

## Experimental Infections of the Thai Domestic Animals with Japanese Encephalitis Virus

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**OBJECTIVE:** The purpose of this investigation was to determine if Thai domestic dogs, waterbuffalo, pigs and cattle may serve as zoonotic viral amplifiers in the epidemiology of Japanese Encephalitis Virus (JEV) in Northern Thailand.

**BACKGROUND:** Evidence that pigs are involved in the epidemiology of Japanese encephalitis has been established by studies performed in Japan. In contrast to Japan, the animal census in Chiangmai, Thailand, has shown that the populations of other domestic species such as dog, waterbuffalo and cattle often approach and in some cases exceed the pig population. Furthermore, pigs in Northern Thailand are born throughout the year, so there is not a larger population of susceptible pigs relative to other species present at the time of the outbreaks of Japanese encephalitis.

If transmission of Japanese encephalitis occurs when mosquitoes carry the virus from an infected animal to man, then several species of domestic animals in the Chiangmai area should be considered as being amplifying hosts in addition to the suspected pig. From data collected in 2 serological surveys of domestic animals in Chiangmai within the last year, waterbuffalo, cattle, dogs, and horses all have a higher percentage of serum HI antibody to JEV than pigs. Each of these animal species produces a number of offspring that are probably susceptible to infection at the time the encephalitis season begins each year. Investigators from Japan isolated JEV from cow's blood (Otsuka S. et al., *Virus* 19 (6):336-339, 1969). However, in a previous study (Gould et al., SEATO Annual Progress Report, 1966 p. 42) conducted at this laboratory, viremia was not detected in cattle following subcutaneous inoculation of JEV. Work by Carey (Ind. J. Med. Res., 56, 1968) confirmed Gould's finding. Other work conducted in Japan (Gresser et al., *Jap. J. Exp. Med.* V. 28, No. 4, 1958 p. 243-248) has shown that horses became viremic after being bitten by JE-infected mosquitoes, and this viremia was sufficient to infect feeding mosquitoes. However, horses are not kept in the villages of Chiangmai and were not studied here. To our knowledge, no investigations of JEV infections in dogs have been reported. Young chickens experimentally inoculated with JEV apparently produce both antibody and viremia, but adult chickens bled in field studies including Chiangmai had no evidence of JEV infection.

In Chiangmai, 3 of 13 field isolates of JEV have been made from mosquitoes trapped while feeding on waterbuffalo or cattle. Ten more JEV isolates were made from mosquitoes collected in light traps placed near bovines and pigs. Culex tritaeniorhynchus, a known JEV vector, and Culex fuscocephala, a potentially important JEV vector, are present in Chiangmai area year round and preferentially feed upon the large domestic animals; they also feed upon man. The analyses of blood in the midguts of these wild-caught JE vector mosquitoes indicate their marked preference for bovine blood. The question of whether or not domestic animals other than the pig may act as important amplifying hosts for JEV must be resolved before sound methods for controlling JE epidemic disease can be formulated. Since dogs, waterbuffalo, pigs and cattles are quite prevalent in the rural villages of northern Thailand, these animals were studied in the laboratory to determine their ability to develop viremia after inoculation with JEV. In addition, the serological responses of these controlled laboratory infections provide a basis for the interpretation of the HI serological patterns found in Chiangmai village animals, from whom no viruses were isolated. Representative HI antibody patterns from indigenous Chiangmai animals are shown in Table 1.

**EXPERIMENTAL ANIMALS:** Two domestic waterbuffalo (Balbulus balbus); #B (female) and #D (male) approximately 11 months old, were obtained from Nakorn Pathom and Ang Thong Provinces, Thailand. These animals and those described below were housed in mosquito-proof rooms throughout the experiment. They received daily feeding and veterinary care.

Three domestic dogs (Canis familiaris); Dog #A (male) born at Vet. Med. Lab. was 11 months old; Dog #3 (male) and Dog #4 (female), 19 months old, came from Din-Daeng dog compound, Bangkok.

Two domestic pigs (Sus scrofa); Pig #226 (male), Pig #229 (female) were 2 1/2 months old when they were bought from Bang Kae District, a suburb of Bangkok.

Two cattle calves (Bos taurus) both males age 4-5 months old (#1 and #2), were born and raised at SMRL.

All of the above animals were selected because they were free of demonstrable HI and NT antibodies to JEV.

**VIRUSES:** JE virus, strain BKM-984-70, SM2, was originally isolated from Culex mosquitoes in Chiangmai. This virus strain was used in every experiment including JEV challenge. Tembusu virus, Strain BKM-4165-70, SM2, was also originally isolated from wild-caught mosquitoes in Chiangmai.

