Hindawi
Journal of Tropical Medicine
Volume 2023, Article ID 9946754, 12 pages
https://doi.org/10.1155/2023/9946754

Research Article

A Study on the Bionomics of Primary Malaria Vectors *Anopheles minimus* and *Anopheles baimaii* in Some States of North East Region of India

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Received 3 March 2023; Revised 29 May 2023; Accepted 5 June 2023; Published 30 June 2023

Academic Editor: Rajib Chowdhury

Northeast region (NER) states of India remain highly malarious due to their geographical and ecotype diversity. Furthermore, rapid urbanization and change in climate are also affecting the vector biology and behavior of the existing species. Hence, a study is conducted in the states of Tripura and Meghalaya to generate data on the bionomics of the prevalent malaria vector species. The data from this study show that primary vectors of malaria *An. minimus* and *An. baimaii* were anthropophagic. However, *An. baimaii* showed a behavioral shift towards zoophagicity (~14%). Insecticide bioassays confirm that these two major vector species are reportedly susceptible to DDT, Malathion, and indicate that intervention by DDT-IRS is effective. Thus, the implementation of appropriate strategies based on this recent information on the bionomics of malaria vectors in NE region of India will provide an opportunity to achieve malaria elimination by date in these states.

1. Introduction

Malaria in India contributes to around 70% of malaria cases and 69% of malaria deaths annually in the South-East Asia Region [1]. Malaria control in India is complex due to the presence of multiple ecotypes and vector systems across the country. In India, 9-10 *Anopheles* species of reported 58 Anopheline species transmit malaria across the country [2]. Distribution is specific to a given ecotype and hence varied behavior [3] and malaria vector control in India is complex as each species has specific bionomic characteristics [4]. Six reported primary malaria vector species are *Anopheles culicifacies*, *An. fluviatilis*, *An. stephensi*, *An. baimaii*, *An. minimus*, and *An. sundaicus* [2, 5–8]. In the Northeast (NE) region of India, *An. baimaii* and *An. minimus* are prominent vector species [9]. *An. minimus* and *An. baimaii* are found in the foothill and forest fringe areas of the NE states [10–12]. Vector diversity of the north east region due to its eco-geography and rich biodiversity is different from peninsular India. Three sibling species, namely, *An. minimus s.s.* (Species A), *An. harrisoni* (Species C), and *An. yaeyamensis* (Species E) of *An. minimus* complex are reported globally [13] and *An. minimus s.s.* (Species A) is reported from NE states (Arunachal, Assam, Meghalaya, Nagaland, and Tripura state) and Odisha state [14–16]. *Anopheles dirus s.l.*, important malaria vector of NE region [17], is a complex of seven isomorphic species, *viz., An. dirus s.s* (Species A), *An. cracens* (Species B), *An. scaloni* (Species C), *An. baimaii* (Species D), *An. elegans* (Species E),

An. nemophilius (Species F) and *An. takaesagansis*. Among them *An. dirus s.s* (Species A), *An. cracens* (Species B), *An. scaloni* (Species C) and *An. baimaii* (Species D) are established vector species while *An. elegans* (Species E), *An. nemophilius* (Species F), and *An. takaesagansis* are not yet incriminated [18]. *Anopheles baimaii* is reported to be prevalent in NE India [17, 19] and was observed that *An. baimaii* supplements the transmission of malaria in the forest fringe area of the NE region during monsoon seasons [20]. *Anopheles annularis,* a secondary vector, is a complex of five morphologically similar species *viz., An. annularis, An. nivipes, An. philippinensis, An. pallidus,* and *An. schueffneri* [21]. Except *An. schueffneri,* other four species of the group are reported from India [3]. However, differentiation among these species is difficult, especially between *An. nivipes* and *An. philippinensis* due to indistinguishable morphological characteristics and hence differentiated using molecular methods based on the ITS2 and D3 genes using polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) [22]. *Anopheles annularis* group is reported as a complex of two cryptic species provisionally designated as species A and species B. Species A was reported to transmit malaria in Assam and Orissa states [21–23]. Species B is not an established vector in India but is considered as vector in some forested eastern states of India, namely, Odisha, Chhattisgarh, and Jharkhand [3, 24].

Insecticide based vector control continues to be the major component for malaria control. During 1950's, DDT, dieldrin, and Hexachlorocyclohexane (HCH) was sequentially used in vector control program. Malathion was introduced in indoor spraying during the late 1960s; subsequently, dieldrin and HCH were banned for use in public health sprays [1, 25, 43]. Currently, DDT (organochlorine), Malathion (organophosphate) and deltamethrin, cyfluthrin, lambda-cyhalothrin, alpha-cypermethrin, permethrin, and bifenthrin (synthetic pyrethroids) are recommended for the control of malaria vectors in India [1]. Improved tools and strategies like artemisinin-based combination therapy (ACT), rapid diagnostic tests (RDTs), long-lasting insecticidal nets (LLINs), indoor spraying of residual insecticides (IRS), and revision of the National Drug Policy for malaria in 2013 acted as key components in reducing malaria and are still effective [26]. This reduction in malaria cases can be attributed to wide-scale implementation of vector control interventions. However, drug resistance in parasites and resistance in malaria vectors to various classes of insecticides are still threats for the disease control programme [1, 27]. As per the WHO Global report on insecticide resistance in malaria vectors 2010–2016, all the major malaria vectors of WHO regions of Africa, the Americas, South-East Asia, the Eastern Mediterranean, and the Western Pacific were found to be resistant against at least one of the four commonly used insecticide classes: pyrethroids, organochlorines, carbamates, and organophosphates [27]. Use of pyrethroids is prominent for the 2 major vector control interventions, indoor residual sprays (IRS), and longlasting insecticidal nets (LLINs) in all malaria endemic states of India [44]. In NE region, DDT-IRS and pyrethroid-LLINs are in use since the last 2 decades and the species was reported susceptible to both DDT and pyrethroid insecticides [9, 28].

