

Countrywide surveillance of malaria vector bionomics and its implications for malaria elimination in India

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Abstract

Background: Mosquito vectors exhibit variable biological characteristics that influence their response to vector control measures. Therefore, updated information on vector bionomics is important for ensuring that the currently used vector control strategies and tools are effective in view of the goal of malaria elimination in India by 2030.

Objective: The objectives of the study were to assess the indoor and outdoor resting proportions of vector species, to know the host biting/feeding preference of vector species, the sites of transmission, and to get the susceptibility status of vectors against insecticides used in public health programme.

Methods: Mosquito collections were made from 13 districts from eight Indian states during 2017 to 2020 using different methods to deduce their densities. After morphological identification in the field, sibling species of *Anopheles* mosquitoes were molecularly identified using polymerase chain reaction (PCR) specific alleles. *Plasmodium falciparum* and *Plasmodium vivax* infections in the vectors were detected out using ELISA and PCR assays. Susceptibility status of the known primary malaria vectors to the insecticides commonly used in the national programme was determined using World Health Organization (WHO) protocol.

Results: *Anopheles culicifacies*, one of the primary malaria vectors, was collected (man hour density 3.1-15.9) from all the states of India except the states in the northeastern region. *An. fluviatilis*, another primary vector, was collected from Madhya Pradesh, Maharashtra, Karnataka and Odisha states. *An. culicifacies* sibling species A was predominant in Haryana and Karnataka, while species C and E were predominant in Madhya Pradesh and Maharashtra. *An. culicifacies* was mainly endophilic in all the states except Madhya Pradesh where proportion of semigravid and gravid mosquitoes was nearly half of the unfed mosquitoes. Human blood index of *An. culicifacies* varied from 0.001 to 0.220 in all the study sites. The sporozoite rate of *An. culicifacies* was in the range of 0.06 – 4.24 except in Madhya Pradesh, where none of the vector mosquitoes was found infected with *Plasmodium* parasite. In the study area, *An. culicifacies* was resistant to DDT (<39% mortality). It has developed resistance also to malathion (49-78% mortality) in all districts except in Angul, Odisha and Palwal, Haryana and to deltamethrin in districts of Maharashtra, Gujarat, Haryana, and Karnataka states.

Conclusions: Our study provides crucial information on prevalence, resting behavior and sibling species composition of malaria vectors in India. It is evident from the study that development of resistance in *An. culicifacies*, the primary vector, to synthetic pyrethroids is increasing in the country. Result of the study also indicated towards the change in the resting behavior of *An.*

culicifacies in Madhya Pradesh but needs larger studies to reaffirm this plausible change. Our findings will be crucial in developing vector control strategies in India, which intends to eliminate malaria by 2030.

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Countrywide surveillance of malaria vector bionomics and its implications for malaria elimination in India

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Abstract

Background: Mosquito vectors exhibit variable biological characteristics that influence their response to vector control measures. Therefore, updated information on vector bionomics is crucial to ensure the efficacy of existing vector control strategies and tools in view of malaria elimination in India by 2030.

Objective: The study aimed to assess the indoor and outdoor resting proportions of vector species, determine the host biting/feeding preference of vector species, the sites of transmission, and to get the susceptibility status of vectors against insecticides used in public health programme.

Methods: Mosquito collections were made from 13 districts across eight Indian states during 2017 to 2020 using different methods to estimate their densities. After morphological identification in the field, sibling species of *Anopheles* mosquitoes were molecularly identified using polymerase chain reaction (PCR) specific alleles. *Plasmodium falciparum* and *Plasmodium vivax* infections in the vectors were detected out using ELISA and PCR assays. We also determined the insecticide susceptibility status of primary malaria vectors using World Health Organization (WHO) protocol.

Results: *Anopheles culicifacies*, one of the primary malaria vectors, was collected (man hour density 3.1-15.9) from all the states of India except the states in the northeastern region. *Anopheles fluviatilis*, another primary vector, was collected from Madhya Pradesh, Maharashtra, Karnataka and Odisha states. *Anopheles culicifacies* sibling species A was predominant in Haryana and Karnataka, while species C and E were predominant in Madhya Pradesh and Maharashtra. *Anopheles culicifacies* was mainly endophilic in all the states except Madhya Pradesh where proportion of semi gravid and gravid mosquitoes was nearly half of the unfed mosquitoes. Human blood index of *An. culicifacies* varied from 0.001 to 0.220 in all the study sites. The sporozoite rate of *An. culicifacies* was in the range of 0.06 – 4.24 except in Madhya Pradesh, where none of the vector mosquitoes was found infected with *Plasmodium* parasite. In the study area, *An. culicifacies* was resistant to DDT (<39% mortality). It has developed resistance also to malathion (49-78% mortality) in all districts except in Angul, Odisha and Palwal, Haryana and to deltamethrin in districts of Maharashtra, Gujarat, Haryana, and Karnataka states.

Conclusions: Our study provides crucial information on prevalence, resting behavior and sibling species composition of malaria vectors in India. It is evident from the study that development of resistance in *An. culicifacies*, the primary vector, to synthetic pyrethroids is increasing in the country. Result of the study also indicated towards the change in the resting behavior of *An. culicifacies* in

Madhya Pradesh but needs further studies to reaffirm this plausible change. Our findings will be crucial in developing vector control strategies in India, which intends to eliminate malaria by 2030.

