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Blood Feeding Patterns of Japanese Encephalitis Vectors-How Zoophilic Mosquitoes Feed on Humans

Nobuko Tuno[1]*, Yoshio Tsuda[2] and Masahiro Takagi[3]

[1]Graduate School of Natural Science and Technology, Kanazawa University, Japan

[2]Department of Medical Entomology, National Institute of Infectious Diseases, Japan

[3]Department of Vector Ecology and Environment, Institute of Tropical Medicine, Nagasaki University, Japan

*Corresponding author: Nobuko Tuno, Graduate School of Natural Science and Technology, Kanazawa University, Japan, Email: tuno@staff.kanazawa-u.ac.jp

ABSTRACT

Japanese Encephalitis Virus **(JEV)** is the most frequent cause of mosquito-borne encephalitis in Asian countries. Several culicine species are potential vectors those are not anthropophilic but are zoophilic. Primary JEV vectors feed mainly on cow (dead end host for JEV), pig (amplifying host), and occasionally on human (dead end host). To find out determinant factors of blood feeding pattern is critical to understand transmission cycle of the disease. We review primary JEV vector blood feeing characteristics by our experimental and field survey conducted in Asian countries. We present that JEV vectors, *Culex tritaeniorhynchus*, *Cx. vishunui*, and *Cx. gelidus*, have innate preference for cow than pig. Contrary to their preference, observed blood fed ratio of pig was often higher than that in cow in the former two species while the latter species, *Cx. gelidus*, stick to feed on cow. In a village where people live with many kinds of animal in their compound, the mosquito abundance was the most affected by cattle abundance not by other animals. We studied micro distribution of pre-biting mosquitoes around a cow to find many of them staying in vicinity of the host. We detected significant spatial autocorrelation at lag distance less than 20m in the *Cx. vishnui* subgroup meaning that they judge preferable host abundance at the spatial scale. We suggest costless control program against JEV vector by village and city planning.

INTRODUCTION

Japanese Encephalitis Virus **(JEV)** is one of arthropod-borne viruses (arboviruses) circulating among wild animals and is the most frequent cause of mosquito-borne encephalitis. JEV which was first isolated in Japan in 1930's [1] is the main cause of viral encephalitis with an estimated annual 68,000 cases in South-East Asia and Western Pacific regions, exposing more than 3 billion people to risks of infection [2]. JEV prevalence is related with rice fields as vector mosquitoes' breeding site and non-human large mammals as blood meal source. It is widely known that primary JEV vectors anthropophilic, but feeding more commonly on pig and cattle than on chicken and human blood [3-8]. If JEV vector mosquito species strictly feed on non-human host or humans, they are no more vectors since JEV is infected by preserving or amplifying hosts to human as dead end host. There is no human to human transmission unlike malaria. Therefore, effective JEV vector should have catholic host preferences, i.e., individual mosquito's bites multiple host species including human, which is quite different requisite compare to anthropophilic malaria vector.

In Asian countries, *Culex tritaeniorhynchus* Giles, *Culex vishnui* sensu lato (sl.), *Culex fuscocephala* Theobald, and *Culex gelidus* Theobald, *Culex whitmorei* (Giles), and *Mansonia uniformis* (Theobald) have been implicated as vectors of Japanese Encephalitis **(JE)** [3-8]. Although these mosquitoes feed more commonly on pig and cattle blood than on chicken and human blood [9-11], their feeding patterns vary depending on host availability. The feeding pattern of mosquitoes is largely influenced by two parameters: (1) innate tendency to respond to particular cues and (2) relative host availability in combination with vector mobile capability. The term "host preference" can be used to describe integration of these parameters [12]. Therefore, studies of the feeding patterns of JE vectors in Asia have produced varying results, depending on the relative abundance of the host population and the sampling procedures used. The relative abundance of pigs compared to cattle can be low in countries dominated by Muslims. In India where the cattle population is higher than the pig population, 86-98% of all the blood meals ingested by the vectors are from cattle hosts [13]. In Okinawa, Singapore, and Taiwan where the pig population is higher than the cattle population, up to 60% of the vector blood meals are from pig hosts [9,14,15].

We have investigated how JEV vector host feeding patterns are realized in Asia, i.e., in Japan, Thailand, and Vietnam. We subject innate host preference and realized feeding habit in the field of primary vector species.