To sustain the gains achieved so far in NE region states, there is a need for the implementation of effective vector control strategies and determine key determinants like biology and bionomical variations of prevalent vector species relevant to transmission dynamics of the disease [29]. Major attributes that have bearing on the success of vector control are biting rhythm and role of outdoor transmission due to changes in vector behavior in view of extensive use of insecticide interventions in addition to existing challenges of multiple insecticide resistance in vectors, sibling species complexes of vectors, etc. Such classified information on bionomic attributes will be of high importance in designing effective vector control strategies [3]. Hence, information on the vector species diversity, bionomics, and their insecticide susceptibility/resistant status is of utmost importance.

In the NE India states, recent information on the bionomics of the prevalent malaria vectors and their role in transmission is lacking and the present study aims to generate information for the development of evidence based sustainable vector control strategy for malaria control. The study is also focused on the possible variations in the behavior of malaria vectors owing to extensive use of insecticide-based interventions in the area that could lead to change in behavior and establish alternate niches such as for outdoor transmission.

2. Methods and Materials

2.1. Study Area. Studies were conducted in selected 2 districts in two Northeastern states *viz.,* Tripura (23.5639°N and 91.6761°E) and Meghalaya (25.4670°N and 91.3662°E). Study area was selected based on the malaria incidence in previous years, 2014–2017. These two states were reportedly of high malaria endemicity among the states of NE region. However, a declining trend in malaria cases and deaths was noticed in the states, relatively more in Tripura state (Table 1).

Entomological surveys were conducted in areas with different ecotypes: plains, hills, and foot hill areas. Two surveys were undertaken during premonsoon and postmonsoon seasons and 3 surveys in peak malaria transmission seasons, during monsoons. During the study period, *i.e.,* August 2017–December 2019, entomological studies were conducted in 16 villages in 4 districts in the two malaria endemic states. Details of the study areas are given in Figures 1 and 2 and Table 2.

Malaria endemic districts, South Tripura and Dhalai, are in the higher malaria receptive state in the NE region. Epidemiological data from 2014 to 2019 showed Tripura contributed 2.56% of the total annual malaria incidence of India (Source: National Centre for Vector Borne Disease Control-NCVBDC). The major population of the state comprises ethnic tribal communities inhabiting remote areas. The terrain is mostly hilly and forested with sparsely distributed population. Annual rainfall range from 2.0 to 2.5 meter and heavy precipitation occur during April–September which coincides with the high malaria transmission period. During the months of March/April and September/October, the climatic conditions remain hot (21–34°C) and humid (70–80%). Thus, the tropical climate favors the breeding, distribution, and longevity of mosquitoes and the transmission of malaria.

Meghalaya state contributes 3.0% of total annual malaria incidence in India (2014–2019) (Source NVBDCP). South Garo Hill and West Garo Hill districts of Meghalaya were

Table 1: Malaria case incidence and *Plasmodium falciparum* (*Pf*) cases of Meghalaya and Tripura, India, 2014–2019 [1].

States	Year	Blood slide examination (BSE)	Malaria cases	*Pf* cases	Death	% *Pf* cases
Meghalaya	2014	437741	39168	37149	73	94.85
	2015	599144	48603	43828	79	90.18
	2016	468254	35024	31773	44	90.72
	2017	421145	16433	14974	12	91.12
	2018	326051	6394	6065	06	94.85
	2019	422237	2615	2364	04	90.40
Tripura	2014	606791	51240	49653	96	96.90
	2015	453298	32525	30074	21	92.46
	2016	351266	10557	9553	15	90.49
	2017	375626	7040	6572	06	93.35
	2018	483982	13079	12600	13	96.34
	2019	619912	12437	11636	01	93.56

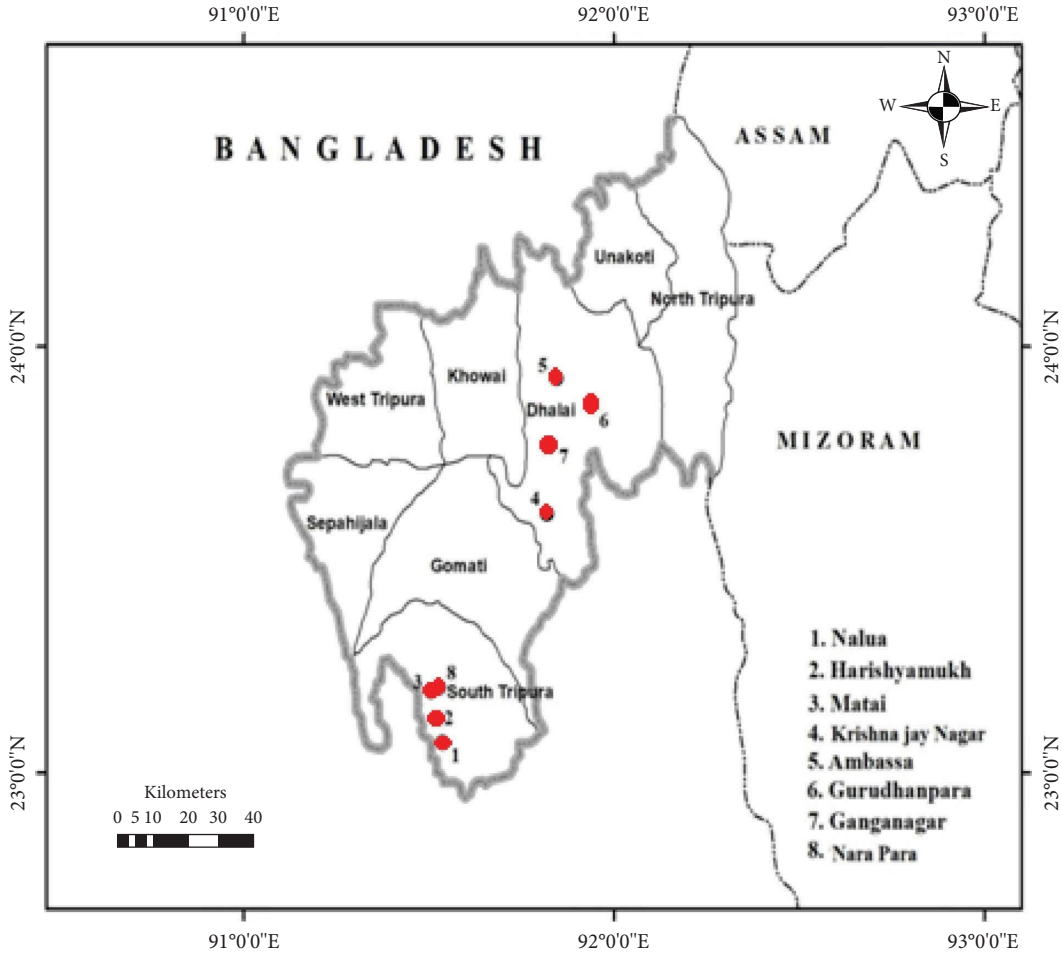

Figure 1: Map of Tripura showing study sites in the surveyed districts in India (2017-18).