Key words: malaria, bionomics, sibling species, insecticide resistance, elimination, India,

Introduction

Malaria remains one of the major causes of morbidity and mortality among the vector-borne diseases. According to the world malaria report 2021, there were 241 million cases and 627,000 deaths due to malaria, mainly in African nations (95% of total cases) [1]. The World Health Organization South-East Asia Region (WHO-SEARO) accounted for around 2% of the malaria burden worldwide. Malaria incidence in this region decreased significantly over the previous two decades, from 23 million in 2000 (18 cases per 1000) to around 5 million in 2020 (3 cases per 1000). India produced the most significant reductions in the WHO-SEARO, from about 20 million cases in 2000 to around 4.1 million in 2020, although it still accounted for 83% of malaria cases and 82% of malaria fatalities in the SEA region [1]. The national program data for the year 2020 revealed that around 82% of malaria cases recorded in India were confined to six states: Odisha, Chhattisgarh, Uttar Pradesh, Jharkhand, Maharashtra, and West Bengal [2].

The overall reduction in malaria burden in India has been attributed primarily to the scaling up of control interventions, such as timely diagnosis by virtue of the availability of rapid diagnostics and microscopy, treatment with artemisinin based combination therapy (ACT) and importantly effective vector control via indoor residual spray (IRS) and long-lasting insecticidal nets (LLINs). In India, there are six primary malaria vectors, namely, *Anopheles culicifacies*, *An. fluviatilis*, *An. minimus*, *An. stephensi*, *An. baimaii* and *An. sundaicus* and the secondary vectors are *An. annularis*, *An. philippinensis*, *An. nivipes* and *An. varuna* [3]. According to the national guidelines, distribution of LLINs target 80% coverage with an average of one LLIN per 1.8 people in all sub-centers reporting an Annual Parasite Incidence of ≥ 1 , whereas IRS is targeted to cover epidemic-prone areas and malaria-affected communities with low access to the health care system [4]. Vector control interventions have been at the forefront and contributed the most to the reduction in malaria transmission.

Vectors exhibit variable biological characteristics that influence their response to vector control measures. In the context of elimination, if the vector control interventions need to remain effective [5], entomological aspects, both physiological and behavioral, of the prevalent malaria vectors that influence malaria transmission are of paramount importance. Further, it would be appropriate to have

recent data on bionomics and efficacy of chemical interventions on the vectors when assessing the effectiveness of current vector control tools and deciding which control strategy to be used in the field.

India is a country with different topographies, climatic conditions and ecosystems resulting in heterogeneity of malaria endemicity [6]. In addition to multiplicity of vectors in India, there is another challenge of vector incursion and vector migration. *Anopheles culicifacies*, a primary malaria vector in India, has extended its range into the northeastern states, establishing itself as a malaria vector in this new territory [7]. Similarly, *An. stephensi*, an urban malaria vector in India, has expanded its presence into regions like Sri Lanka, Djibouti, Ethiopia, and Sudan in Africa [8]. The emergence of these vectors in new areas underscores the importance of ongoing entomological surveillance to monitor and address these evolving challenges. Migration of different malaria vectors across nearby locations, ecological successions, and colonization to new habitats through evolutionary adaptation to eco climatic changes has been re-ported in many instances in India [9]. An entomological surveillance study to monitor *Aedes* invasion was carried out in Guilan Province of Iran having several ports of entry. Though no specimen of *Ae. aegypti* or *Ae. albopictus* was collected but importance of such regular surveillance was recognized [10]. On similar lines, a study is underway in India by Indian Council of Medical Research's Vector Control Research Centre to investigate the relationship between human and mosquito mobility and its ramifications on control of mosquito borne infections. The study will unravel the complex phenomenon of dispersion and movement of short and long distance cargo and human transportation along with its surrounding travel infrastructure (ports, stations markets etc.). Recently, there has been a growing demand for extensive nationwide vector surveillance studies [11]. The Indian Council of Medical Research (ICMR), New Delhi, is the nodal research organization under the Ministry of Health and Family Welfare to carry out biomedical research in the country. ICMR, via its permanent institutes situated in various malaria-endemic states, has carried out significant entomological research over the past several decades and contributed immensely to the understanding and dissemination of the science of vector biology in the country, especially in the context of malaria elimination goals [12]. The current multi-center study was conceptualized and formulated by ICMR. In consultation with experts, common objectives were framed, and a standard methodology to study vector bionomics was devised. The current study was carried out by ICMR's four research institutes and their field stations located in various parts of the country during 2017 to 2020 with the objectives to assess (i) the indoor and outdoor resting proportions of vector species in relation to time, (ii) host biting/feeding preference, biting rhythm and peak biting activity of vector species during different seasons, (iii) the

sites of transmission, and (iv) the susceptibility status of vectors to different insecticides.

Methodology

Study sites: The study was carried out by four institutes of the ICMR, namely, the National Institute of Malaria Research, Delhi, and its field units located at Bengaluru and Nadiad, National Institute of Research in Tribal Health, Jabalpur, Madhya Pradesh, Regional Medical Research Centre, Bhubaneswar, Odisha and Regional Medical Research Centre, Dibrugarh, Assam. Thirteen districts from eight Indian states with different ecotypes, vector distribution, insecticide susceptibility status of vectors etc. were included in the study. In each district, we selected one or more blocks (sub-district level) with varying epidemiological situation and in each block, we selected 2-3 villages. Each village was having a characteristic one ecotype such as forest, foothill, riverine or plains. All the four prevailing seasons, pre-monsoon, monsoon, post-monsoon, and winter were covered during the study. Figure 1 shows the criteria, number of states, districts, blocks/CHCs, and villages selected for the study. The location of the participating institutes and the districts covered by each institute is depicted in Figure 2.