INNATE HOST PREFERENCE AND HOW IT IS DISTORTED

Primary JEV vectors have reported to feed on pigs and cattle than on chickens and humans [12,13]. Pigs are amplifying host while cattle's are dead end host for JEV, thus, blood meals of which is taken by vector mosquitoes is critical in the disease transmission. We conducted host

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preference test with the two animals and field samplings to know innate preference and identified blood meal source in wild mosquitoes.

We conducted the study of release and recapture test and light trap catches on Mae Joh University campus, in Chiang Mai, where various animals including cattle and pigs were kept and JEV vectors were found in abundance. The study site has been described in detail elsewhere [16,17].

Innate Preference

Two experimental nets were erected to put animal bait inside, a cattle or a pig, or both, in which wild-collected or laboratory-raised mosquitoes are released and recaptured to evaluate mosquitoes host preference by blood fed ratio [16,17]. As the result, all three species, Cx. *tritaeniorhynchus*, *Cx. vishnui* and *Cx. gelidus* fed on the cow in significantly higher proportions (65.2-66.1%) than they did on the pig (42.4-56.6%). Meanwhile, when they were exposed to both animals in a net, they fed on the cow almost 10 times (39.0-45.3%) of that on the pig (2.4-5.3%) [16]. The result showed higher preference for cow than pig in the JEV vectors but the difference is not very big when they have no choice. When we released mosquitoes those fed on or attracted to cow or pig, they inclined to bite the same host to which they had originally been attracted. However, the offspring of pig-fed or cow-fed mothers did not show such differences but show uniform preference for cow [17]. Therefore, the three JEV vector species show physiological or behavioural conditioning in the host preference rather than genetic variability at least in the choice of the two mammal hosts, cow and pig.

Observed Blood Feeding

We collected 34,708 mosquitoes in light traps baited with dry ice in animal sheds, i.e., cows, pigs, chickens, and sheep and goats, as to represent wild mosquitoes feeding success[16]. Not in harmony with the feeding experiments, *Cx. tritaeniorhynchus* and *Cx. vishnui* got more meals from the pigs than from the cows whereas *Cx. gelidus* fed on the cows more than the pigs in significantly higher proportions. We divided the light trap catches into three periods according: early night (18-22hr), middle night (22-02hr) and late night (02-06hr). Interestingly, fed ratio of *Cx. tritaeniorhynchus* and *Cx. vishnui* caught in pigsty showed drastic increase in later periods, whereas those in cowshed did not show such acute increase. On the other hands, fed ratios of *Cx. gelidus* that fed on the cows in significantly higher proportions than on pigs were rather stable in course of night. This is our speculation that species-specific biting hours may relate to plasticity in biting behavior in mosquitoes. *Cx. gelidus* has fixed feeding preference that stick to feed on cattle, while *Cx. tritaeniorhynchus* and *Cx. vishnui* those also prefer cows but show more flexible feeding. The two species showed higher fed ratio in later hours in pigsty, they might have abandoned their innate preference but compromised with available host. What physiological or behavioral conditioning controls their biting behavior will be discussed later.

HOST ANIMAL DISTRIBUTION CHANGES RISK OF HUMAN

We presented that host availability influences the choice of host may differ among vector species. Naturally, a question arises if the host animal distribution changes risk of a person being bitten by JEV vectors. Dose it increases our risk or reduces keeping animals in vicinity of humans? There was an ideal place to explore the answer for the question. We conducted a field investigation to elucidate the relationship between the host species and mosquito distributions within a rice production area in northern Vietnam. We determined the mosquito and host abundance in 50 compounds and the host abundance in an additional 29 compounds in order to determine the correlation between the mosquito and vertebrate host density.

