

Ecology of Arboviruses in Thailand

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OBJECTIVE: To determine how the transmission of arboviruses is sustained via natural cycles, with particular emphasis on the role of known or suspect vertebrate hosts.

PROGRESS: In the past year, work under this project included:

- (1) studies of virus transmission in bat populations, and
- (2) studies of suspect wild-vertebrate hosts of JE and Wesselsbron viruses.

Transmission of S-19-B Virus in Wrinkle-lipped, Free-Tail Bats (*Tadarida p. plicata*)

Additional studies of S-19-B virus transmission were undertaken in 1970-71 after circumstantial evidence suggested that human infection with the virus occurs. Of people surveyed who worked in one cave, 20% (3/14) were found to possess neutralizing antibody to S-19-B virus (SMRL Report dated April 1970, p. 54). Cave workers have reported that people invariably become sick soon after entering the cave for the first time with an illness that may last as long as a month and which is characterized by extreme weakness, headache and joint pains. Another reason for this study was to gain some idea of how other viruses might be transmitted among bats. Japanese and St. Louis encephalitis and rabies viruses have been isolated from bats. In Thailand, one study indicated that 12% (38/323) of bat sera neutralized JE virus (SMRL Report dated 15 April 1968). Recently, a number of sera from wrinkle-lipped bats (24) and from the tomb bat *Taphozous theobaldi* (5) were tested for neutralizing antibodies for chikungunya and Sindbis viruses after diluting plasmas 1:50.

Chikungunya plaques were reduced 53% by one plasma from a tomb bat. Sindbis plaques were reduced as much as 82-83% by several sera of wrinkle-lipped bats. Although considerable work with viral agents in bats has been conducted in laboratories, little is known of the mechanisms of their transmission in feral bat populations.

A. Neutralizing-antibody prevalence.

After neutralization of S-19-B virus was found in 30-35% of sera from wrinkle-lipped bat populations (SMRL Report dated April 1970, p. 46-49), work was conducted to determine if seasonal trends in antibody prevalence could be demonstrated. Bats (60-70) were collected in February, April, July and October 1970 and in March 1971 from a cave located at Wat Khao Wong Kot, Ban Me, Lopburi Province, where collecting was accomplished more easily than at caves surveyed previously. Plasma from bats was tested in PRNT at 1:10 and at 1:50 dilutions. Statistical evaluation of the results has not been completed, but preliminary analysis of the data indicated that the prevalence of neutralization particularly increased in March and April. Transmission of S-19-B virus may increase about that time.

B. Potential amplifying hosts.

Bats, of course, are the prime suspects as natural amplifying hosts of S-19-B virus. However, *Rattus rattus*, which also live in caves, have been shown to possess serum-neutralizing capabilities against the virus (SMRL Report dated April 1970, p. 54). Thus an experiment was done in which 20 *R. rattus* were inoculated subcutaneously with $5 \times 10^{2.8}$ mouse LD₅₀ of S-19-B virus and subsequently examined for viremia and

neutralizing antibody. Blood samples were taken by cardiac puncture prior to, 3 days after and 30 days after inoculation with virus. Bloods taken on day 3 were ground in chilled mortars, mixed with diluent to give a 1:5 dilution of whole blood, and centrifuged at 1,400 xg (4°C) for 15 minutes. Supernatant was inoculated into suckling mice. Virus was not recovered. Plasmas from blood specimens taken before and 30 days after inoculation of virus were tested in PRNT at 1:50 dilution. Of 10 plasmas from males, one exhibited 54% plaque reduction on day 30, whereas 28% plaque reduction was observed in the pre-inoculation plasma. Of 10 plasmas from females, one gave plaque reduction of 75% and another of 96% on day 30, whereas no plaque reduction occurred by pre-inoculation plasmas. These results suggest that *R. rattus* may be immunized or occasionally infected with S-19-B virus but probably do not function as amplifying hosts.

Antibody prevalence in adult wrinkle-lipped bats appeared to increase in March and April, which is the time of year when large numbers of young, presumably non-immune bats appear. Thus, an attempt was made to get some indication of whether the young might function as amplifying hosts. A collection of 250 suckling bats was made on 13 April 1971 from the large cave on Khao Lom Phat, where S-19-B virus was first isolated. Collecting was accomplished simply by searching the floor of the cave for bats which had fallen from the ceiling and upper reaches of the cave wall, where they normally occur. Large numbers were falling at the time. The 250 baby bats were found in 2 1/2 hours. After falling, the young climb upwards towards the ceiling, but probably few survive. The collection appeared to contain a considerable number of weak individuals in various stages of starvation. The probability of picking up sick young bats seemed good, since sick individuals would be the mostly likely to lose their grip or be knocked from the ceiling. The young bats were carried down the mountain, placed in plastic bags and frozen on dry ice.

