

**A Study of Japanese Encephalitis Virus in Chiangmai Valley: The Role of Small Wild Vertebrates  
in Virus Transmission**

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**BACKGROUND:** Several large field studies have provided evidence that wild birds participate in the maintenance and transmission of group B arboviruses in nature. For example Ardeid birds migrating to Japan have been implicated in re-introducing JEV into that country every year. Migrating birds probably play an important role in maintaining the transmission of St. Louis encephalitis virus in the Western Hemisphere. In Thailand Dr. Tom Yuill found that many species of birds netted in Bang Phra, Southeast Thailand, have neutralization antibody to JEV as measured in a metabolic inhibition test (SMRL Annual Report, 1968). For example 5 of 49 Passer montanus (house sparrow) had JEV neutralizing antibody in their serum, while 1 of 31 tested had antibody to Wesselsbron. Moreover Yuill inoculated 5 P. montanus subcutaneously with JEV and measured JE viremia for 5 days in high titers of  $10^5$  infectious units/ml blood in one of the five birds. Other wild caught small vertebrates were also challenged with JEV, including birds (Bulbuls and Pegu sparrows) mammals, (mice, roof rats, bats) and reptiles (lizards) but only the Pegu sparrow in addition to the house sparrow developed a demonstrable viremia.

The sum total of the previous work caused us to focus attention on birds, and in particular, the sparrow, as a possible important participant in JEV ecology in Chiangmai Valley of Northern Thailand. P. montanus is the most abundant bird in Chiangmai Valley, where it roosts and nests in large flocks in buildings and trees adjacent to houses and pig pens. This small bird does not migrate and breeds nearly 10 months of the year, so that presumably virus susceptible and relatively immobile fledglings are being constantly introduced into the environment. Thus if birds do in fact participate in an enzootic JEV cycle in Chiangmai, no bird species is as promising a host as P. montanus. We therefore elected to study this species intensively. Fortunately their low, active flight pattern made them easy to observe and to catch in mist nets.

**PROGRESS:** A. Serological studies of P. montanus

In the 1970-1971 SMRL annual report we reported an evaluation of the micro culture plaque reduction neutralization test (micro-PRNT) using LLC-MK2 cell cultures. Statistical analysis revealed that the micro-PRNT was as precise and reproducible as the standard macro-PRNT. Both tests seemed more accurate measuring low-titered rather than high-titered antiserum. Representative results are shown in Table 1. These results indicated the feasibility of using the micro PRNT to measure the expected low-titered neutralizing activity of bird serum. The micro test offered the further advantage of requiring only 0.025 ml of serum per test, which was an important requirement, since in most instances no more than 0.1 ml of blood was obtained from each bird.

Birds were caught in mist nets raised in the 4 study villages, bled through jugular venipuncture, aged (immature or mature), banded, and released. Collected blood was diluted to 1.0 ml with balanced salt solution, and sent frozen to Bangkok where it was stored at  $-20^{\circ}\text{C}$ .

The sera, diluted in the field to 1:10, and later in the laboratory to 1:20, were titrated against JEV (Nakayama) without further dilution. The exact dilution giving 50% plaque reduction was calculated from a statistical table (supplied by the Japan National Institute of Health) which utilizes the percent plaque reduction found at the serum dilution tested in the micro-PRNT. If seronegative, each serum is expressed as <math><1:10</math>, and if sero-positive, as  $\geq 1:10$ . If the titer was  $\geq 1:20$  the sera was listed as positive twice, once in a  $\geq 1:10$  and again in a  $\geq 1:20$  dilution.

First a comparison was made of the percent positive serum in a large sample of immature ( $\leq 4-6$  months old) and mature birds, irrespective of the date bled. Mature birds were netted in each of the 10 months of the study (June 1970 through November 1970, January 1971 through April 1971). Immature birds were sampled each month except January, February and April. These prevalence data are shown in Table 2.

Nearly the same percentage of positive serum was found in mature and immature birds. Therefore it was not necessary to segregate serological data according to the age of the bird. Moreover, if specific antibody is being measured by the micro-PRNT, the data suggests that antibody is being acquired within the first 4 to 6 months after hatching, with no serological evidence of additional JEV antigenic exposure over the next 4.5 years, the life span of a mature bird. However, definitive interpretation of these results is hampered by a lack of information about the persistence of possible neutralizing antibody in birds, and the transmission of maternal antibody to offspring.