**MOSQUITOES:** First generation (3-4 days old) laboratory-raised progeny of wild-caught Culex tritaeniorhynchus from Bangkhen District, a suburb of Bangkok, were infected by allowing them to feed on JE viremic baby chicks overnight. Each 1 day old chick had been inoculated subcutaneously with JEV (1700 PFU) 2 days previously. Blood-engorged mosquitoes were collected and maintained on 5% glucose and water for 12-13 days in the insectary at Entomology Department before transmission attempts were made. After each transmission attempt, fed mosquitoes were individually triturated and tested for virus in MK2 cell culture and suckling mice. Groups of uninfected Culex tritaeniorhynchus females were induced to feed on dogs and water buffalo on day 2, 3, 4 post infective mosquito feeding (PIMF). Engorged mosquitoes from this feeding were kept for 10 days and tested for virus as above. Attempts to transmit JEV to uninfected mosquitoes were not done in the pig and cattle experiments due to a shortage of mosquitoes.

**SEROLOGICAL TESTS:** Five ml of blood was drawn at various intervals after JEV challenges. The sera were tested for the presence of HI antibodies to group B arboviruses present in Thailand i.e. dengue 1-4, JEV, Tembusu, and Wesselsbron.

**VIRUS ISOLATION SYSTEMS:** LLC-MK2 cell culture (direct and delayed plaque method) and suckling mice were used. Serum, separated from the clotted blood, was inoculated into 2 bottle cultures of MK2 cells (0.3 ml/bottle). Heparinized whole blood was also drawn from each experimental animal at various intervals and immediately inoculated into 2 litters of 1-2 day old white, Swiss mice (8 suckling mice per litter, 0.02 ml of blood I.C./mouse). The inoculated mice were observed daily for sickness and death for a period of 21 days. Brains of sick mice were passed at least twice before the specimens were collected for virus identification. Neutralization tests were done on virus isolated from animal blood in order to confirm JEV viremia.

**PROGRESS:** Dog and water buffalo study: Presumably-JE infected mosquitoes were allowed to feed on dogs and waterbuffalo for 2 hours on each of 2 consecutive nights. Blood engorged mosquitoes were then collected, recorded, and individually tested for virus. Then blood was drawn from each animal every 12 hours for 7 days and each blood specimen was tested for virus in MK-2 cell culture and suckling mice. The results are present in the first 2 columns of Table 2.

Data in Table 2 show that very low level viremia (3 PFU/ml blood) was detected in Dog #A lasting less than 24 hours at 48th hour post-infective mosquito feeding. However, none of the 8 uninfected mosquitoes that fed on this dog at the time of viremia was later found to be infected. No viremia was detected in the other 2 dogs and 2 waterbuffalo.

The experimental design and results of the serological studies are summarized in Tables 3, 4, 5.

Buffalo #B (Table 3) had only low titer serological responses to 3 consecutive inoculations with JEV. With a very high dose of JEV in the fourth challenge ( $4 \times 10^9$  PFU), HI titer to JEV rose only to 1:40 at day 7 with low titer cross reactions to some other group B antigens. Buffalo #D (Table 4) had similar low and transient serological responses.

This weak and transient antibody response probably reflects a response to the large amount of antigen injected repeatedly rather than virus replication in vivo. The low HI titers and cross reactive antibodies in these experimentally challenged animals reproduce the serological pattern noted in animals bled in Chiangmai area (see Table 1). On this basis, it is likely that water buffalo in Chiangmai are repeatedly inoculated with JEV by vector mosquitoes. The meagre antibody responses, the absence of detectable viremia, and the inability to infect mosquito vectors support the conclusion that waterbuffalo are not important amplifying hosts of JEV.

Dog #A (Table 5) which developed detectable viremia showed significant HI antibodies to JEV and dengue 4 from day 7 PIMF but it is impossible to tell whether antibody responses are due to infection by mosquito feeding or to JEV inoculation. Dog #3 shows similar heterologous responses to dengue 4 after mosquito and inoculation challenges. Dog #4 shows only low titered and transient antibody response after inoculation challenge. The serological patterns for the 3 dogs are similar to the serological patterns observed in Chiangmai dogs (Table 1).

JEV viremia was detected in 1 of 3 dogs bitten by JE infected mosquitoes but the viremia was brief, low titered and did not infect susceptible mosquito vectors. Therefore, it is less likely that dogs are as important JEV amplifying hosts as pigs reported below.

PIG STUDY: Experimental procedures employed were similar to the waterbuffalo and dog experiment reported above, except that Tembusu virus rather than JEV was given as a second virus challenge to pigs initially challenged to JEV. Tembusu virus has been recovered from Chiangmai mosquito species that are known to bite pigs.