selected for the study surveys and these districts reported the highest malaria cases. The major population comprises largely tribal groups living in difficult-to-reach areas of hilly and forested foothill terrain. Major breeding habitats for mosquitoes in plain and foothill areas are waterlogged paddy fields, and shallow irrigation in channels while in forested areas, breeding habitats are water streams, puddles with desiccated leaves, jungle pools, and depressions in the ground including animal footprints in forest areas. Climatic conditions are suitable for the breeding and survival of mosquitoes. Total annual rainfall ranges from 2.5 to 4.5 meters; heavy rainfall occurs from May to September. In some foothill areas, villages are generally affected by flash floods during the rainy seasons. Average temperature ranges from 23-28°C, and relative humidity remains >70%.

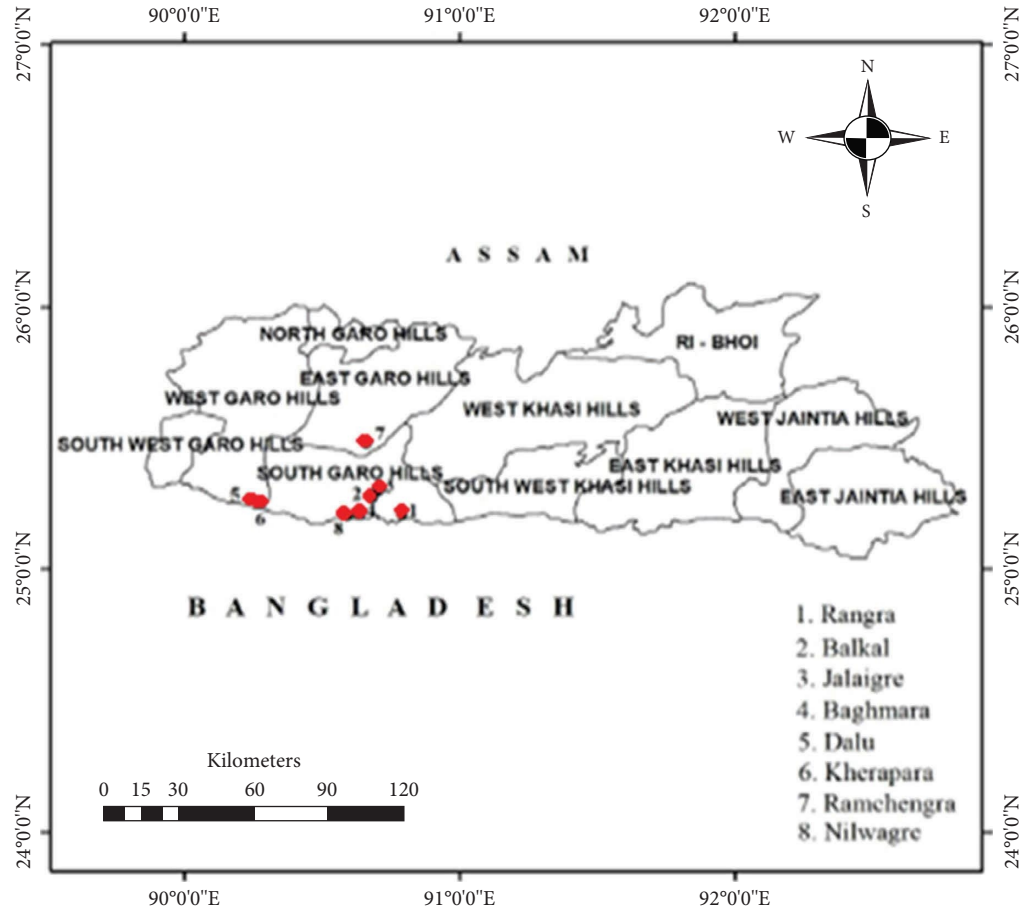

Figure 2: Map of Meghalaya showing study sites in the surveyed districts in India (2017-18).

Table 2: Details of the surveyed areas in the two states, Tripura and Meghalaya, and their ecotypes, India (2017-18).

Sl. no.	State	District	Villages/areas, co-ordinates	Ecotype(s)
1	Tripura	Dhalai	Ganganagar (23.77670, 91.82770)	Foot hill
			Gurudhanpara (23.85935, 91.94334)	Forested hill
			Krishnajay Nagar (23.602282, 91.819430)	Plain
		South Tripura	Ambassa (23.92578, 91.84610)	Plain
			Matai (23.19117, 91.50628)	Plain
			Hrishyamukh (23.12811, 91.52254)	Plain
			Nalua (23.07421, 91.54485)	Foot hill
			Nara para (23.241005, 91.524521)	Forested hill
2	Meghalaya	South Garo Hills	Nilwagre (25.166642, 90.845782)	Foot hill
			Rangra (25.31632, 90.41890)	Foot hill
			Ramchengra (25.447220, 90.638415)	Foot hill
			Balkal (25.27577, 90.67816)	Hill
			Jalaigre (25.187770, 90.577991)	Hill
			Baghmara (25.19410, 90.63809)	Hill
		West Garo Hills	Dalu (25.20913, 90.23189)	Foot hill
			Kherapara (25.34415, 90.21505)	Hill

2.2. Mosquito Collections. Different methods were employed for the collection of female anopheline species from the study sites *viz.*, hand catch using mouth aspirator and flashlight; CDC-light traps, human baited double net trap (HDN), and pyrethrum spray sheet collection [46, 47]. However, maximum number of mosquitoes was collected by the use of mouth aspirators and the minimum numbers by the use of human baited double bed net trap (HDN). From indoors, maximum mosquitoes could be collected using CDC-light traps. Collections were made indoors in human dwellings, cattle sheds, and pig sty and from outdoors from resting sites such as from thatched roofs (hay), bamboo fencing, wall, and vegetation. Field collections of adult *Anopheles* mosquitoes were made in morning hours (6.00 to

8.00 am), evening hours (6.00–7.30 pm), and some collections from dusk to dawn (6.00 pm to 6.00 am) using light traps and mouth aspirators. Quantitatively, hand catch densities were expressed as man-hour density (PMHD) calculated using the following formula.