Study Area

We conducted the study in an area where JE transmission is endemic in Hatay Province that lies 60 km northeast of Hanoi in northern Vietnam [18]. For the study area, we selected a part of the village that lay adjacent to the rice fields in order to minimize the effect of the flight distance of the mosquitoes between the breeding sites and the hosts. A compound comprised a human residence and a shed for the animals. Thus, the people lived in close proximity, in a few meter, to their livestock. We collected adult mosquitoes from 50 adjacent compounds in June, in 2003. There were 370 humans, 787 pigs, 48 cattle, 3852 chickens, 144 dogs, and 141 ducks in the study area during the study period. We collected mosquitoes indoor and outdoor from each compound. Pyrethrum-Spray Collections **(PSC)** [19] was carried out from the compounds between 19-23hrs. We hung a light trap in each compound at a point that was approximately equidistant from the host animals of various species in that compound. The mosquito collection was divided into an early night period (19-23hr) and a late night period (23-09hr). We applied autocorrelation analysis to identify the optimum spatial resolution for correlating environmental factors and mosquito distribution. We used Moran's I correlogramto determine whether there was a significant clustering of mosquitoes in the study area [20,21].

Cattle Increase Human Risk

The most dominant species found indoors was *Cx. quinquefasciatus* (not JEV vector), followed by the *Cx. vishnui* subgroup and *Cx. gelidus* that was the most dominant species found outdoors. We applied PCR analyses to a part of samples to find, 79% of the captured specimens were identified as *Cx. tritaeniorhynchus* and 21%, as *Cx. vishnui*. We described the two species as the *Cx. vishnui* subgroup here because a few of specimen classified as *Cx. tritaeniorhynchus* were *Cx. vishnui* by PCR. The number of the *Cx. vishnui* subgroup and *Cx. gelidus* mosquitoes was larger in the outdoor collections. Both the *Cx. vishnui* subgroup and *Cx. gelidus* had mainly fed on cattle and pigs even those sampled indoor (Table 1). These species had also fed on human blood. The number of human blood meal of the *Cx. vishnui* subgroup positively related with the number of the cow kept in the compound. Thus, we found that presence of cow increase human risk to be bitten

by mosquitoes (Figure 1). *Cx. quinquefasciatus* had mainly fed on human and chicken blood. Mixed blood meals were detected in 15 (9%) of the 164 *Cx. vishnui* subgroup mosquitoes, 3 (4%) of the 70 *Cx. gelidus* mosquitoes, and 16 (5%) of the 299 *Cx. quinquefasciatus* mosquitoes. The mixed blood meal combinations were as follows: 2 of the *Cx. vishnui* subgroup mosquitoes had ingested human and cattle blood and 13 of these mosquitoes along with 3 of the *Cx. gelidus* mosquitoes had ingested cattle and pig blood. A variety of mixed blood meal combinations was detected in the *Cx. quinquefasciatus* mosquitoes as follows: 3 had ingested human and pig blood; 2, human and cattle blood; 5, human and chicken blood; 2, pig and cattle blood; 1, pig and chicken blood; 2, cattle and chicken blood; and 1, human, pig, and chicken blood. It shows us the possibility of any kind of arbovirus may start to spread.

Mosquito Abundance and Environmental Factors

We found that (1) the number of female JE vectors exhibited a positive relationship with the number of cattle hosts while not with the number of pigs and (2) the critical factor affecting the number of mosquitoes varied with the mosquito species. *Cx. gelidus*, *Cx. tritaeniorhynchus*, and Cx. *vishnui* are exophilic, i.e., they remain outdoors. The blood meal hosts of the JE vectors collected indoors were identified to predominantly be cattle and pigs (Table 1). This suggests that the structure of the houses in the study area permitted entry of the JE vectors before and after feeding. The number of cattle hosts was a significant factor for the indoor collections of the *Cx. vishnui* subgroup, whereas the proximity of these hosts to the breeding sites (the minimum distance between a compound and the nearest rice field) was less significant. This indicates that the distribution of the *Cx. vishnui* subgroup was not constrained by their breeding site in the village. The number of *Cx. gelidus* mosquitoes was mainly affected by the proximity to the breeding sites and slightly affected by the number of cattle hosts; this result is also consistent with our previous result that *Cx. gelidus* prefer cows rather than pigs or chickens as hosts [16,17]. However, our results implied that the proximity between the available hosts and the breeding sites is a more critical factor than host preference for *Cx. gelidus*. It has been reported that this species breeds in a variety of habitats in Malaysia [22]. In the study area, people washed animal sheds and thus created polluted ground pools that served as a larval habitat for *Cx. gelidus*. In cases where there is proximity between the hosts and the breeding sites, the mosquitoes do not need to disperse long distances; this may be an important factor for the species with limited flight ability. The number of female *Cx. quinquefasciatus* mosquitoes positively correlated with the proximity to the breeding sites. *Cx. quinquefasciatus* is reported to breed in any type of habitat that contains water ranging from fresh and clear water to polluted water with decayed organic matter [23]. In the study area, the larval habitats of *Cx. quinquefasciatus* were assumed to locate within the village, similar to those of *Cx. gelidus*. Human blood comprised 76% of the diet of this species. The number of female *Cx. quinquefasciatus* mosquitoes did not correlate with the abundance of any animal hosts.

