.	0/16	TNTC ⁷	—	—
10 ⁻¹	0/16	TNTC	0/16	45,52
10 ⁻²	0/16	TNTC	3/16	17,36
10 ⁻³	1/16	79,90	14/16	2,5
10 ⁻⁴	12/16	10,13	16/16	0,1
10 ⁻⁵	16/16	2,2	16/16	0,0
Control	16/16	0,0	16/16	0,0
	<u>1 LD₅₀ = 2.4 PFU</u>		<u>1 LD₆₅₀ = 0.7 PFU</u>	

1. Isolated from human brain and passed 3 times in S.M.
2. Isolated from mosquitoes and passed once in MK₂ cell culture.
3. Total number of suckling mice surviving IC inoculation over the total number of mice inoculated.
4. PFU/0.3 ml inoculum.
5. 1 LD₅₀ = 10^{-3.6} dil, 0.02 ml IC/mouse
6. 1 LD₅₀ = 10^{-2.8} dil.. 0.02 ml IC/mouse
7. Plaques too numerous to count.

Table 4. Death of JEV-Inoculated Mice Rechallenged with JEV

Dilution of JEV (BKM-977) ¹	Number of Surviving Mice			
	<u>Day 0</u> Inoc. ² with JEV (BKM-977)	<u>Day 14</u>	<u>Day 28</u> Re-inoc. with JEV ³	<u>Day 35</u>
10 ⁻⁴	16	0	—	—
10 ⁻⁵	16	0	—	—
10 ⁻⁶	16	0	—	—
10 ⁻⁷	16	0	—	—
10 ⁻⁸	16	0	—	—
10 ⁻⁹	16	3	3	0
10 ⁻¹⁰	16	14	14	0
11 ⁻¹¹	16	14	14	0
Control	16	16	16	0

1. Isolated from C. tritaeniorhynchus; second S.M.¹ passage
2. 0.02 ml IC/mouse of 20% S.M. brain
3. Nakayama strain (100 LD₅₀/mouse IC)

Table 5. JE Virus Strains Isolated from Chiangmai Mosquitoes (from April 1, 1970 to March 31, 1971)

Virology Log No.	Mosq. Species	Passage	Identification Results			Reisolated ³ from mosq. pool
			Titer ¹ (PFU/0.3 ml)	Reciprocal PRN titer against JEV antiserum ²		
1. BKM-438-70	C. tri	MK2-1	7 × 10 ²	1,280	yes	
2. BKM-775-70	C. tri	S.M.1	1.5 × 10 ⁹	1,600	N.A.	
3. BKM-977-70	C. tri	S.M.2	1 × 10 ⁹	1,000	yes	
4. BKM 984-70	C. fusco	S.M.2	2.8 × 10 ⁹	1,100	N.A.	
5. BKM-1022-70	C. fusco	S.M.2	5.6 × 10 ⁹	3,900	yes	
6. BKM-1074-70	C. tri	S.M.2	1 × 10 ⁹	600	N.A.	
7. BKM-1137-70	C. tri	MK2-1	3.2 × 10 ⁴	1,000	yes	
8. BKM-1096-70	C. tri	MK2-1	2 × 10 ⁴	640	yes	
9. BKM-1410-70	C. tri	S.M.3	1 × 10 ¹⁰	2,000	yes	
10. CMT-678-70	C. tri	MK2-3	1 × 10 ⁵	2,560	N.A.	
11. BKM-4018-70	C. gelidus	S.M.2	2.4 × 10 ⁹	2,560	N.A.	
12. BKM-4035-70	C. gelidus	S.M.2	2.0 × 10 ⁹	2,560	N.A.	
13. BKM-4088-70	C. gelidus	S.M.2	2.5 × 10 ⁹	1,600	N.A.	

1. PFU/0.3 ml of 20% S.M. brain or MK2 culture fluid

2. Homologous antiserum titer = 1:4500.

3. In MK2 cell culture or S.M. . N.A. = not attempted.

Table 6.
Viruses Other than JEV Isolated from Chiangmai Mosquitoes Caught
from 1 April 1970 to 31 March 1971