MHD = total mosquitoes collected/number of persons × time spent in hours.

2.3. Morphological Identification. Wild caught female mosquitoes are identified to species based on species specific morphological characteristics [30–32]. The identified mosquitoes were classified as unfed, full-fed; semigravid, and gravid mosquitoes based on the abdominal condition and held separately in plastic cups for processing.

2.4. Sibling Species Identification. Sibling species composition of primary vectors, *i.e., An. minimus* and *An. baimaii*, was determined by the molecular methods. Individual mosquitoes were stored in isopropanol after morphological identification and insecticide bioassay. Molecular assay was carried out by isolating the genomic DNA of mosquitoes by DNeasy Blood and Tissue Kits (Qiagen, Germany) as per manufacturer's instructions. Screening of the samples was performed by PCR using allele specific primers including 28S rDNA gene, ITS2 gene, and mitochondrial COII subunit gene [33–35].

2.5. Host Feeding Preference. Full-fed abdomens were punctured and smeared in FTA® card (Flinders Technology Associates card) and dried. DNA isolated using DNeasy Blood and Tissue Kits (Qiagen, Germany) from the blood smear of FTA card was used as a template for multiplex PCR assay following Kent and Norris protocol [36], to determine the source of blood meal of the vectors and determine host feeding preference.

2.6. Insecticide Susceptibility Test. Field-collected identified mixed-age vector mosquitoes were exposed to WHO prescribed discriminatory concentration of insecticide-impregnated papers and kits following the WHO method [37]. Mosquitoes were exposed to DDT-4.0% and malathion-5.0% impregnated papers in a room maintained at $27 \pm 2^{\circ}$C and 60–70% relative humidity both during one-hour exposure period and later for 24 hours holding period. During the surveys, densities of mosquitoes were low and exposures were made in low numbers. As the density of mosquitoes were not sufficient, we conducted only a preliminary study with the available mosquitoes using only 1 replicate (15–20 mosquitoes) for test and control exposure, respectively. From areas having very low vector densities, at least 10–15 mosquitoes per test were used to perform the bioassays. Percent mortality to the given insecticide was calculated from the number of dead and alive mosquitoes at the end of 24 hr holding period using the formula:

Percent Mortality = total number of dead mosquitoes × 100/total number of exposed mosquitoes.

If the mortality in control replicates was between ≥5% and ≤20%, test mortality was corrected using the Abbott's formula for corrected percent mortality is determined using the formula [48]. If the control mortality exceeds more than 20%, the tests are discarded.

$$\text{corrected percent mortality (CPM)} = \frac{[(T - C)100]}{(100 - \text{C})}, \quad (1)$$

where T is the mortality in test replicates and C is the mortality in control replicates.

3. Results

3.1. Breeding Habitat of the Anopheline Mosquitoes. It was observed that different mosquito species preferred different type of habitats for breeding influenced by abiotic conditions such as rainfall, humidity, temperature, pH, presence of the vegetation, and the kinetics of water flow. that act as determinants for their breeding and propagation. The details of habitat-wise larval collection are given in Table 3.

Breeding habitat of 9 *Anopheles* species were identified including the two primary vectors of malaria, *An. baimaii* and *An. minimus*. Preferred breeding habitat of *An. baimaii* include stagnant water bodies in shady places such as pot-holes, fallen leaves, and depression in the forest areas (e.g., animal foot print and tire tracks), pools in dry streams beds, ground pools, and small water bodies in the deep forested areas including Jhum cultivated hill areas. However, *An. minimus* breeding sites were found in foothill areas having slow-moving streams with grassy margins or in drains adjacent to rice fields with the perceptible flow of water.

Secondary vectors, *An. philippinensis/An. nivipes* prefers to breed in stagnant water bodies with vegetation. Other anopheline species, *An. annularis, An. vagus*, *An. kochi, An. splendidus,* and *An. hyrcanus*, breed in almost every possible breeding site with stagnant water. Tropical climate, high humidity, and high to medium rainfall are preferred by these species for breeding and prevalence. Possible breeding sites include paddy fields, ponds, jungle pool, and animal footprints.

3.2. Entomological Survey of the Adult Anopheles Mosquitoes. A total of 1890 *Anopheles* mosquitoes were collected in the surveys. Indoor collections comprised 30.52% ($n = 577$) and proportion of outdoor collection 69.48% ($n = 1313$) of the total collection. From outdoors, 62.90% of mosquitoes were collected using mouth aspirator ($n = 826$) and 37.09% were collected using CDC light traps ($n = 487$). From indoors of the total collected 577 mosquitoes, 52.51% were collected using CDC light traps ($n = 303$), 33.62% were collected using Pyrethrum spray sheet collection ($n = 194$), and the remaining 13.86% ($n = 80$) were collected using human baited double bed net traps. During the survey period, the density of the two primary vectors, *An. minimus* and *An.*

Table 3: Breeding habitat of different *Anopheles* mosquitoes, name of study area, India (2017-18).

Species	Paddy field	Ponds	Animal footprints	Stagnant water	Slow streams
An. baimaii			+	+	
An. minimus					+
An. philippinesnsis	+	+			
An. vagus	+	+	+	+	
An. jeyporiensis	+	+	+	+	
An. annularis	+	+	+	+	
An. kochi	+	+	+	+	
An. splendidus	+	+	+	+	
An. hyrcanus	+	+	+	+	

baimaii, was found in low densities and in isolated pockets across the study sites. *Anopheles minimus* could be collected in 9 out of 16 villages and in less numbers in Meghalaya. However, in both the districts of Tripura, *An. minimus* were collected predominantly from indoor resting sites in morning collections indicating its strong endophilicity. Overall, 15.42% of *An. minimus* comprised the total indoor collection of *Anopheles* mosquitoes. On the other hand, high numbers of *An. baimaii* were collected from Nilwagre in South Garo Hills and Naurapara in South Tripura. *Anopheles baimaii* contributed 4.5% proportion of the total indoor collections and 6.16% of the total outdoor collections. High density of *An. annularis, An. vagus*, and *An. hyrcanus* was observed in all the four districts of Tripura and Meghalaya while *An. splendidus, An. karwari An. varuna, An. kochi,* and *An. aconitus* were found in lesser densities in all the study sites. MHD is given in Figure 3 and Table 4.