HOW DO MOSQUITOES ACHIEVE PLASTICITY IN THEIR BLOOD FEEDING

We presented that primary JEV vectors prefer cattle rather than pig although realized blood feeding does not necessary coincide with their host preference depending on the species-specific plasticity. The *Cx. vishnui* subgroup showed more flexibility than did *Cx. gelidus*. The distribution of the latter species is supposed to be limited in vicinity of its breeding site whereas, that of former is not limited to its breeding site, i.e., rice fields. It implied us if there are any relationships between plasticity in host preference and movability from breeding sites in vector species. In relation to the subject, we studied an interesting behavior, called pre-biting resting. Pre-biting resting made us to speculate if vector breeding site affect to their blood feeding plasticity. Mosquito blood-feeding behavior is composed of several phases: search of host, attraction to host, attacking, feeding, and resting. Among these phases, a marked interval between the appearance of mosquitoes nearby and their alighting on the bait is called 'pre-biting rest' [23] or 'pre-attack resting' [12]. Pre-biting rest has been reported on several taxa [19] including An. leucosphyrus subgroup [24,25], *An. dirus* [26], *An. gambiae* s. l. [27], *Cx. quinquefasciatus* [28], *Cx. tritaeniorhynchus* [29] and *Mansonia* species [30,31]. The biological significance of pre-biting rest had not elucidated. We suggested evolutional significance of the pre-biting rest by studying of mosquito spatial distributions around a bovine [32].

Study Site and Method

Spatial distribution of fed and unfed mosquitoes around a bovine host was field-studied in November 1997 at countryside of Northern Thailand. Around a tethered host, 10 bamboo sticks were arranged each in furrows, 1m apart. All mosquitoes resting on the sticks were collected and identified their feeding status and the location from the cow between 19 to 24hr for 8 nights with and without the host cow.

Pre-Biting Rest

In total, 1566 mosquitoes of 25 species in 5 genera were captured. *Anopheles aconitus* was most abundant, followed by *An. peditaeniatus*, *Cx. vishnui* and *Cx.* pseudo*vishnui*. There was no difference of the mosquito abundance among directions. Mosquitoes distributed randomly before their approach to the cow. We found that more unfed mosquitoes were collected at sites closer to the host, i.e., pre-biting rest was observed, and fed ratio showed negative correlation with mosquito density. Thus, fed mosquitoes were almost constant in number despite of the fluctuation in the daily number of mosquitoes captured. We also found that inter-specific heterogeneous distribution of fed and unfed mosquitoes around the host could be clustered into two groups. One group, represented by five species, engorged the host indiscriminately, while the other, represented by seven species, aggregated around the host within a close distance of 1-4m with lower proportions of fed mosquitoes. Characteristic of mosquito blood feeding is that amount of blood is so huge compared with mosquitoes' consumption. That is that mosquitoes do not

need to hurry to bite in fear of shortage of blood. Possible limiting factors can be available host's body surface area [12] or more likely host defense triggered by excessive attack. The negative correlation between mosquito density and their feeding success caused by density-dependent defensive behavior of hosts has been reported [32]. If it is a general rule, pre-biting rest may be adaptation for mosquitoes to avoid aggressive host defense. But if so, why pre-biting resting behavior is observed only in a part of mosquito species? In fact, our result showed that the density-dependent fed ratios were not observed in all the species. Now we go back to the two different feeding groups. We listed specific breeding place and adult host preference to find if there is any common character within group but are different between groups. We found that the second group members, aggregated around the host with lower fed proportions, utilizes larger sizes of breeding site, such as rice field, pond, swamp and stream compared to the other. Meanwhile, there seems to be no clear difference in their host preference between the groups. If host animals' defensive behavior depends mosquitoes density means that mosquito species of which population is large have more experience of it. In this point of view, members consisting of the group, utilize larger breeding site, must have evolutionally been selected by these density dependent selection. Gathering these, pre-biting rest would be a meaningful period for the mosquito to evaluate if they attack hosts or not. Gillies [34] clarified that carbon dioxide generally attracts host-seeking mosquitoes. Most mosquitoes would be attracted by general host cues like carbon dioxide and heat, however, several species wait for the host in closest distance where general cues would be highest. Why mosquitoes can hold themselves from attacking to the host in the very closest place. We suggest that it is a kind of density effect. The closer to the host, the more mosquito present as well as stimuli from the host. Mosquitoes may evaluate their density by wing vibration or odor that we have not tried to identify yet.