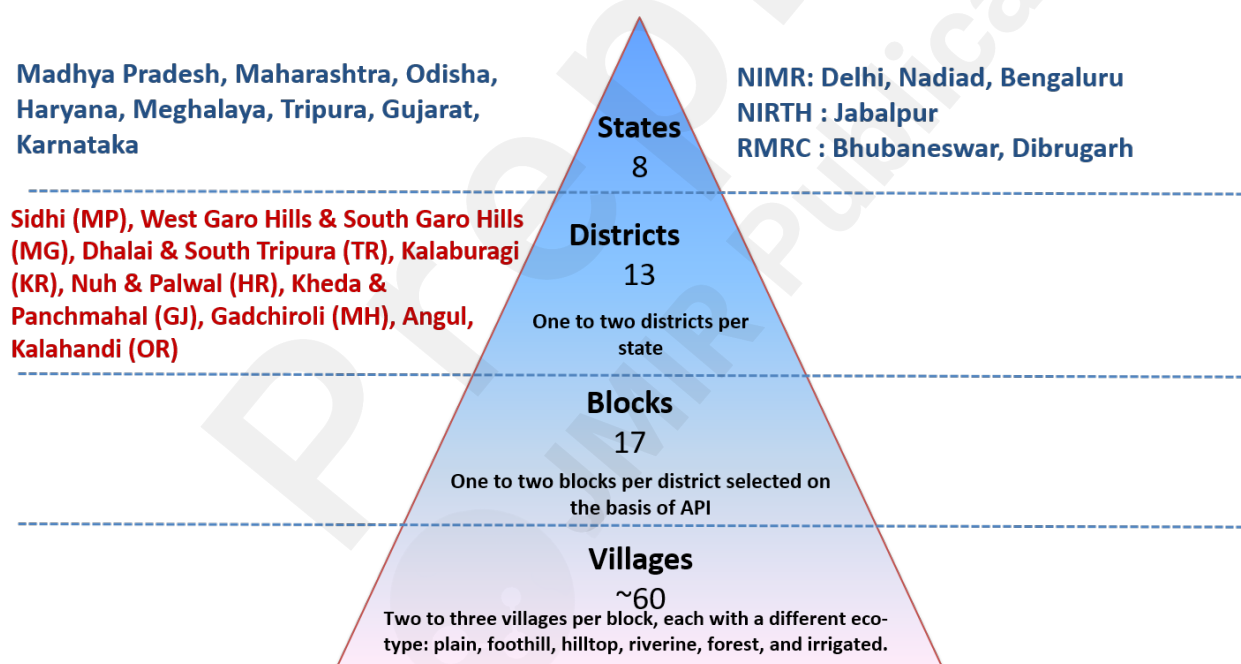


Figure 1: Criteria for study site selection. Abbreviation: GJ-Gujarat; HR-Haryana; KR-Karnataka; MG-Meghalaya; MP-Madhya Pradesh; MH-Maharashtra; OR=Odisha; TR-Tripura, NIMR-National Institute of Malaria Research; NIRTH-National Institute for Research on Tribal Health; RMRC-Regional Medical Research Center)