In the laboratory, bats were thawed and disinfected with alcohol. The brown fat on the back, when present, was removed from each bat and pooled with a piece of the liver. Tissue pools were ground in chilled mortars, mixed with 2 c.c. of diluent and centrifuged at 1,400 xg (4°C) for 15 minutes. Supernatant was inoculated into suckling mice. Isolates were identified by neutralization with S-19-B specific rabbit sera. Six of the 250 tissue pools produced strains of S-19-B virus. Thus young bats do become infected and could function as amplifying hosts of S-19-B virus.

C. Virus in bedbugs.

The tropical-cave environment would seem favorable for any of several modes of virus transmission (by aerosol, by contact with urine, feces, and saliva, or by vector), since bat populations measured in millions are aggregated in a small, inclosed, warm and humid space. Environmental conditions are relatively constant the year round. However, the potential for arthropod-borne transmission seemed very high in those caves housing wrinkle-lipped bats. Hemophagous arthropods in close association with the bats included fleas, ticks and bedbugs. Bedbugs were particularly abundant and were the only arthropods observed to bite man, causing itchy welts that lasted 7-10 days. Consequently, our search for potential vectors concentrated on the bedbugs, even though they have never been implicated as vectors of viruses. In addition, the bat tick *Ornithodoros hermsi* (identified by H. Stark) was investigated, but fleas, mites and other ectoparasites of bats were not studied.

On 4 April 1970, 1000 bedbugs and 350 *O. hermsi* were collected from the cave at Ban Me, Thailand, and subsequently examined for virus. Pools of up to 5 ticks or 50 bedbugs were ground in chilled mortars mixed with 1 1/2 cc diluent, and centrifuged at 2,000 xg (4°C) for 10 minutes. Supernatant was inoculated into suckling mice. No virus was recovered from the 65 pools of bat ticks tested. However, 20% (4/20) of bedbug pools yielded strains of virus which were identified subsequently as S-19-B. The original inoculants killed suckling mice in about 4 days. It could not be determined if the virus strains isolated originated from bat blood within engorged bedbugs or from infected bedbug tissues, as engorged and non-engorged individuals were mixed in pools. The absence of virus in pools of engorged *O. hermsi* would favor the latter hypothesis. To clear up the question, a second collection of bedbugs was made, this time from the cave on Khao Lom Phat, Kaeng Khoi District. On 13 April 1971, approximately 1150 bedbugs were taken off rocks on the floor of the cave and frozen when alive. On 14 April 1971, a native

assistant delivered a collection of 950 bedbugs which he had made the previous night, but all the bedbugs had been dead for several hours when delivered. Collections of 13 and 14 April were processed separately. In each, pools of up to 50 bedbugs were prepared in which engorged and non-engorged bedbugs were separated. Pools were processed as above.

Isolations of S-19-B virus were made from non-engorged and engorged bedbugs (Table 1). The frequency of isolation was greater from pools of non-engorged than from pools of engorged. Virus was recovered from bedbugs which were alive when frozen and from bedbugs which had been dead several hours before freezing. In fact, the collection of bedbugs which was frozen hours after death produced more strains than did the collection of live-frozen bedbugs.

The overall frequency of virus isolation from pools of bedbugs collected on Khao Lom Phat on 13-14 April 1971 was 43% (Table 1), or more than twice the rate from the collection of bedbugs made from the cave at Ban Me on 4 April 1970 (20% of pools tested). Suckling bats were not observed at Ban Me on 4 April 1970, and probably we arrived just as the birth of young was beginning. Of female adult bats collected at Ban Me on 4 April, 33% (15/46) were lactating and showed evidence of recently having given birth (bloody and torn vagina). 56% (26/46) were lactating and in late pregnancy, and 11% (5/46) were not lactating nor obviously pregnant. The lower rate of virus isolation from bedbugs collected on 4 April 1970 possibly occurred because we sampled the beginning of a period when virus amplification and transmission were increasing but had not yet increased to the extent present in the cave on Khao Lom Phat on 13-14 April 1971. In effect, both serological data from adult wrinkle-lipped bats and isolation data from bedbugs suggest that S-19-B transmission increases in the period when bats are born.

D. In-cave transmission: sentinel infection.

That virus infection occurs in bedbugs and suckling bats, neither of which are found outside of caves, seems good evidence that transmission of S-19-B virus occurs within the cave environment. Other proof of in-cave transmission has been obtained from sentinel animals. Virus isolation from sentinels was reported previously (SMRL Report dated April 1970, p. 54), although the identity of the isolates was not stated. Recently (2-5 March 1971) an experiment using weanling mice as sentinels was done in the cave at Ban Me. Pairs of mice, in 31 cylindrical cages, were distributed throughout the cave and exposed for 3 days to hemophagous arthropods, aerisols, dropping feces and urine. Mice that died were frozen. Those surviving (38) were returned to SMRL and observed for 2 weeks. A number of them died or became sick with a paralysis which affected the muscles on only one side of the body, either right or left, and which centered in the hind limb. Sick mice demonstrated a peculiar rolling behavior when disturbed.