In Tripura, density of primary and secondary vector species, *i.e., An. minimus, An. baimaii, An. annularis,* and *An. philippinensis/An. nivipes,* and other nonvector species were high in the month of August, *i.e.,* during monsoon seasons. In Meghalaya, the densities of vector and nonvector mosquitoes were high in the month of September. In both the states, the density of mosquitoes decreased substantially during January/February with receding temperature and rainfall (Figures 4 and 5).

Present studies were undertaken in 16 villages in different ecotypes, *i.e.,* plain, foothill, and forested. *Anopheles minimus* was found in 9 villages in low densities, and overall MHD was, respectively, 0.80 and 0.60. In Tripura and Meghalaya, *Anopheles baimaii* was collected from 8 of the 16 and only from two villages Nilwagre of Meghalaya and Naurapara of Tripura. Overall MHD was 1.00 and 0.80 in Tripura and Meghalaya, respectively. Increased MHD was found for other anopheline and in all villages, *An. hyrcanus* (24.20–20.40), *An. vagus* (25.00–19.40), and *An. annularis* (29.80–24.20).

3.3. Blood Meal Preference Analysis. From the blood meal collected in the FTA card, DNA was isolated using the Qiagen DBS kit and protocol. Blood meal analysis was performed for the primary vectors: *An. minimus* and *An. baimaii*. Multiplex PCR was carried out following Kent and Norris. Blood meal analysis showed that 76.2% to 78.5% of *An. minimus* ($n = 28$) and 85.71% of *An. baimaii* ($n = 25$) collected during our surveys from various locations were human blood fed, *i.e.,* the species are primarily anthropophagic. Other blood meal sources included bovine, pig, goat, and more than one species. Detail of the analysis is depicted in Figures 6(a) and 6(b).

3.4. Sibling Species Composition. Individual mosquito samples were subjected to molecular assay to detect sibling species. Screening for sibling species revealed that only *An. baimaii* and *An. minimus s.s* and morphologically similar *An. varuna* of *An. minimus* complex were present in study sites in both states. PCR gel image is shown in Figure 7. Diagnostic product fragment size for *An. baimaii* is 540 bp, *An. minimus* is 184 bp, *An. varuna* is 252 bp, and *An. jeyporiensis* is 346 bp.

3.5. Insecticide Susceptibility Test. Since the density of primary vectors *An. baimaii* and *An. minimus* was not adequate to perform tests according to WHO standard guidelines, a study was conducted using the number of available mosquitoes that could be collected. Susceptibility tests for *An. minimus* were performed against DDT (4%) in Dhalai (Tripura) and South Garo Hill district (Meghalaya). Observed percent knockdown (KD) was 100% in Dhalai district and 60% in South Garo Hills and recorded 100% mortality at the end of 24 h holding period. The KDT_{50} (median knockdown time) ranged between 33.65 (CI 95%: 25.45–51.99) and 43.06 (95% CI: 32.62–66.36) minutes. The lowest KDT_{50} being 33.65 and highest KDT_{50} was 43.06. Similarly, KDT_{90} ranged between 76.74 (95% CI: 50.43–407.43) and 115.53 minutes (95% CI: 72.11–604.22).

Bioassay against DDT was performed for *An. baimaii* in Dhalai, South Tripura, and South Garo Hill. Percent knock down of *An. baimaii* for DDT was found to be 100%. For Malathion, assays were performed in South Garo Hills and South Tripura, percent knock down was 90% in South Garo Hill and 100% in South Tripura. However, the mortality was 100% after 24 hour holding time.

Knock-down assay of *An. baimaii* for DDT showed that the lowest KDT_{50} was 23.12 and KDT_{90} was 43.22 minutes. The assay also showed that the highest KDT_{50} and KDT_{90} values were 29.48 and 47.74 minutes, respectively. For Malathion 5%, the KDT_{50} was observed to be 20.30–22.30 minutes and KDT_{90} was observed to be 38.75–40.77 minutes.