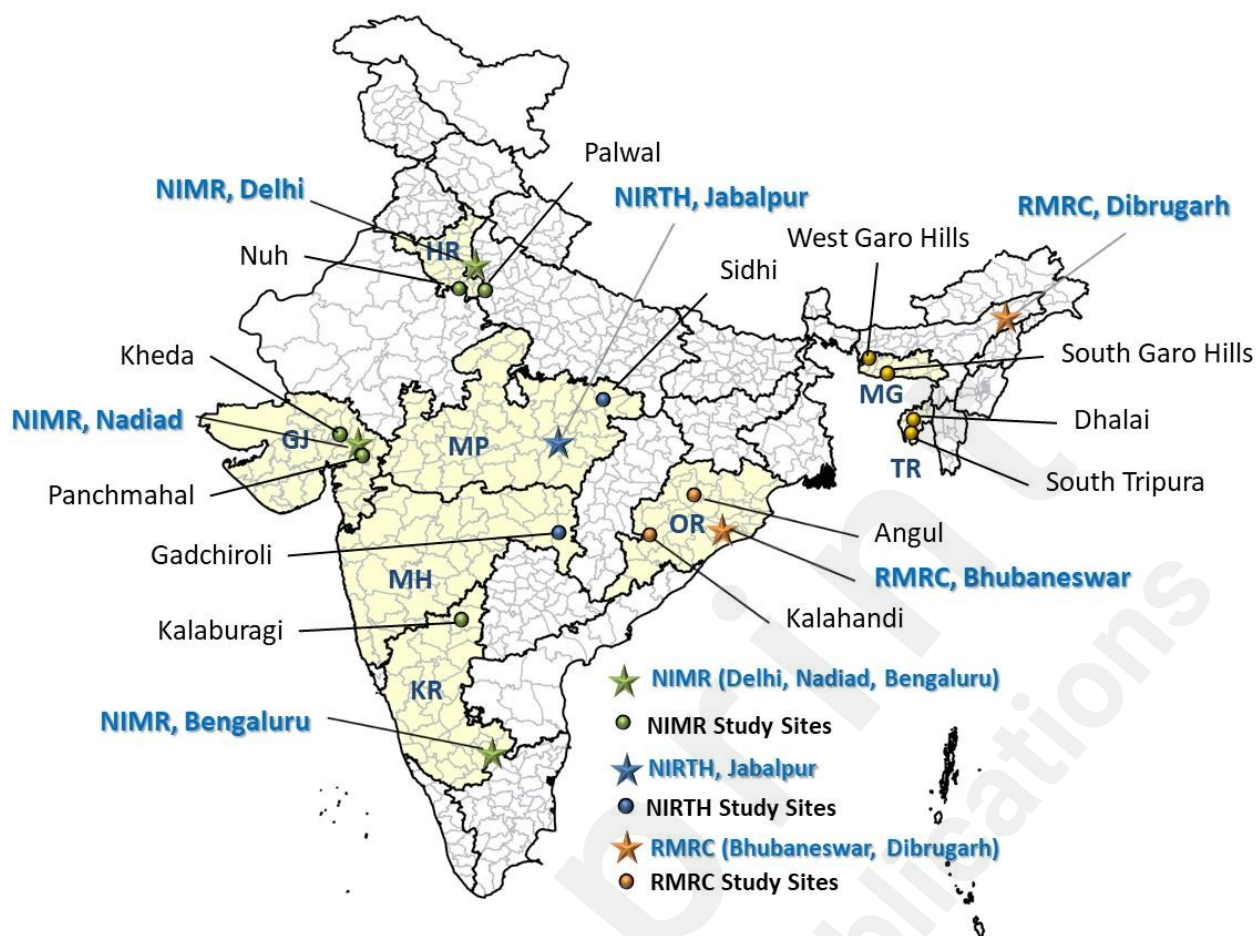


Figure 2: Location of participating institutes and study sites (Abbreviation: GJ-Gujarat; HR-Haryana; KR-Karnataka; MG-Meghalaya; MP-Madhya Pradesh; MH-Maharashtra; OR=Odisha; TR-Tripura; NIMR-National Institute of Malaria Research; NIRTH-National Institute for Research on Tribal Health; RMRC-Regional Medical Research Center)

Ethical clearance – Following community sensitization, verbal approval was obtained from the chiefs and elders of the communities for mosquito collection in selected villages. Written consent was also obtained from the persons involved as human baits. As the study protocol involved human landing collections which could have led to biting of the human baits by mosquitoes. Attempts were made to collect mosquitoes as soon as they landed on the host before engorging to avoid the actual biting. The human baits were given prophylaxis as part of malaria prevention measures. Ethical approval for the study was obtained from the ethics committee of the respective participating Institutes.

Entomological surveys: The different methods used for collection of adult anopheline

mosquitoes from the study sites are as follows:

Indoor resting collections: Anopheline resting indoors were caught by hand using a mouth aspirator and a flash light. Four human dwellings (HD) and four cattle sheds (CS) were searched in each village for mosquitoes in the morning from 0600 to 0800 hrs., spending 15 minutes in each structure [13]. The standard morpho-taxonomic keys were used to identify mosquito species collected from the field [14]. From the number of female mosquitoes, per man-hour density (MHD) of each vector species was computed as per formula given under data analysis section.

Light trap collections_(CDC light trap): Adult vector density was also monitored using light traps. Light traps were hung inside human dwellings near eaves, sleeping hosts, and door for mosquito collection from 1800 to 0600 hrs. Outdoor collection was also done by hanging traps closer to the houses, as well as in the open, away from the houses. The collected mosquitoes were kept in cartons with wet towels at the bottom and outside during transportation to maintain a temperature of $27\pm 2^{\circ}\text{C}$ and a relative humidity (RH) of $80\pm 10\%$. From the light trap collections, vector density i.e. number of females collected per trap-night indoor or outdoor was calculated.

Pyrethrum spray collection (PSC): Another method employed in the study for collecting adult mosquitoes that were resting indoors was pyrethrum spray collection (PSC). Most, if not all, of the *Anopheles* mosquitoes resting indoors were collected using this procedure in the morning hours (0800–1000 hrs.). The entire floor of the room (human dwelling) was covered with a white cotton sheet, and by spraying the complete room with 0.1-0.2% pyrethrum spray with flit sprayer, all mosquitoes resting inside the room were knocked down onto the sheet. The mosquitoes collected were kept in petri-dishes lined with wet cotton or filter paper and transported to the laboratory. This method gave the total number of mosquitoes/species resting per house or structure.

Mosquito landing collections on human baits: A whole night collection of mosquitoes landing on human bait was carried out from dusk to dawn (1800 to 0600 hrs.). Landing collection was made hourly but it was continuous for 12 hours. Collection was made from exposed legs of human bait. Attempts were made to collect mosquitoes as soon as they landed on the host before engorging to avoid the actual biting. Mosquito collections from each hour were kept in a separately labeled paper cup covered with a mosquito net. These collections were used for estimating the man-biting/landing density and determination of entomological inoculation rate (EIR) of vector species.

Laboratory processing

Mosquito species identification: Mosquitoes collected by different methods were morphologically identified to different *Anopheles* species using the taxonomic keys of Nagpal and Sharma (1995) [14].

Sibling species identification: The mosquito specimens stored in isopropanol were subjected to molecular identification of sibling species of *An. culicifacies* and *An. fluviatilis* according to the method of Goswami et al., 2006 and Singh et al., 2004, respectively [15,16].

Blood meal preferences: Blood from the stomach of the fully-fed mosquitoes obtained from the field was collected on Whatman No. 1 filter paper to identify the source of blood meal against human and bovine anti-sera Human blood index (HBI) of *An. culicifacies* was determined using the Gel diffusion technique [17].

Vector incrimination: Mosquitoes obtained from different collection methods were examined for vector infection with human malaria parasites. The head and thoraces of the mosquitoes were dissected and stored in isopropanol at -20°C until use. Polymerase chain reaction (PCR) and ELISA are the two main methods used for detection of sporozoite in mosquitoes and both were used in our study. Vector incrimination was done by the ELISA-based method to detect species-specific circumsporozoite antigen (CSP) of *Plasmodium falciparum*, *P. vivax* 210, and *P. vivax* 247 using the protocol described by Wirtz et al. (1985, 1989) [18,19] and/or by employing diagnostic PCR for the detection of malaria parasite. We tested 4067 *An. culicifacies* mosquitoes by PCR and 4164 *An. culicifacies* by ELISA. We used specific antibodies against circumsporozoite protein (CSP) which allows species identification of *Plasmodium* parasite as well subtyping of *P. vivax* sporozoites in ELISA. PCR was performed using the nested PCR protocol described by Snounou et al. (1993) [20].