The monthly prevalence data listed in Table 3 shows that seropositive serum ( $\geq 1:10$ ) was found in birds 9 out of the 10 months sampled. The percent seropositive serum seems to occur in a cyclic fashion characterized by 2 peaks, one peak occurring in the rainy season of July thru September, and the other in the dry season, January and February. The absolute serum titers were low: in the large majority of those few serum titrating  $\geq 1:20$ , the titers ranged between 1:20 and 1:40. No serum exceeded a titer of 1:80-1:100.

Seropositive birds were found in 3 of the 4 village study sites which included Sanpatong, Maerim, and Saraphi. Birds from Sankampaeng were seronegative, but the sample size was small, representing less than 5% of the total sera tested.

The serum obtained from 3 migratory birds, Cocomantis merlinus (2) and Cocomantis sonnerattii (1) (shrikes) were titrated and found to be negative.

Of over 1000 birds caught, banded and released, 75 were recaptured 1 to 3 times over subsequent months. An attempt was made to demonstrate seroconversion to JEV in 71 of these birds. Fifty-nine were caught twice, 11 were netted 3 times and 1 bird was trapped 4 times. All were bled 0.1 ml after each capture and then released. The 2 or more serum samples obtained from each bird were titrated by micro-PRNT at the same time.

We tested 151 sera obtained from the 71 birds. Thirty of these birds provided 42 sera which were seropositive at  $\geq 1:10$ , 6 birds provided 10 sera which were also positive at  $\geq 1:20$ . The percent seropositive at titers of  $\geq 1:10$  (27.8%) and  $\geq 1:20$  (6.6%) are higher than the percent positive sera obtained from single bleeding of 660 birds. Only 1 bird, an adult when first caught, converted from  $<1:10$  in September 1970 to  $\geq 1:20$  in March 1971. The titer of the March 1971 serum in this bird was 1:25. The 29 other seropositive birds had titers that remained fixed (rise or fall of no more than 1 serum dilution) over 1 to 6 months. If we are indeed measuring antibody and regard seroconversion to JEV as a marker for JEV infection, then the birds sampled provide very little evidence of having been infected during the 10 month study period. This period covers part of the time when JEV was being actively transmitted to man and domestic animals in the villages.

Next we determined if the JEV neutralizing activity in P. montanus was specific for JEV. Serum from the January, February, March, and April bleeds (Table 3) were selected at random. Some sera were retitered against JEV and others were run against Wesselsbron or dengue 4 virus; others were titered against 2 or 3 of the test viruses if enough serum was available. The results are shown in Table 4. It is apparent that a factor exists in bird serum that neutralizes Wesselsbron and dengue 4 virus. A striking 26.4% of serum neutralized dengue 4 at titers > 1:10. A lower percentage of sera neutralized JEV and Wesselsbron. The titers against the 3 group B arboviruses were low, and no serum titer exceeded 1:80—1:100. In order to confirm the non-specific neutralizing activity of bird serum, we selected 31 sera still available from 14 JEV seropositive birds that had repeated bleedings. Each month of the study was represented in these sera, and both immature and mature birds of both sexes. Twelve of 14 birds were seropositive ( $\geq 1:10$ ) against dengue 4, and 13 were seropositive against Wesselsbron. Two birds with fixed titers to JEV and Wesselsbron had titer rises to dengue 4. These results confirm the low titer non-specific cross-reactivity of P. montanus serum. Moreover, if we reasonably assume that dengue-4 does not infect birds, then titer rises to dengue-4 in 2 birds suggests that we are measuring either virus neutralizing factors other than antibody, or cross-reactive antibody raised against an infection by another group B arbovirus. A reasonable candidate virus would be Tembusu, a Group B arbovirus recovered in Chiangmai Valley and thought to infect birds. Attempts to plaque Tembusu for the micro-PRNT have been unsuccessful.

HAI tests on selected small wild vertebrate sera collected in Chiangmai.

A complete list of small wild vertebrates trapped and bled in Chiangmai during 1970—1971 was printed in the 1970—1971 SMRL Annual Report. A small sample including P. montanus sera, were selected for HAI testing against JEV, dengue 1—4, and Chikungunya HA antigens. The results, found in Table 5 reveal that many tree sparrow and lizard sera contained low-titered inhibitors against the 5 test group B arbovirus antigens; higher-titered inhibitors were found to Chikungunya. It is likely that these inhibitors represent acetone resistant non-specific inhibitors of HA rather than antibody; because of an inability to distinguish between these 2 alternatives, no further testing of small wild vertebrate serum was made by HAI.