The brains from dead or sick sentinel mice were examined for virus by thawing, extracting with needle and syringe, grinding in chilled mortar, mixing with 2 1/2 cc of diluent, centrifuging at 1,400 xg (4°C) for 20 minutes, and inoculating the supernatant onto drained LLC-MK 2 cell monolayers. Monolayers were incubated 90 minutes at 37°C, washed, replenished with media and observed 3 weeks for cytopathic effect (CPE). Those with evidence of CPE were passaged into fresh LLC-MK 2 cultures. Isolates were identified, as before, with specific rabbit sera.

Of 19 mice examined, 3 gave isolates of S-19-B virus. Thus, the potential for transmission of S-19-B virus existed at Ban Me in March 1971, well before new-born bats appeared.

Sentinel weanling mice provide a valuable tool for investigating temporal cycles in or the conditions which influence transmission of S-19-B virus.

E. Studies in a small cave.

The work reported thus far was conducted in two large caves, one at Ban Me and another on Khao Lom Phat, in which large populations of bats and bedbugs were present at all times. Fluctuations in these populations possibly occurred, but they were not obvious to casual observation. However, work was done

in a small cave on Khao Lom Phat where the density and species composition of bat and bedbug populations varied greatly. The small cave was visited several times. On 3 September, 1969, only a few bats were seen in the cave. Upon returning on 8 December, many thousands (perhaps hundreds of thousands) of bats were present, and it was not possible to work in the cave due to the high concentration of ammonia then present. Most of the bats were Tardarida plicata. On 10 February 1970, several thousand bats were found in the cave but almost all were the tomb bat Taphozous theobaldi. By 1 April 1970, there was a sizable population made up by Taphozous theobaldi (49%), Taphozous melanopogon (13%) and Tardarida plicata (38%). About 60% of the female tomb bats were pregnant, whereas none of the wrinkle-lipped bats were. We visited the small cave for the last time on 11 April 1970, when few if any bats were present.

What looked like two species of bedbugs were seen in the small cave on 1 April 1970. Neither type of bedbug has been identified as yet, but the smaller variety looked like the bedbugs found in larger caves. The larger bedbugs, found in crevices on the wall, were essentially 100% engorged. A collection (595) of the large bedbugs was made, from which 119 pools of 5 bedbugs each subsequently were prepared and examined for virus using the method described previously. No virus was recovered. Four pools killed mice through 2 passages, but no virus was found by direct or delayed plaquing procedures using second passage mouse brain. Nor was CPE observed in fluid LLC-MK2 cell-culture.

Whereas bedbugs were abundant in the small cave on 1 April 1970, when bats were present in considerable numbers, few were detected on 11 April 1971 when bats were absent. The smaller type of bedbug was found but was scarce; the larger kind was absent. In contrast, very large populations of bats and bedbugs were found in the big cave on Khao Lom Phat at the time (13 April 1971). These observations lead to several questions concerning the geographic distribution of S-19-B virus. Does S-19-B virus survive in smaller caves where host and potential vector populations fluctuate wildly? The small cave approaches, in some degree, conditions of bat roosts in buildings. In Malaysia, wrinkle-lipped bats characteristically occur in trees and buildings, not in large cave concentrations as in Thailand. Secondly, if virus does not survive the year round in small caves, is the virus introduced into them (or other habitats) at certain times? These questions can perhaps be answered by employing sentinel mice in small caves to determine if and when transmission occurs and by examining bats and arthropods for virus.

SUMMARY

S-19-B virus may be an unrecognized cause of human illness in Thailand. Through studies of its ecology, an unique opportunity exists for determining how viruses can be transmitted in feral bat populations. Work to date suggests that transmission is seasonal, increasing when young bat are present, that the young function as amplifying hosts, and that bedbugs may be a vector.

Experimental Inoculation Of Fence Lizards (Calotes spp.) With JE Virus.

Data derived from the serological survey completed at Bang Phra, Thailand, in 1968, suggested that Calotes lizards might function as hosts of JE virus; 19% (44/233) of sera from C. versicolor and 55% (12/22) of sera from C. mystaccus reduced metabolic-inhibition of BHK-21 cells by JE virus (i.e. showed evidence of neutralizing antibody). In 1971, an experiment was done to determine if these animals have viremia following inoculation with JE virus.