Insecticide susceptibility tests: Vector susceptibility to the insecticides used in the national control programme was tested once during peak abundance of vector species, following the WHO guidelines [21]. Field collected, preferably from unsprayed villages/houses, mixed-age vector mosquitoes were exposed to the WHO papers impregnated with insecticides at diagnostic concentration (DDT 4%, malathion 5%, similarly WHO recommended discriminating concentration of different synthetic pyrethroids) using WHO test kit. For a test, 100 mosquitoes, in four/five replicates (20-25 mosquitoes per replicate) for treatment and 50, in two replicates (25 mosquitoes per replicate), for control were exposed. Tests were carried out in a room with no insecticide contamination and maintained at $27\pm 2^{\circ}\text{C}$ temperature and $80\pm 10\%$ relative humidity both during exposure and during 24 h holding period. From the total number of alive and dead mosquitoes in the replicates, percent mortality was calculated post 24 h of holding. If control mortality was between 5% and 20%,

Abbott's formula was used to correct the treatment mortality. According to the WHO criteria, mortality of $\geq 98\%$ was considered to be 'susceptible'; $<90\%$ - 'resistant'; and between 91-97% - 'possible resistance'.

Data analysis

All data generated during the study were fed in to computer on MS Excel, and the following parameters were analyzed.

- a) Man hour density (MHD) is the number of mosquitoes collected by one person for one hour and is calculated taking into account the total number of mosquitoes (n) collected, time spent in minutes (t), and the number of persons involved in the collection (p). $MHD = n \times 60 / t \times p$.
- b) The proportion of fed *Anopheles* mosquitoes found to contain human blood were used to calculate Human blood index (HBI).
- c) The proportion of female *Anopheles* mosquitoes carrying sporozoite in their salivary glands were used to calculate Sporozoite rate.
- d) The human landing density (HLD) was defined as the ratio of the total mosquitoes captured landing on human bait for a period to the total person-nights used for the same period.
- e) The entomological inoculation rate (EIR) was calculated from human landing/biting catches as the product of the human landing density (HLD) and sporozoite rate of mosquitoes.

Results

The results of *Anopheles* species collected by different collection methods, resting, pyrethrum spray, light trap and human landing at different sites are described below and summarized in Table 1.

Table 1: Malaria vector density by various collection methods in districts of India

State	District	Species	per manIndoor resting	oom)Pyrethrum spray	ht) Light trap outdoor	ght) Light trap indoor	an perHuman landing	in perHuman Landing	/Animal bait collection
Gujarat	Kheda	<i>An. culicifacies</i>	3.15	1.92	3.9	3.1	1.85	-	-
	Panchmahal	<i>An. culicifacies</i>	4	2.46	3.08	1.41	1.04	-	-
Madhya Pradesh	Sidhi	<i>An. culicifacies</i>	9.47	3.94	3.88	-	0.5	0.6	3.11
Haryana	Nuh	<i>An. culicifacies</i>	15.95	10.96	0.41	-	-	4.2	-
	Palwal	<i>An. culicifacies</i>	15.46	36.03	1.02	-	-	3.3	-
Maharashtra	Gadchiroli	<i>An. culicifacies</i>	12.1	3.7	0.06	0.06	0	0	1.5
Karnataka	Kalaburagi	<i>An. culicifacies</i>	5.86	2.3	0.48	3.71	12	-	12
Odisha	Kalahandi	<i>An. culicifacies</i>	-	-	1.1	4.5	-	-	-
	Angul	<i>An. culicifacies</i>	-	-	1	2	-	-	-
Gujarat	Kheda	<i>An. stephensi</i>	0.17	0.06	0	0	0	-	-
	Panchmahal	<i>An. stephensi</i>	0.025	0.016	0	0	0.125	-	-
Haryana	Nuh	<i>An. stephensi</i>	8	20.44	2.92	-	-	4.5	-
	Palwal	<i>An. stephensi</i>	21.39	45.03	8.5	-	-	4	-
Madhya Pradesh	Sidhi	<i>An. fluviatilis</i>	0.74	0.33	0.49	-	0.01	0.01	0.24
Karnataka	Kalaburagi	<i>An. fluviatilis</i>	0.06	0.06	2.96	18.6	22.4	-	22.7
Odisha	Kalahandi	<i>An. fluviatilis</i>	-	-	2.3	3.8	-	-	-
	Angul	<i>An. fluviatilis</i>	-	-	0.5	1.2	-	-	-

The symbol – indicates ‘Not done’

* Man hour density (MHD): the number of mosquitoes collected by one person in one hour

Density and proportion of indoor resting *Anopheline* vectors

Madhya Pradesh (Sidhi district): *Anopheles culicifacies* and *An. fluviatilis* vectors were prevalent with a MHD of 9.47 (62%) and 0.47 (4%), respectively (Table 1). In addition, 13 more *Anopheles* species were collected, including *An. annularis*, a recognized secondary vector. Majority of *An. culicifacies* (92.3%) and

An. fluviatilis (81%) were collected from cattle sheds. July and August recorded the highest density of anopheline including the two vector species, whereas May and June had the lowest (figure 3).