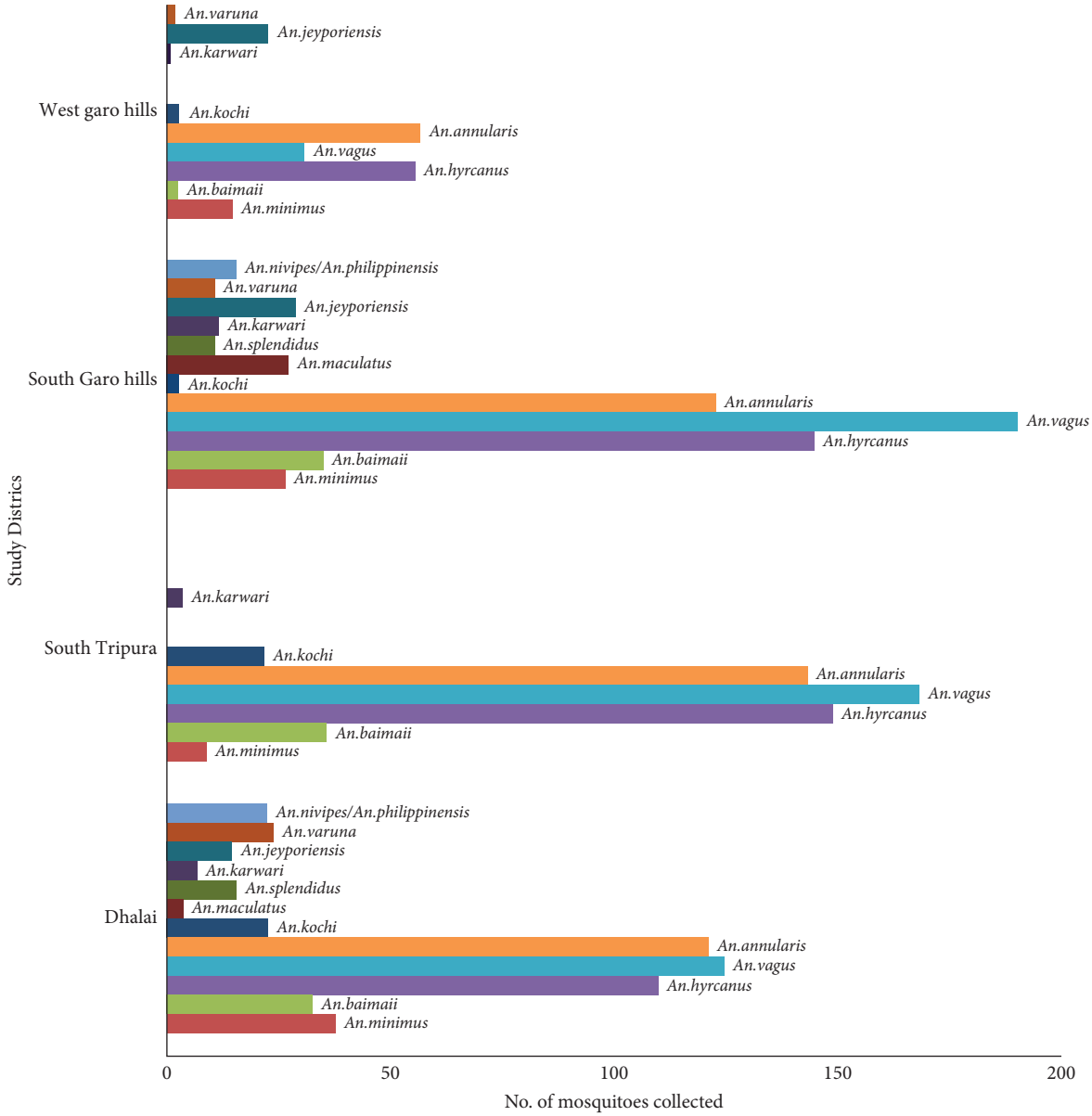

Figure 3: Species-wise mosquito abundance in the surveyed districts, India (2017-18).

Table 4: Density of *Anopheles* mosquitoes in the study area, India (2017-18).

Mosquito species	*Man hour density of Anopheles mosquitoes*	
	Tripura	Meghalaya
An. minimus	0.80	0.60
An. baimaii	1.00	0.80
An. hyrcanus	24.20	20.40
An. vagus	25.00	19.40
An. annularis	29.80	24.20
An. kochi	3.40	4.60
An. maculatus	2.20	1.40
An. splendidus	0.60	0.80
An. karwari	1.20	1.00
An. jeyporiensis	0.40	0.60
An. varuna	0.40	0.80
An. philipinensis/An. nivipes	1.20	0.40

4. Discussion

Tripura and Meghalaya states, situated in the NE Region of India, are the major contributors of malaria cases in India (NVBDCP data). The population in the study area was predominantly tribal and lives in poor socioeconomic conditions [38] and most of the families live in one room often in mud/bamboo houses. Previous studies suggest that most of the cases are reported from areas in difficult terrains, far away from the primary health centre; a similar observation was also made during this field study [19]. Present studies were undertaken in 16 villages in 2 states: Tripura and Meghalaya, in different ecotypes, *i.e.,* plain, foothill, and forested. *Anopheles minimus* was found in 9 villages in low densities, and overall MHD was, respectively, 0.80 and 0.60 in Tripura and Meghalaya. *Anopheles baimaii* was collected form 8 of the 16 villages but could be collected only from two

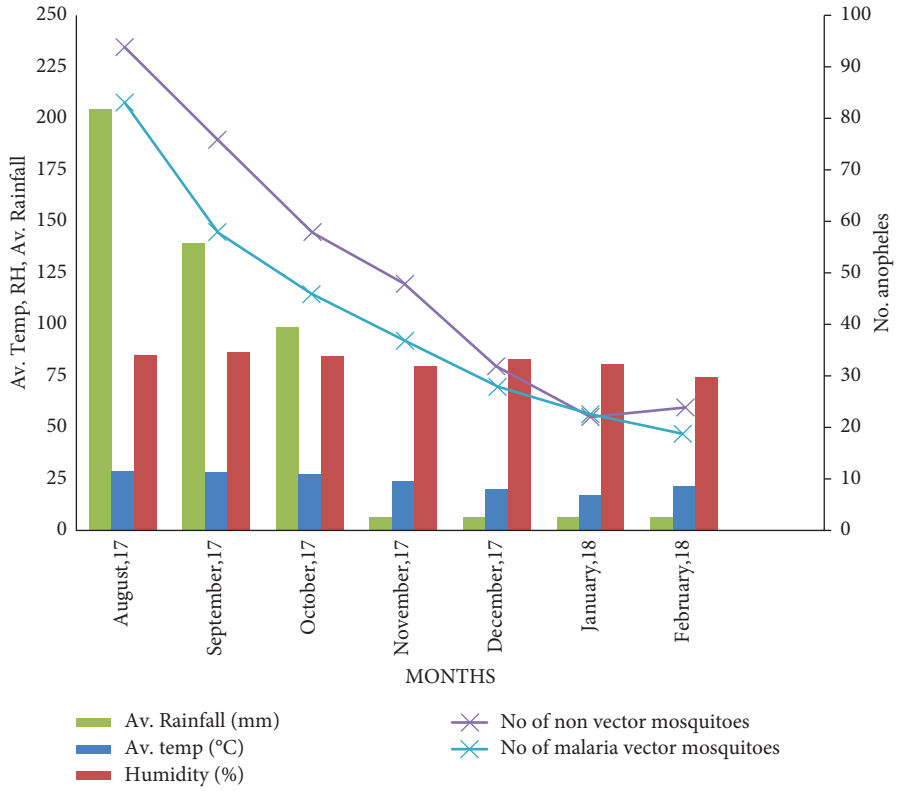

Figure 4: Relative abundance of anopheline mosquitoes in relation to meteorological parameters of Tripura state, India (August 2017–February 2018).