Following JEV challenge, pig #229 developed high titered JE viremia for about 3 days (Table 2); pig #226 did not circulate detectable virus. No antibody response was detected in the non-viremic pig #226 (Table 6) but a high titered, broadly reactive antibody response occurred in the viremic pig #229, with highest titers to JEV. Following Tembusu challenge 44 days after JEV, pig #226 developed specific Tembusu antibody, while pig #229, previously sensitized to group B antigen, had an anamnestic hetero-specific antibody response with highest titers to JEV. Because Tembusu viremia was not monitored, it is unknown whether Tembusu replicated in pigs. However, JEV seems to be capable of replicating vigorously in pigs.

The HI antibody patterns noted after both virus infections in Pig #229 are similar to those found in indigenous Chiangmai pigs (Table 2). On the other hand, no pig in Chiangmai had antibody patterns that resembled pig #226 after Tembusu infection. Thus there is no clear evidence that a group B arbovirus other than JEV infects pigs in Chiangmai.

CONCLUSIONS: 1) Of all the animals studied, the pig is the most likely candidate for a JEV amplifying host. It shows a vigorous antibody response indicative of virus replication in vivo and also circulates virus at high titer ( $10^2$  PFU/ml blood) for at least 3 days.

2) Dogs are shown to be susceptible to JE infection. In contrast to the pig, their low titered HI antibody responses suggest a more limited virus replication in vivo which is also reflected by the low and transient viremia of less than 24 hours. Moreover, only 1 of 3 dogs was found to be viremic and none of the susceptible mosquitoes fed on this animal became infected. Thus, the dog would not be considered as a good amplifying host for JEV.

3) The waterbuffalo seem to be poor hosts for JEV replication giving low serological responses only after repeated JEV challenges by high titered inoculums. In addition, viremia was not detected by frequent titration of the animal's blood for 7 days. None of the susceptible mosquitoes which fed on these animals became infected.

4) The serological patterns in these experimental animals resembling those noted in the Chiangmai Valley for these animals provide indirect evidence that JEV is infecting the indigenous animals in Chiangmai.

5) The preliminary results of the cattle experiment in progress shows no detectable viremia for 7 days after infective mosquito feeding. Cattle, like waterbuffalo, thus appear not to be good amplifying hosts for JEV.

Table 1.  
Representative HI Antibody Patterns. Indigenous Animal Sera, Chiangmai, 1970.

Animal	Reciprocal of HI titer against						
	D1	D2	D3	D4	JEV	TEMB	WESS
buffalo	10	10	10	20	80	40	20
cattle	0 <sup>(1)</sup>	0	10	10	40	20	10
pig	160	160	320	640	2560	1280	1280
dog	10	20	40	320	640	160	40
horse	0	0	0	20	40	20	10
cat	0	0	0	10	80	10	0
chicken	10	20	20	40	160	640	80
duck	20	20	40	80	320	1280	80

(1) Titer 0 = <1:10; D1-4, = dengue 1-4, TEMB = Tembusu, WESS = Wesselsbron

Table 2.  
JEV Transmission Experiments in Dogs, Water buffalo, Pigs and Cattle

	Dog		Water buffalo		Pig		Cattle		
	#A	#3	#4	#B	#D	#226	#229	#1	#2
No. of presumably —JE infected mosquitoes that fed <sup>(1)</sup>	2	2	2	1	4	0	5	12	11
No. of fed mosquitoes found to be infected <sup>(2)</sup>	2	1	2	1	4	—	5	3	4
Viremia detected (hours after mosquito feedings)	48	— (3)	—	—	—	—	36,48, 60,72,84	—	—
Level of viremia detected (PFU/ml of blood)	3	—	—	—	—	—	approx. 100	—	—

- (1) Presumably JE infected mosquitoes were 12-13 days post infectious blood meal  
(2) Presumably—JE infected mosquitoes which became engorged after feeding on the above domestic animals were individually triturated and tested for virus immediately in MK2 cell culture and suckling mice (I.C.)  
(3) No virus detected

Table 3.  
HI Response of Thai Water buffalo #B to JEV Challenges

Days after JEV Challenge				Reciprocal of HI Titer against:						
1st	2nd	3rd	4th	D1	D2	D3	D4	JEV	Tembusu	Wess.
0 <sup>(1)</sup>				0 <sup>(5)</sup>	0	0	0	0	0	0
7	0 <sup>(2)</sup>			0	0	0	0	0	0	0
	7			0	0	0	0	20	0	0
	14			0	0	0	0	20	10	0
	21			0	0	0	0	20	10	0
	38			0	0	0	0	10	0	0
	90	0 <sup>(3)</sup>		0	0	0	0	0	0	0
		7		0	0	0	0	20	10	0
		21		0	0	0	0	20	10	0
		30	0 <sup>(4)</sup>	0	0	0	0	10	10	0
			7	10	0	0	0	40	20	10
			28	10	0	0	10	20	20	10