Gujarat (Kheda and Panchmahal districts): Three vector species, viz. *An. culicifacies*, *An. stephensi*, and *An. fluviatilis* were collected. *Anopheles culicifacies* was dominant in Kheda and Panchmahal, with a MHD of 3.15 and 4.0, respectively. The MHD of *An. stephensi* and *An. fluviatilis* was lesser than 0.2. In addition, three other *Anopheles* species, *An. subpictus*, *An. vagus* and *An. annularis* were collected. *Anopheles culicifacies* density was greater in cattle sheds in Panchmahal than in Kheda, although overall, anopheline density was higher in human dwellings in Kheda than in Panchmahal. Two peaks of *An. culicifacies* densities were observed in Gujarat, i.e. February-April, and July-August.

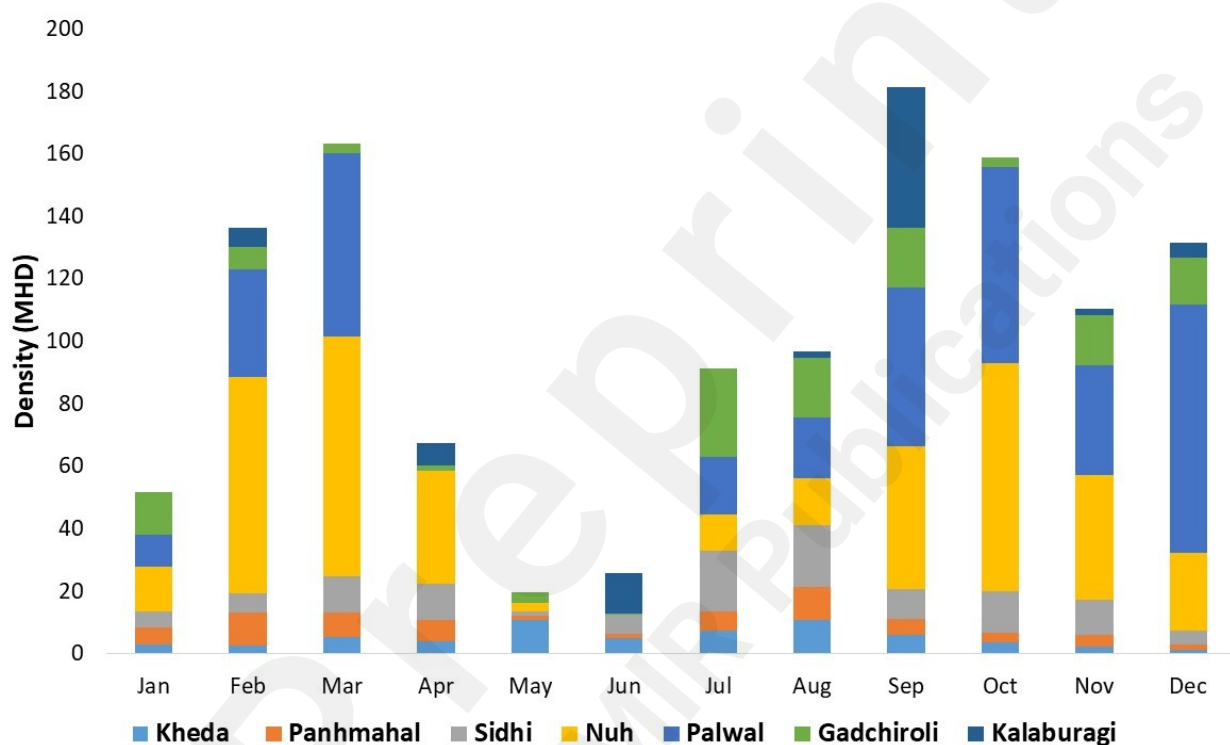


Figure 3: Monthly density of *An. culicifacies* collected from selected districts of India

Karnataka (Kalaburagi district): *Anopheles culicifacies*, *An. stephensi*, and *An. fluviatilis* were the three vector species recorded in this area, while the other *Anopheles* species viz., *An. annularis*, *An. vagus*, *An. nigerrimus*, *An. tessellatus*, and *An. barbirostris* were also prevalent. The MHD of *An. culicifacies* in human dwellings ranged from 0 to 69, whereas in cattle sheds the MHD ranged from 1 to 54. *An. fluviatilis* MHD ranged from 0 to 1 in human dwellings and 0 to 2 in cattle sheds. *An. culicifacies* density was the highest between June and September, whereas the density of *An. fluviatilis* was the highest during November to December.

Maharashtra (Gadchiroli district): *Anopheles culicifacies* was the predominant species, with *An. fluviatilis* and *An. stephensi* being collected in negligible numbers. *Anopheles subpictus*, *An. annularis*, *An. vagus*, *An. nigerrimus*, *An. pallidus*, *An. splendidus*, and *An. barbirostris* were among the other collected anopheles species. *Anopheles culicifacies* MHD was 12.1 (ranged from 11.5 to 12.7), with a substantially more significant percentage in cattle sheds than in human dwellings. *Anopheles subpictus* and *An. culicifacies* accounted for more than 95% of all anopheline, and both were perennial species, with the highest density in August.

Haryana (Nuh and Palwal districts): *Anopheles culicifacies* and *Anopheles stephensi* were collected from cattle sheds. Density of these vector species was generally low but increased considerably during post-monsoon and onset of winter, with a MHD ranging between 8 and 21. Among the other anopheline, *An. subpictus* was found in high densities, followed by *An. annularis* and *An. pulcherrimus*. The monthly MHDs of *An. culicifacies* and *An. stephensi* showed high variations in different seasons. Two peaks of densities of *An. culicifacies* were observed, i.e. in March-April and September-October. *Anopheles stephensi* showed first peak in January-April and a more prominent peak during September-December. The peak densities of *An. culicifacies* were in March (48.5) and October (29.5).