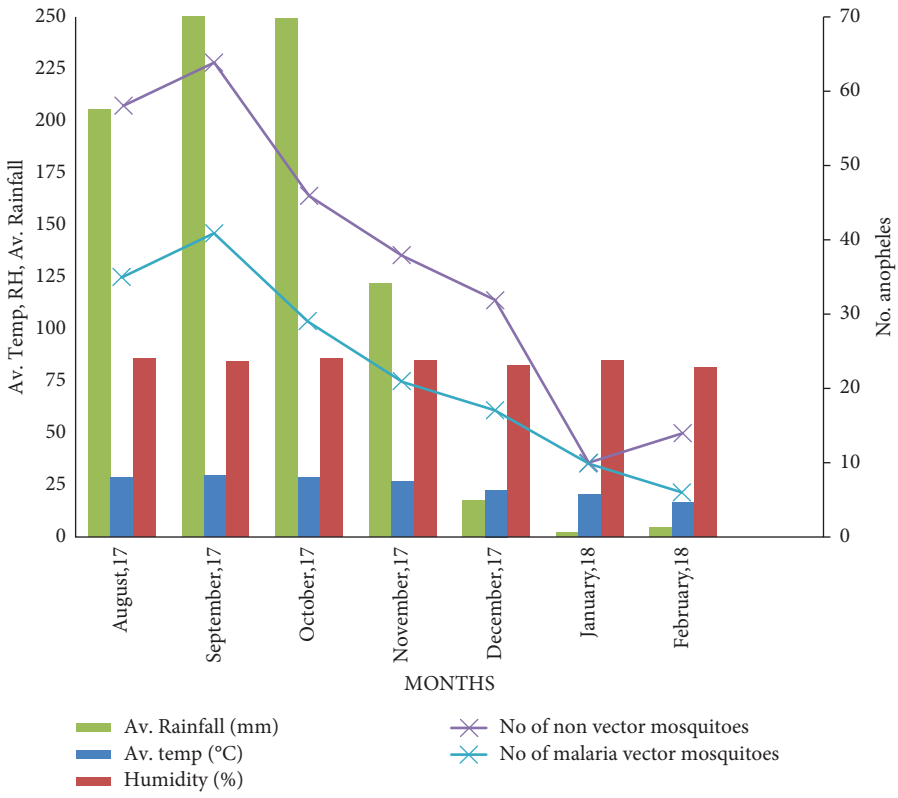

Figure 5: Relative abundance of anopheline mosquitoes in relation to meteorological parameters of Meghalaya state, India (August 2017–February 2018).

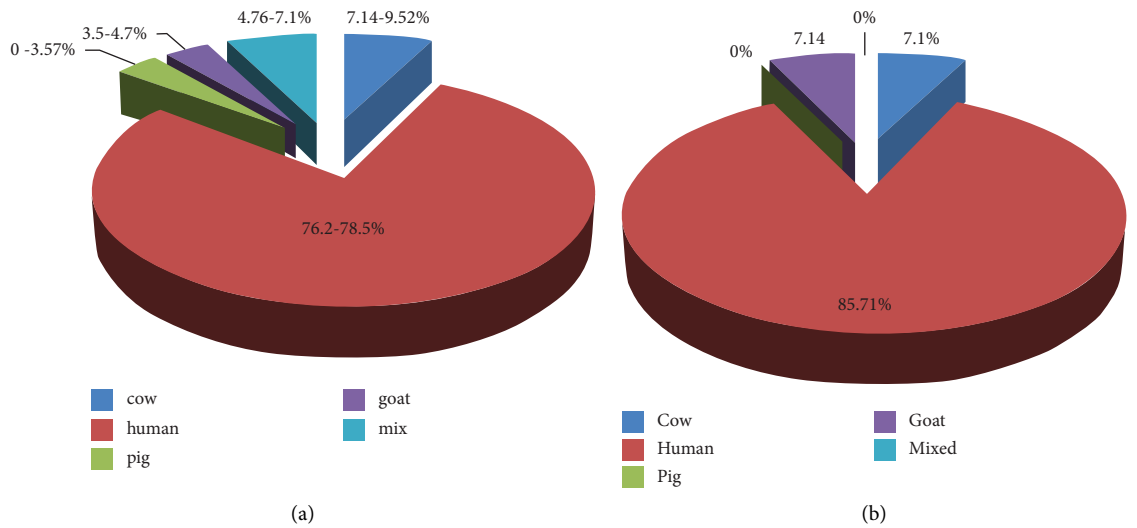

FIGURE 6: Pie diagram of blood meal analysis of *Anopheles minimus* (a) and *Anopheles baimaii* (b) in Tripura and Meghalaya states of, India, 2017-2018.

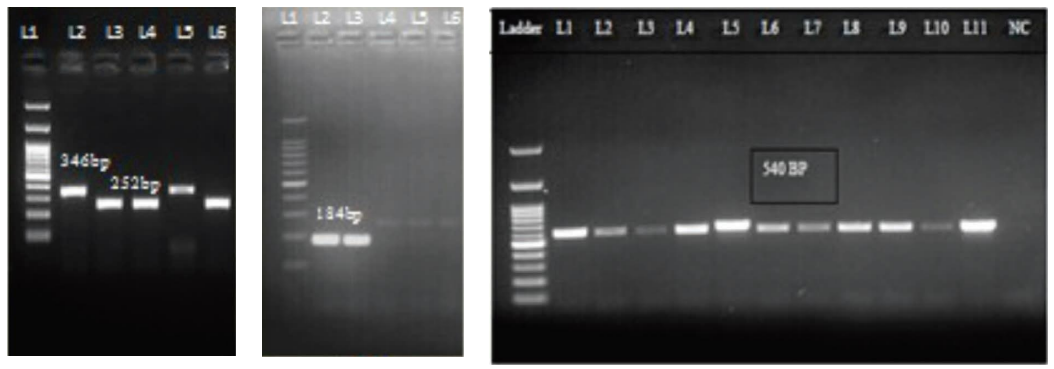

FIGURE 7: Allele-specific PCR gel image of anopheline mosquitoes. Lane1-ladder (1 kb); L2 and L5 *L: An. jeyporiensis* (346 bp); L3, L4, and L6: *An. varuna* (252 bp) of picture (a). L2 and L3: *An. minimus* (184 bp) of picture (b). D2 region PCR assay gel picture of *An. baimaii*;Lane1-ladder (1 kb), *An. baimaii* (540 bp) of picture (c).

villages Nilwagre of Meghalaya and Naurapara of Tripura. Overall MHD was 1.00 and 0.80 in Tripura and Meghalaya, respectively. Increased MHD was found for other anophelines and in all villages, *An. hyrcanus* (24.20 – 20.40), *An. vagus* (25.00 – 19.40), and *An. annularis* (29.80 – 24.20).