#### B. Virus isolation attempts from Passer montanus

We attempted to isolate an infectious agent from 297 blood clots and 136 spleens and livers of P. montanus netted in January, February, and March. After removal from the bird, the tissues were frozen immediately in sealed glass containers and shipped in dry ice to Bangkok where they were stored at -90°C. The clot and organs were thawed, triturated in BAPS with mortar & pestle, centrifuged 10,000 RPM  $\times$  30 minutes, and the supernatant from the clot and organs inoculated separately IC into 6—8 suckling mice. In addition 855 Passer serum stored at -20°C and thawed inadvertently several times were injected into suckling mice. No infectious agents were isolated from the blood clots, organs, or serum.

#### C. Zootropism of Vector Mosquitoes for P. montanus.

The biting preference of proven mosquito vectors, C. gelidus, C. tritaeniorhynchus, C. fuscocephala for Passer montanus was examined by Drs. Joe Marshall and D.J. Gould. Sweep vacuum collections were made near bird nests, and mosquito traps baited with live tree sparrows were placed in various habitats in order to attract vector mosquitoes. The data is summarized in Table 6. Two engorged C. gelidus were collected by sweep vacuum near bird roosts, but they did not contain ingested bird blood. No vector mosquitoes were attracted to the bird-baited traps placed in a variety of ecological sites in Chiangmai Valley. Thus we could not demonstrate attraction of JEV mosquito vectors to P. montanus. However these studies were carried out in the dry season when mosquito populations were at their nadir and therefore may not accurately reflect the biting pattern during the rainy season when vectors are abundant.

SUMMARY OF P. MONTANUS STUDY: The sum total of the serological, virological and entomological studies provides no convincing evidence that P. montanus is a maintaining or amplifying host for JEV in Chiangmai Valley. The JE virus neutralizing and HAI activity in bird serum was low titered and cross-reactive with at least 2 other group B viruses; the neutralizing activity could represent a non-specific inhibitor of arboviruses rather than antibody. No viruses were isolated from many samples of bird tissues, and a biting preference of the 3 mosquito vectors for P. montanus could not be demonstrated.

#### D. Baby chickens as sentinels for JEV.

This study was the work of James E. Williams, CPT, MSC. He evaluated the suitability of baby chickens as a more convenient alternative to the pig as a sentinel for JEV in Chiangmai Valley. During the rainy season approximately 200 chicks, 3 days old, were bled, caged, and put into trees (4 feet above the ground) in the Chiangmai study villages for a period of 7 days. The chicks were bled several weeks later and their serum was titered by HAI and macro-culture PRNT. No neutralizing antibody was found. A small number of sera were HAI positive to JEV, but converted to HAI negative ( $<1:20$ ) following repeat acetone extraction. The results indicate that the vector mosquitoes were not infecting sentinel chicks.

#### E. Serological Study of Bats:

A small percentage of bats caught throughout the year in Japan circulate JEV in their blood, apparently store JEV in brown fat, and their serum neutralizes JEV in low titers. Bats are thus considered to be reservoir hosts for JEV in Japan. Accordingly, 54 bats collected in Chiangmai were examined for the presence of JEV neutralizing activity in their serum.

Five species of bats were collected in banana groves and roof roosts in the study villages during April, May and June, 1970. The species are Cynopterus sphinx, Rousettus leschenaulti, Eonycteris spelaea, Taphozous longimanus, and Scotophilus kuhlii. The first 3 species are fruit eaters, while the last 2 species are insectivorous. All but 7 of the 54 bats collected were fruit eaters. The bats were bled through cardiac puncture and the serum shipped to SMRL. The sera were diluted 1:10 and 1:40 for the micro-PRNT against JEV, diluted 1:10 and 1:20 for titration against dengue 4 and Wesselsbron. The absolute titers were calculated as they were for P. montanus sera; results are shown in Table 7. Of 54 sera tested, 26 (48%) were positive to JEV at a titer  $\geq 1:10$ , and 14.8% & 9.3% of these sero positives titered  $\geq 1:20$  and  $\geq 1:40$ , respectively. The percentage of JEV seropositive serum is almost 5 times higher for bats than P. montanus. Bat sera did not neutralize Wesselsbron but some did neutralize dengue 4. However only 9 of 26 JEV positive ( $\geq 1:10$ ) sera cross-reacted with dengue 4; and only 9 of 17 dengue 4 positive sera ( $\geq 1:10$ ) neutralized JEV. Thus many sera reacted monospecifically with JEV or dengue 4. The large percentage of serum reacting specifically and at relatively high titers ( $\geq 1:40$ ) with JEV, suggests that these bats may have been previously infected with JEV. The dengue 4 neutralizing factor most likely represents either cross-reactive antibody produced by a serologically related bat virus, or a non-specific serum inhibitor of dengue-4 plaque formation.