Tripura (Dhalai and South Tripura districts) and Meghalaya (West Garo Hills and South Garo Hills districts): *Anopheles minimus* and *An. baimaii*, the primary vectors in the states, were collected in the study districts of Tripura and Meghalaya. *An. minimus* was collected from 9 of the 16 selected villages but their densities were low, particularly in the state of Meghalaya. Besides the primary vectors, *An. annularis*, *An. vagus*, *An. varuna* and *An. jeyporiensis* were the other anopheline collected. In Tripura, *Anopheles* species density was higher in August, while in Meghalaya it was in September.

Odisha (Angul and Kalahandi districts): The proportion of *An. culicifacies* and *An. fluviatilis* was higher in Kalahandi than in Angul, and an increased density of *An. culicifacies* was observed in both the districts. During the study period, 15 and 18 different *Anopheles* species were collected in Kalahandi and Angul, respectively. *An. fluviatilis* density was significantly higher during winter than during rainy and summer seasons in both the districts. In contrast, the densities of *An. culicifacies* were considerably higher during rainy than post-monsoon and winter seasons.

Anopheline density from spray sheet collections

The districts of Palwal and Nuh in Haryana had the highest density of *An. culicifacies*, with 36.0

and 10.9 mosquitoes captured per room respectively. The per-room density of *An. culicifacies* in the remaining districts (Sidhi, Kheda, Panchmahal, Gadchiroli and Kalaburagi) ranged from 1.9 to 3.9. Similarly, collections from Palwal (45.0) and Nuh (20.4) districts showed high densities of *An. stephensi*, while in Kheda (0.02) and Panchmahal (0.06) districts in Gujarat its density was low. *Anopheles fluviatilis* was collected from Sidhi (Madhya Pradesh) and Kalaburagi (Karnataka) districts with a per-room density of 0.33 and 0.06, respectively.

Anopheline density from light trap catches

Density of vectors recorded from light trap collections, i.e. number per trap- night, both indoors and outdoors are given in Table 1. *Anopheles culicifacies* density indoors and outdoors was in the range of 0.4-3.9 mosquitoes per trap- night except in Gadchiroli where the density was negligible. *Anopheles stephensi* was collected only from light traps set outdoors in Palwal (8.5 mosquitoes/ per trap- night) and Nuh (2.9 mosquitoes per trap- night) districts. *Anopheles fluviatilis* was collected from both indoor and outdoor traps in Kalaburagi, Kalahandi and Angul and only from indoor traps in Sidhi. In all these districts, the density of *An. fluviatilis* was below 3.8 with the exception of Kalaburagi, where it was 18.6 from indoor trap collections..

Abdominal condition of vector species in different collections

Madhya Pradesh (Sidhi district): In Kusumu CHC, in indoor human dwelling collections (hand catch and pyrethrum spray collections), the proportion of semigravids (SG) plus gravid (G) of *An. culicifacies* was 32.8% and in Sumaria CHC, it was 31.16%, while in the two CHCs, the proportion of unfeds (UF) plus fullfeds (FF) respectively was 65.75% and 69.07%.

Gujarat (Kheda and Panchmahal districts): In Panchmahal, in March, May and June the proportion of UF+FF was higher while in all the remaining months SG+G proportion was higher ranging from 51% to hundred. Similarly, in Kheda district, the proportion of SG+G category was similar to Panchmahal except in January, October and December. With a few monthly variations the data suggest a predominantly endophilic behavior of *An. culicifacies* in the two districts.

Karnataka (Kalaburagi district): In Kalaburagi district in Karnataka, the proportion of SG+G in indoor human dwelling collections in all three villages, Laxmipurwadi (60.4%), Shankerapurwadi

(81.9%) and Muglegaon (65%), was higher than UF+FF suggesting that *An. culicifacies* continues to be endophilic. Similarly, the proportion of SG+G of *An. fluviatilis* was significantly higher than UF+FF in village Lashmipuriwadi also indicating its endophilic behavior. From the collection of other villages, no conclusion can be drawn as only a few specimens of *An. fluviatilis* were collected from Muglegaon village and in Shankerpurwadi, collection was nil.

Maharashtra (Gadchiroli district): In Gadchiroli, in human dwellings the proportion of SG+G of *An. culicifacies* (from resting and pyrethrum spray collections) was 57.6% in Ahiri CHC and 46.1% in Dhanora CHC indicating its endophilic behavior in the district without much difference between two CHCs.

Blood meal preferences:

Human blood index (HBI) of *An. culicifacies* recorded in different study sites is shown in Figure 4.