Anopheles minimus breeding is reported to occur in seepage water from irrigation streams [39] paddy fields and shallow water wells [40] and grow normally between 16 and 35°C and avoid ovi-position in polluted waters [41]. The species predominantly breeds during the monsoon season due to the abundance of breeding streams in the foothill areas and also in the winter in low numbers, showing its adaptation to varied environmental conditions [49]. In the present study, *An. minimus* was found breeding in slow moving streams and showed no association with other *Anopheles* species. As the flight range of the species is limited, this species was found to be transmitting malaria mainly in the foothill villages in proximity to breeding sites.

In these studies, *An. baimaii* was found predominantly breeding in natural or man-made depressions on the ground having stagnant water under shade in the forested areas. Unlike *An. minimus*, *An. baimaii* was found breeding in association with six *Anopheles* species, namely, *An. vagus, An. jeyporiensis, An. annularis, An. kochi, An. splendidus,* and *An. hyrcanus*. Some of the breeding habitats created in monsoons were found dry by postmonsoon season leading to decrease in number of breeding habitats and consequently decrease in mosquito density.

Members of *An. annularis s.s.* and *An. philippinensis/An. nivipes* are reported as the secondary vectors of malaria in the NE region [9, 42]. Generally, densities of the vector mosquitoes as well as nonvector mosquito density were high during monsoon season due to abundance of breeding places and preferable climatic conditions [43]. In the present study, *An. annularis s.s.* was found in high density throughout the year but peak breeding was observed during monsoon season similar to the finding of previous study.

Anopheles annularis s.s. was found to be breeding in a variety of breeding sites with stagnant water. Adult mosquitoes were collected from indoors and outdoors. On the other hand *An. philippinensis/An. nivipes* were found mostly breeding in paddy fields with grassy margins. Availability of the breeding habitats throughout the year justified its presence in all seasons. Effect of the temperature do not act as a major factor on the availability of vector hence these species were found throughout the year, and during rainy season which is also the peak transmission season, density of these anopheles mosquitoes increases. Previous studies indicate that *An. baimaii* has been reported from all the states of North-East region, India [19]. *Anopheles. bamaii* was previously reported to be exophilic in nature and *An. minimus* to be highly endophilic which is in concurrence to our findings.

Blood meal analysis during the study period confirms the anthropophagic behavior of both the primary vectors, *An. minimus* and *An. baimaii*. In this study, shift in the feeding behavior of *An. baimaii* to zoophagicity was observed with 85.71% anthropophagic compared to 92.3% previously reported [44]. This indicated the possible change in the blood feeding behavior of the species which can be attributed to the vertical pulsation ability, *i.e.,* the ability to feed on alternate host like goat, pig, or cattle other than human host due to changes in the environmental condition described by Dev and Sharma [2]. This change in behavior can be related to the irritability of *An. baimaii* to insecticides in LLINs and IRS to avoid lethal contact. This change in feeding behavior will be advantageous for the decrease in malaria transmission in view of its reported behavior being both exophagic and exophilic [45].

The investigation was carried out to identify the sibling species of *An. minimus* complex, of the reported 3 species only *An.minimus s.s.* (Species A) is found prevalent in NE Region; however, a morphologically similar species, *An. varuna* was present in the study area. On the other hand, *An. baimaii* (Species D of the *An. dirus* complex) is prevalent in the NE Region. However, few identified samples of *An. baimaii* have shown variation in the molecular assay. PCR assay on the ITS2 gene shows amplification of 850 bp band which resembles the "Species X" of *An. dirus* complex described earlier [19]. Hence, further investigation is needed to confirm the density, distribution, and role in the transmission of this "unknown" species in the NE region.

Insecticide susceptibility tests on the wild-caught mosquitoes showed that *An. minimus* and *An. baimaii* were 100% susceptible to DDT, the insecticide that is in use in IRS. *Anopheles minimus*, recorded 60–100% knock-down within 1-hour exposure in the study sites while it was 90–100% knock-down in *An. baimaii* was found to have a percentage in the study sites for the insecticide DDT. This trend in knockdown mortalities indicated increased susceptibility to DDT in *An. baimaii*. To Malathion, *An. baimaii* was reported as completely susceptible. The sustained susceptibility to DDT, Malathion, and to pyrethroids (unpublished data) is advantageous for the efficacy of insecticidal interventions in use, IRS, and LLINs.

In view of the eradication goal set by the Asia-Pacific Leaders Malaria Alliance, Malaria Elimination Roadmap, and National Framework for Malaria Elimination in line with the WHO Global Technical Strategy for Malaria 2016–2030 to eliminate malaria by the year 2030, it is pertinent that the gains towards malaria elimination in northeast region be sustained further and implement proactively appropriate strategies for disease elimination.

5. Conclusion

Tripura and Meghalaya are the high malaria prevalence states of NE India. Both *An. minimus* and *An. baimaii* showed anthropophagic behavior. However, a slight shift (~14%) in *An. baimaii* towards zoophagic behavior is advantageous for impact on decreased transmission of disease. Collection using mouth aspirators yielded maximum number of mosquitoes from Outdoors and maximum from indoors using CDC-Light traps. Insecticide susceptibility tests confirmed these two major vector species are susceptible to DDT and Malathion till date unlike other vector species and indicate their efficacy of ongoing interventions like DDT-IRS. Thus, for sustenance of gains more effective implementation of appropriate strategies based on this recent information on bionomics of the two major malaria vectors in north east region of India will provide good opportunity to achieve malaria elimination by date in majority states in this region.

Data Availability

The datasets of the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors would like thank MO, SMO, and health workers of respective districts of the states who supported during field surveys and provided valuable information related to visited places. This research was funded by ICMR, under Grant no. 6/9-7(161)/2017-ECD-II.

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