Viral isolation was attempted on 12 bats trapped at a large cave near Chiangdao, a town north of Chiangmai or at the field laboratory in Chiangmai City in January-February 1971. Six insectivorous bats, Scotophilus sp., were collected in the cave and 6 fruitivorous bats, Cynocephalus sp. from the field laboratory. Brain suspensions from the bats were inoculated intracerebrally in suckling mice but no isolates were made. The sample tested was certainly not adequate to eliminate the involvement of bats in dry season maintenance of JEV; a more extensive survey is indicated if their role as a reservoir of JEV is to be definitely established.

Table 1.  
Comparison of the micro and macro PRN tests

<u>JEV Antiserum</u>	Reciprocal of Antibody Titer			
	Micro-PRNT		Macro-PRNT	
	<u>1st test</u>	<u>2nd test</u>	<u>1st test</u>	<u>2nd test</u>
Monkey V141	55	40	80	28
Monkey V145	62	72	48	78
Rabbit 1	2560	1100	10240	6400

Table 2.  
Serological\* Results: Mature and Immature Passer montanus

<u>Bird</u>	<u>No. Birds Tested</u>	Serum titers vs. JEV	
		<u>≥ 1:10 (%)</u>	<u>≥ 1:20 (%)</u>
Immature <sup>x</sup>	244	20 (8.2)	8 (3.3)
Mature <sup>xx</sup>	346	36 (10.4)	10 (2.8)

\* 50% plaque reduction by micro-culture PRNT  
 x less than 4 to 6 months old (pre-molt)  
 xx 6 months to 4-5 years

Table 3.  
 Monthly Prevalence of JEV Neutralizing Activity in Passer montanus serum

<u>Date Bled+</u>	<u>No. Birds</u>	<u>Serum Titer vs JEV</u>			
		<u>No. <math>\geq</math> 1:10</u>	<u>%</u>	<u>No. <math>\geq</math> 1:20</u>	<u>%</u>
June 1970	43	0	0	0	0
July 1970	45*	5	11.1	4	8.9
August 1970	62 <sup>xx</sup>	7	11.9	6	10.0
September 1970	72 <sup>xxx</sup>	4	5.5	0	0
October 1970	46	1	2.2	0	0
November 1970	139	10	7.2	3	2.2
January 1971	95	19	20.0	6	6.3
February 1971	59	15	25.4	3	5.1
March 1971	62	3	4.8	0	0
April 1971	37	3	8.1	0	0
Total	660	67	10.2	22	3.3

+ No trapping done in December 1970 or May 1970 & 1971.

\* 41 sera run by macro-plaque PRNT

xx 2 " " " " " "  
 xxx 27 " " " " " "

Table 4.

Comparison of JEV, Wesselsbron and Dengue 4 Serum Neutralizing Titers in Mature Passer montanus

Month Bled	Serum Titer vs					
	JEV		Wesselsbron		Dengue 4	
	$\geq 1:10$ (%)	$\geq 1:20$ (%)	$\geq 1:10$ (%)	$\geq 1:20$ (%)	$\geq 1:10$ (%)	$1:20$ (%)
Jan	$\frac{15^{(x)}}{82}$ (18.3)	$\frac{5}{82}$ (6.1)	$\frac{0}{15}$ (0)	$\frac{0}{15}$ (0)	$\frac{8}{15}$ (53)	$\frac{2}{15}$ (13.3)
Feb	$\frac{14}{58}$ (24)	$\frac{3}{58}$ (5.2)	$\frac{8}{48}$ (16.6)	$\frac{2}{48}$ (4.2)	$\frac{12}{47}$ (25.5)	$\frac{6}{47}$ (12.7)
Mar <sup>xx</sup>	$\frac{0}{50}$ (0)	$\frac{0}{50}$ (0)	$\frac{1}{21}$ (4.8)	$\frac{0}{21}$ (0)	$\frac{2}{21}$ (9.5)	$\frac{0}{21}$ (0)
April	$\frac{2}{37}$ (5.4)	$\frac{0}{37}$ (0)	$\frac{2}{18}$ (11.1)	$\frac{0}{18}$ (0)	$\frac{5}{18}$ (27.8)	$\frac{3}{18}$ (16.7)
Total	$\frac{26}{227}$ (11.5)	$\frac{8}{227}$ (3.5)	$\frac{11}{102}$ (10.8)	$\frac{2}{102}$ (2.0)	$\frac{27}{101}$ (26.4)	$\frac{11}{101}$ (10.9)