- (1) 1 JEV—infected mosquito fed on water buffalo #B on day 0
- (2) Subcutaneous (SC) inoculation with JEV (BKM-984-70,  $6 \times 10^5$  PFU)
- (3) Sc. inoculation with JEV ( $2.5 \times 10^7$  PFU)
- (4) Sc. inoculation with JEV ( $1 \times 10^9$  PFU at 4 sites)
- (5) Titer 0 =  $< 1:10$

Table 4.  
 HI Response of Thai Water Buffalo #D to JEV Challenges

Days after JEV challenge				Reciprocal HI Titer against						
1st	2nd	3rd	14th	D1	D2	D3	D4	JEV	Tembusu	Wess.
0 <sup>(1)</sup>				0 <sup>(5)</sup>	0	0	0	0	0	0
7	0 <sup>(2)</sup>			0	0	0	0	0	0	0
	7			0	0	0	0	0	0	0
	14			0	0	0	0	0	0	0
	21			0	0	0	0	0	0	0
	38			0	0	0	0	10	0	0
	90	0 <sup>(3)</sup>		0	0	0	0	0	0	0
		7		0	0	0	0	0	0	0
		21		0	0	0	0	0	0	0
		30	0 <sup>(4)</sup>	0	0	0	0	10	0	0
			7	10	10	0	0	40	40	20
			28	0	0	0	0	40	20	20

- (1) 4 JEV—infected mosquitoes fed on water buffalo #D on day 0
- (2) Sc. inoculation with JEV ( $6 \times 10^5$  PFU)
- (3) Sc. inoculation with JEV ( $2.5 \times 10^7$  PFU)
- (4) Sc. inoculation with JEV ( $1 \times 10^9$  PFU at 4 sites)
- (5) Titer 0 = <1:10

Table 5.  
HI Responses of Thai Dogs to JEV Challenges

Days after challenge		Reciprocal HI titer against						
1st	2nd	D1	D2	D3	D4	JEV	Tembusu	Wess.
<u>Dog #A</u>								
0 <sup>(1)</sup>		0 <sup>(3)</sup>	0	0	0	0	0	0
7	0 <sup>(2)</sup>	0	0	0	0	0	0	0
	7	0	0	0	40	40	10	10
	14	10	0	0	40	40	10	10
	21	10	0	0	40	40	10	10
	38	0	0	0	10	20	10	10
<u>Dog #3</u>								
0 <sup>(1)</sup>		0	0	0	0	0	0	0
7	0 <sup>(2)</sup>	0	0	0	0	0	0	0
	7	10	0	10	40	160	10	10
	14	10	0	10	40	160	10	10
	21	10	0	10	40	160	10	10
	38	0	0	0	20	80	10	10
<u>Dog #4</u>								
0 <sup>(1)</sup>		0	0	0	0	0	0	0
7	0 <sup>(2)</sup>	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0
	21	0	0	0	20	20	10	0
	38	0	0	0	10	10	0	0

- (1) JE-infected mosquitoes fed on dog #A, #3, and #4  
(2) Sc. inoculation with JEV ( $6 \times 10^5$  PFU/dog)  
(3) Titer 0 = <1:10

Table 6.  
HI Responses of Thai Pigs to JEV and Tembusu Challenges

Days after challenge		Reciprocal HI titer against						
1st	2nd	D1	D2	D3	D4	JEV	Tembusu	Wess.
Pig # 226								
0 <sup>(1)</sup>		0 <sup>(3)</sup>	0	0	0	0	0	0
7		0	0	0	0	0	0	0
14		0	0	0	0	0	0	0
21		0	0	0	0	0	0	0
44	0 <sup>(2)</sup>	0	0	0	0	0	0	0
	7	0	0	0	0	0	10	0
	14	0	0	0	0	0	40	0
	21	0	0	0	0	0	20	0
	35	0	0	0	0	0	0	0
Pig # 229								
0 <sup>(1)</sup>		0	0	0	0	0	0	0
7		0	0	0	10	80	20	0
14		0	0	0	80	320	40	20
21	(2)	10	20	10	80	320	40	40
44	0	20	20	20	40	160	40	40
	7	20	20	20	80	160	80	40
	14	40	40	40	160	640	160	80
	21	40	40	40	80	160	80	80

- (1) JE-infected mosquitoes probed on Pig #226 and fed on Pig #229. JEV viremia was detected in Pig #229 from 36 to 84 hours after mosquito feeding (av. titer =  $10^2$  PFU/ml. blood)
- (2) Sc. inoculation with Tembusu virus ( $1.5 \times 10^3$  PFU/pig)
- (3) Titer 0 = <1:10