Virology Log No.	Entomol. Log No.	Mosq. Species	Plaque in MK2 ¹
1. BKM-705-70	CM-1594	<i>C. vishnui</i> subgroup	yes ²
2. BKM-1160-70	CM-1778	" "	yes ³
3. BKM-4165-70	CM-6183	" "	no
4. BKM-1052-70	CM-1771	<i>C. fuscocephala</i>	no
5. BKM-1072-70	CM-1773	" "	no
6. BKM-1125-70	CM-1768	" "	yes ³
7. BKM-1126-70	CM-1769	" "	yes ³
8. BKM-1165-70	CM-1772	" "	no
9. BKM-1188-70	CM-1777	" "	no
10. BKM-2804-70	CM-4395	" "	no
11. BKM-2849-70	CM-4410	" "	no
12. BKM-3148-70	CM-4496	" "	no
13. BKM-3716-70	CM-5771	" "	no
14. BKM-1006-70	CM-1882	<i>C. tritaeniorhynchus</i>	no
15. BKM-1048-70	CM-1766	" "	no
16. BKM-1095-70	CM-1767	" "	no
17. BKM-1098-70	CM-1765	" "	no
18. BKM-1100-70	CM-1772	" "	yes ⁴
19. BKM-1065-70	CM-1759	<i>C. gelidus</i>	no
20. BKM-1142-70	CM-1816	" "	yes ³
21. BKM-3136-70	CM-4788	" "	no
22. BKM-3990-70	CM-5883	" "	no
23. BKM-4116-70	CM-6274	" "	no
24. BKM-1028-70		<i>Aedes lineatopennis</i>	no
25. BKM-1088-70	CM-1789	" "	yes ³
26. BKM-1122-70	CM-1783	" "	no
27. BKM-1173-70	CM-1782	" "	yes ³
28. BKM-2063-70	CM-2938	<i>Aedes mediotineatus</i>	yes ²
29. BKM-1064-70	CM-1918	<i>Aedes vexans</i>	yes ³

1. Isolated and passed 2-3 times in suckling mice and then titrated in MK2 cell cultures
2. Medium clear plaque
3. Small hazy plaque
4. Large irregular plaque

Table 7. Preliminary Identification of Tembusu Virus Strains Isolated from Chhengmai Mosquito Pools in 1970

Virology No.	Mosquito Species	CFI titre		Anti-Dengue 2	HI titre ²		PRNT in MK2 cells	L.N.I. in S.M. ⁴
		Anti-grp A	Anti-grp B		Anti-grp B	Anti-Tembusu		
BKM-4165-70	C. vishnui	1:8	≥ 1:64	1:32	320/320	2560/5120	no plaque	1.5/3.0
BKM-1142-70	C. gelidus	<1:4	<1:4	<1:4	160/320	1280/5120	> 40 ³	2.1/3.0
BKM-4116-70	C. gelidus	<1:4	> 1:64	1:32	160/320	640/5120	no plaque	not done

1. Mouse hyperimmune group A and B ascitic fluid and monkey dengue 2 antiserum vs 8 units unidentified virus CF antigen prepared as 20% S.M. brain suspension.

2. Antiserum vs $\frac{8 \text{ HA units unidentified virus}}{8 \text{ HA units Tembusu virus}}$ at HA pH optimum = 6.0

3. 1:40 dilution of Tembusu antiserum produces 95% plaque reduction.

4. Log Neutralization Index in suckling mice; $\frac{\text{unknown}}{\text{homologous (Tembusu)}}$

Table 8.
Ether and Sodium Desoxycholate Resistant Viruses Isolated from Chiengmai Mosquitoes in 1970

Virus Log No.	Ether sensitivity tests		Ether sensitive	Sod. Desoxycholate tests		Desoxycholate sensitive
	(Mouse LD50)			(Mouse LD50)		
	Before R _x	After R _x		Before R _x	After R _x	
BKM-1064-70	10 ^{-4.7}	10 ^{-3.3}	no	10 ^{-4.5}	10 ^{-4.4}	no
BKM-1052-70	10 ^{-3.9}	10 ^{-3.9}	no	10 ^{-3.2}	10 ^{-3.1}	no
BKM-1065-70	10 ^{-3.0}	10 ^{-3.2}	no	10 ^{-2.9}	10 ^{-2.8}	no
BKM-1072-70	10 ^{-3.6}	10 ^{-4.1}	no	10 ^{-3.6}	10 ^{-3.1}	no
BKM-1100-70	10 ^{-4.3}	10 ^{-4.0}	no	10 ^{-4.4}	10 ^{-4.3}	no
BKM-1165-70	10 ^{-5.8}	10 ^{-4.9}	no	10 ^{-6.0}	10 ^{-3.5}	yes
BKM-2804-70	10 ^{-5.0}	10 ^{-5.1}	no	10 ^{-4.7}	10 ^{-5.1}	no
BKM-2849-70	10 ^{-4.2}	10 ^{-4.3}	no	10 ^{-4.3}	10 ^{-4.1}	no
BKM-3716-70	10 ^{-6.3}	10 ^{-6.1}	no	10 ^{-6.9}	10 ^{-6.3}	no
COXSACKIE-BI	10 ^{-4.0}	10 ^{-4.2}	no	10 ^{-6.3}	10 ^{-6.9}	no
BKM-977-70 (J.E. Virus)	10 ^{-8.0}	≤10 ^{-1.0}	yes	10 ^{-8.5}	≤10 ^{-1.0}	yes