(x)  $\frac{\text{Total birds positive}}{\text{Total birds tested}}$

xx Includes 10 Immature birds

Table 5.  
HAI Tests on Small Wild Vertebrate Sera, Chiangmai

<u>Animal</u>	<u>No. Serum Tested</u>	<u>No. Sera HAI positive<sup>x</sup> to:</u>					
		<u>D1</u>	<u>D2</u>	<u>D3</u>	<u>D4</u>	<u>JE</u>	<u>Chik*</u>
1. <i>Passer montanus</i> (tree sparrow)	20	0	4 <sup>xx</sup>	1	1	1	2
2. <i>Calotes mystaccus</i> 20 (gingka lizard)	20	1	7 <sup>xxx</sup>	1	3	2	5
3. <i>Bandicota indica</i> (bandicoot)	14	0	0	0	0	0	0
4. <i>Rattus rattus</i> (roof rat)	7	0	0	0	0	0	0

x titer  $\geq$  1:20  
 xx 4 of 4 sera titered 1:40  
 xxx 1 of 7 sera titered 1:40

\* titers ranged from 1:20 to > 1:160.

Table 6.  
Zootropism of Vector Mosquitoes: Bird Bait Trap and Sweep Vacuum  
Mosquito Collections, Chiangmai

<u>Location</u>	<u>Collection</u>	<u>Trap Nights</u>	<u>Mosq. Species</u>	<u>No. Mosq. Collected</u>	<u>No. Fed</u>
1. Chiangmai City (Buildings)	Sweep—vac <sup>x</sup>	2(11—12 Feb 71)	<i>C. quinquefasciatus</i>	95	2
			<i>M. uniformis</i>	1	0
			<i>E. luzonensis</i>	1	0
			<i>C. gelidus</i> <sup>xxx</sup>	5	2 <sup>xxx</sup>
2. Chiangmai City (Trees, roofs, porches)	Bait trap <sup>xx</sup>	7(12 Feb to 20 Mar) 29 traps/night	<i>C. quinquefasciatus</i>	95	89
3. Chiangmai Villages (Vegetation near houses, cow & pig pens <sup>xxx</sup> )	Bait trap	8(14 Feb 71 to 25 Mar 71) 29 traps/night	<i>C. quinquefasciatus</i>	1	1
4. Chiangmai Valley (Marshes or rice fields <sup>xxxx</sup> )	Bait trap	3(23—25 Mar 71) 7 traps/night	<i>C. bitaeniorhynchus</i>	17	11
			<i>C. vishnui</i> subgrp.	2	0
			<i>C. nigropunctatus</i>	2	0

x Collections made adjacent to *P. montanus* roosts & nests.

xx Each trap baited with 2—6 live *P. montanus* overnight.

xxx JEV vector; blood meal identified as bovine (1/mosq.) and non-reactive (1 mosq.) when tested against battery of antisera, including anti—bird (sparrow) serum.

xxxx preferred habitat for 3 vector mosquitoes species.

Table 7.  
Group B Arbovirus Serology<sup>(x)</sup> in Bats: Chiangmai 1970

Virus	Serum Titer		
	≥ 1:10 (%)	≥ 1:20 (%)	≥ 1:40 (%)
JEV	$\frac{26^{(xx)}}{54}$ (48)	$\frac{8}{54}$ (15)	$\frac{5}{54}$ (9)
Wesselsbron	$\frac{0}{52}$ (0)	—	—
Dengue 4	$\frac{17}{48}$ (35)	$\frac{3}{51}$ (6)	$\frac{0}{51}$ (0)

x 50% plaque reduction by micro-culture PRNT

xx  $\frac{\text{Total sera positive}}{\text{Total sera tested}}$