C. versicolor (20) and C. mystaccus (8) were collected from Bang Phra. Lizards were inoculated with 5,000 mouse LD₅₀ of JE virus, administered as a 0.1 cc injection into the coelomic cavity via the abdomen. Tissue samples were taken from 5 C. versicolor and 2 C. mystaccus after 2, 4, 11 and 36 days. The thorax was opened and blood taken from the ruptured heart with needle and syringe. In addition, most of the liver, minus the gall bladder, and the spleen were removed from each animal.

Whole blood from each lizard was ground with 1 cc of diluent in chilled mortars. Suspensions were centrifuged at 4°C for 20 minutes at 1,400 xg. Supernatant was inoculated into suckling mice (0.02 ml IC/ea). Liver and spleen of each animal were pooled and ground together in chilled mortars with 3 cc diluent, centrifuged and inoculated into mice, as above.

No virus was recovered from any specimens.

Potential of Rodents as Hosts of JE and WESS Viruses.

As described in last year's report, an investigation of rodents gave little evidence for neutralizing antibody against JE and Wesselsbron (WESS) viruses. Tests by tube-neutralization or by plaque reduction were done on 209 rodent sera. Two sera of Rattus rattus produced 72-74% reduction in JE plaques at a 1:10 plasma dilution, but no plaque reduction occurred at a 1:20 dilution. Four sera from R. rattus gave > 50% reduction in WESS plaque at 1:10 dilution, but only 0-15% plaque reduction occurred at 1:20.

During the past year, additional rodent sera, collected by H. Stark, were tested in PRNT. The presence of neutralizing ability for WESS virus was confirmed for R. rattus at the 1:10 plasma dilution (Table 2). That specific antibody to WESS virus was present, rather than non-specific inhibitory substances, remains an important unanswered question.

Laboratory experiments were undertaken to better assess the potential of rodents as hosts of JE and WESS viruses. In the first experiment, young bandicoots (Bandicota indica) were inoculated with JE virus. The animals used were born and raised in the laboratory and thus had not been exposed to natural infection. Bandicoots were inoculated subcutaneously on the left lateral surface of the thorax with 0.1 cc of solution containing JE virus or, in the case of controls, diluent. Blood samples were taken prior to and at 3, 6, 11, 16 and 27 days after inoculation by heart puncture. Plasma, removed from blood cells by centrifuging at 1,400 xg (4°C) for 10 minutes, was used in PRNT. Specimens giving > 50% plaque reduction at the 1:10 dilution were tested subcutaneously at higher plasma dilutions. Plasmas taken before and 27 days after inoculation were used also in HI tests against JE, CHIK, and 4 dengue antigens. Blood cells from samples taken 3, 6, and 11 days post-inoculation were examined for virus by making 10% dilutions which were inoculated onto drained monolayers of BHK-21 cells in tubes. Cell cultures were incubated at 37°C for 2 hours, washed and replenished with media. Tubes were subsequently examined for CPE over nine days.

No virus was recovered from blood samples; HI tests with all antigens were negative (titers < 1:20). In PRNT, numerous sera showed > 50% plaque reduction at the 1:10 dilution of plasma. However, no clear pattern of response was found; the distribution of positive sera was not typical of a response to infection. All sera positive at 1:10 gave < 50% plaque reduction at 1:20, except one plasma from a control animal. The numerous positives at the 1:10 plasma dilution perhaps resulted from non-specific neutralization of virus. Similar results in a serological survey, where animals often can be bled only one time, could be misleading if interpreted as evidence of specific antibody. For this reason, serological surveys for JE and WESS antibodies should include tests of plasma diluted to 1:20 or more to establish neutralizing abilities. Other experiments were undertaken. R. rattus, R. exulans and Menetes berdmorei (striped ground squirrel) have been inoculated with JE virus, and R. rattus and B. indica have been inoculated with WESS virus. Results of these tests are pending.

Table 1. Virus Isolations from Pools of Bedbugs from the Large Cave on Khao Lom Phat.

	<u>Total pools</u>	<u>Pools positive</u>	<u>Percent positive</u>
Bedbugs frozen when alive (13 April 1971).			
Engorged:	17	3	18
Non-engorged:	6	2	33
Bedbugs dead several hours prior to freezing (14 April 1971).			
Engorged:	16	10	63
Non-engorged:	3	3	100
Total bedbugs.			
Engorged:	33	13	39
Non-engorged:	9	5	56
All pools:	42	18	43

Table 2.
Wesselsbron Virus Neutralization by Sera from Rattus rattus Collected
in Pak Thong Chai District, Nakorn Ratchasima Province

Specimen No.	Date Collected	Percent Plaque Reduction		
		Sera 1:10	Sera 1:20	Sera 1:40
1365	25 Sep 69	55	35	28
1424	31 Oct 69	51	48	9
2355	8 May 70	66	31	6
2359	8 May 70	52	37	42