DISCUSSION

Lastly, we integrate our gained result to construct a suggestion how to control JEV vectors. We presented experimentally that JEV vectors, *Cx. tritaeniorhynchus*, *Cx. vishunui*, and *Cx. gelidus*, have innate preference for cow than pig, and likes and dislikes was clearer when they could choose between the two animals. Contrary to their preference, observed blood fed ratio of pig was often higher than that in cow in the former two species while the latter species, *Cx. gelidus,* stick to feed on cow in Thailand. In a village in north Vietnam where people live with many kinds of animal in their compound, the mosquitoes abundance was most affected by cattle abundance. Abundance of the *Cx. vishnui* sub group related positively with cattle abundance (Figure 2). Number of human blood meal in the species increased with the number of cattle in the compound (Figure 1). While abundance of *Cx. gelidus* and *Cx. quinquefasciatus* were primary affected by closeness to their breeding sites [18]. Their distribution was grounded by their breeding grounds [18]. We studied micro distribution of pre-biting mosquitoes including the *Cx. vishnui* subgroup around a cow to find many of them staying in vicinity of the host. We discussed adaptive aspect of pre-biting resting [32]. Wada observed nocturnal biting activity of *An. sinensis* and *Cx. tritaeniorhynchus*

by various methods, the counts of mosquitoes alighting on pigs and those on plates or tapes set near dry ice baits in Japan [29]. In his study *Cx. tritaeniorhynchus* showed a sharp peak of their nocturnal activity by methods of dry ice baits, however, counts of alighting on pigs did not show such tendency but were almost constant. He attributed this deviation to pre-attacking rest. The dry-ice baited counts indicated only the flight activity rhythm of mosquitoes that differs from the counts of mosquitoes attacking the pigs, and the deviation is explained by the counts of pre-attacking resting mosquitoes.

We show schematic illustration of blood feeding of JEV vector in Figure 2. Primary vectors aggregated around cow to yield high density of mosquitoes to let them do pre-biting rest. A part of them will make success to get blood meal from cattle, but more than a half always remain to be unfed. Certain portion of vectors would convert to bite pigs and humans and chickens in smaller ratio with more than a half as unfed status [18] (Table 2). The mixed blood meals and the high ratio of unfed mosquitoes tell us that blood feeding is not easy for vectors. Our studies of JEV vector suggest that it is important where we keep cattle from human residence. It is critical to reduce our risk to suffer JEV. It has proved in Japan in 1960's. We had thousands of JEV cases until 1960' in Japan, but with the last outbreak in 1966, we became free from JVE cases. There are several reasons for the drastic reduction, national human immunization program, pigsties and cowsheds were isolated from human dwellings [1]. We still have reports that naïve piglet kept in pigsties change into JEV preservers every year, but we have few opportunities to get contacts with JEV vectors.

Cx. tritaeniorynchus can disperse long distances so that control program of JEV vector should be planned in larger scales. It is most desirable to establish human dwellings isolated from the other kind of animals especially cow and pig as we did in Japan. But it is hard to make a social change in short period. What we can practice without much cost is (1) to keep cattle remote from human, (2) to keep cattle and pigs separate. This spatial isolation can be less than 100m. We detected significant spatial autocorrelation at lag distance less than 20m in the *Cx. vishnui* subgroup meaning that they judge preferable host abundance at the spatial scale [18] *Cx. gelidus*, the other important JEV vector species, are less movile. It is needed to clear their breeding sites in vicinity of human habitats. Costless control will be possible against JEV vector by village and city planning.

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