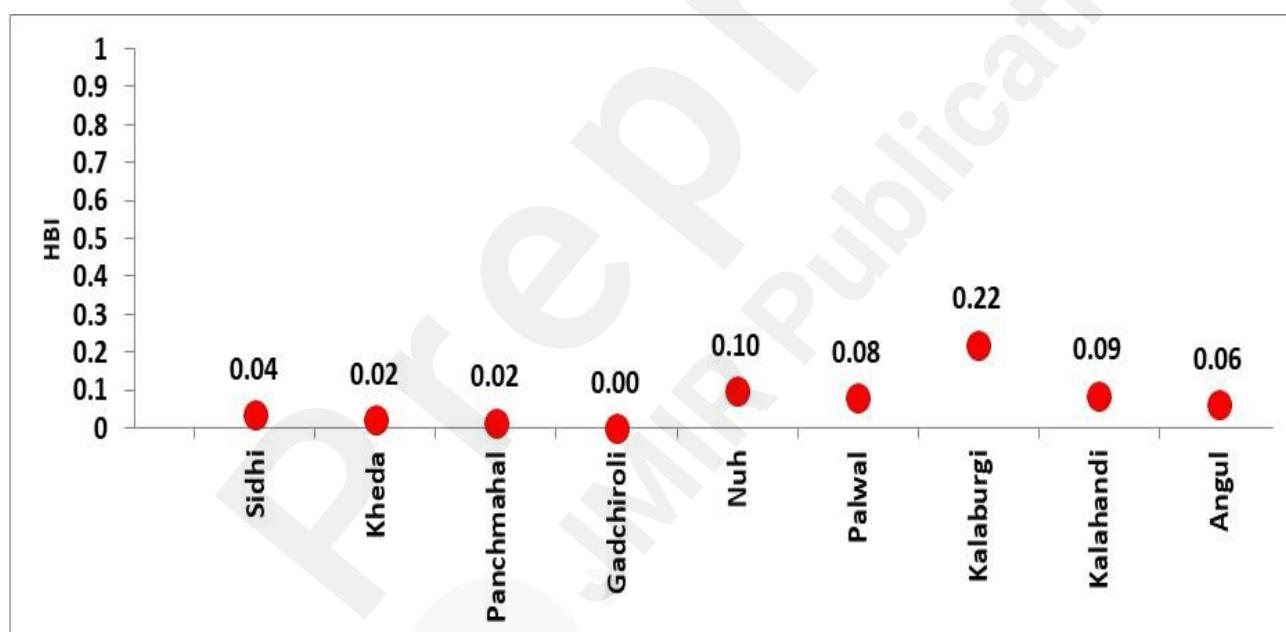


Figure 4: Human blood index (HBI) of *An. culicifacies*

Madhya Pradesh (Sidhi district): The HBI of *An. culicifacies* was 0.03 and of *An. fluviatilis* was 0.09.

Gujarat (Kheda and Panchmahal districts): The HBI of *An. culicifacies* in the canal irrigated area of Kheda district was 0.02, whereas, in the riverine area, none contained human blood. In Panchmahal district, HBI was 0.016 in canal irrigated area and 0.01 in riverine area.

Karnataka (Kalaburagi district): The HBI of *An. culicifacies* and *An. fluviatilis* was 0.22 and 0.06, respectively.

Maharashtra (Gadchiroli district): The HBI of *An. culicifacies* was 0.10 in Gadchiroli district of

Maharashtra.

Haryana (Nuh and Palwal districts): The HBI of *An. culicifacies* was low, 0.09 in Nuh and 0.07 in Palwal. Similarly, the HBI of *An. stephensi* was also low in the two districts, 0.01 and 0.004, respectively.

Tripura (Dhalai and South Tripura districts) and Meghalaya (West Garo Hills and South Garo Hills districts): The blood meal analysis showed a higher HBI of two vector species; *An. minimus*- 0.76 – 0.78 and *An. baimaii*- 0.85, indicating their higher anthropophagic behavior in northeast India. The HBI of *An. jeyporiensis* was also found to be higher (0.66-0.75) there.

Odisha (Angul and Kalahandi districts): The HBI of *An. fluviatilis* was higher in both the districts indicating the species was primarily an anthropophagic one. The HBI observed in Kalahandi was 0.52, and in Angul, it was 0.32. In contrast, *An. culicifacies* was found to be primarily a zoophagic mosquito as the observed HBI was 0.086 and 0.062, respectively, in the two districts.

Sibling species composition:

The proportion of *An. culicifacies* sibling species B, C, and E was 23%, 44.4%, and 33%, respectively in Sidhi district of Madhya Pradesh (Figure 5). In Kheda district of Gujarat, sibling species A/D was 12%, and B/C/E was 82%, while in Panchmahal district of same state, the corresponding proportion was 8% and 91%, indicating the predominance of sibling species B/C/E over A/D in both the districts. In Kalaburagi district of Karnataka, *An. culicifacies* was mostly comprised of species A (57.2%) and species B (42.8%). The proportion of three sibling species S, T, and U of *An. fluviatilis* was found to be 4.6, 94.2, and 1.2%, respectively in this state.

A total of 1050 specimens of *An. culicifacies* were analyzed in Maharashtra (Gadchiroli district) for the sibling species composition, sibling sp. C was predominant (43.9%), followed by species E (25%). In Haryana (Nuh and Palwal districts) *An. culicifacies* species A which is an efficient vector was predominantly constituting 99.4% in Nuh and 99.4% in Palwal.



Figure 5: Sibling species composition of *An. culicifacies*

Vector infection

Madhya Pradesh (Sidhi district): A total of 1038 *An. culicifacies* and 27 *An. fluviatilis* were screened for sporozoite positivity using PCR assay, and none was found positive (Figure 6).

Gujarat (Kheda and Panchmahal districts): In Kheda district, 916 *An. culicifacies* were analyzed and 12 were detected with malaria parasites (7 *Pf* positive, 5 *Pv*) with a sporozoite positivity rate of 1.31%. In Panchmahal district, four *An. culicifacies* (3 *Pf* positive, 1 *Pv*) were found positive (n= 900) with a sporozoite positivity rate of 0.44%.

Karnataka (Kalaburagi district): In total, 1305 *An. culicifacies* and 691 *An. fluviatilis* were screened and the sporozoite rate was 0.08% and 0.14%, respectively.

Maharashtra (Gadchiroli district): Out of 3029 *An. culicifacies* tested, there were only two positives showing a sporozoite positivity rate of 0.06% .

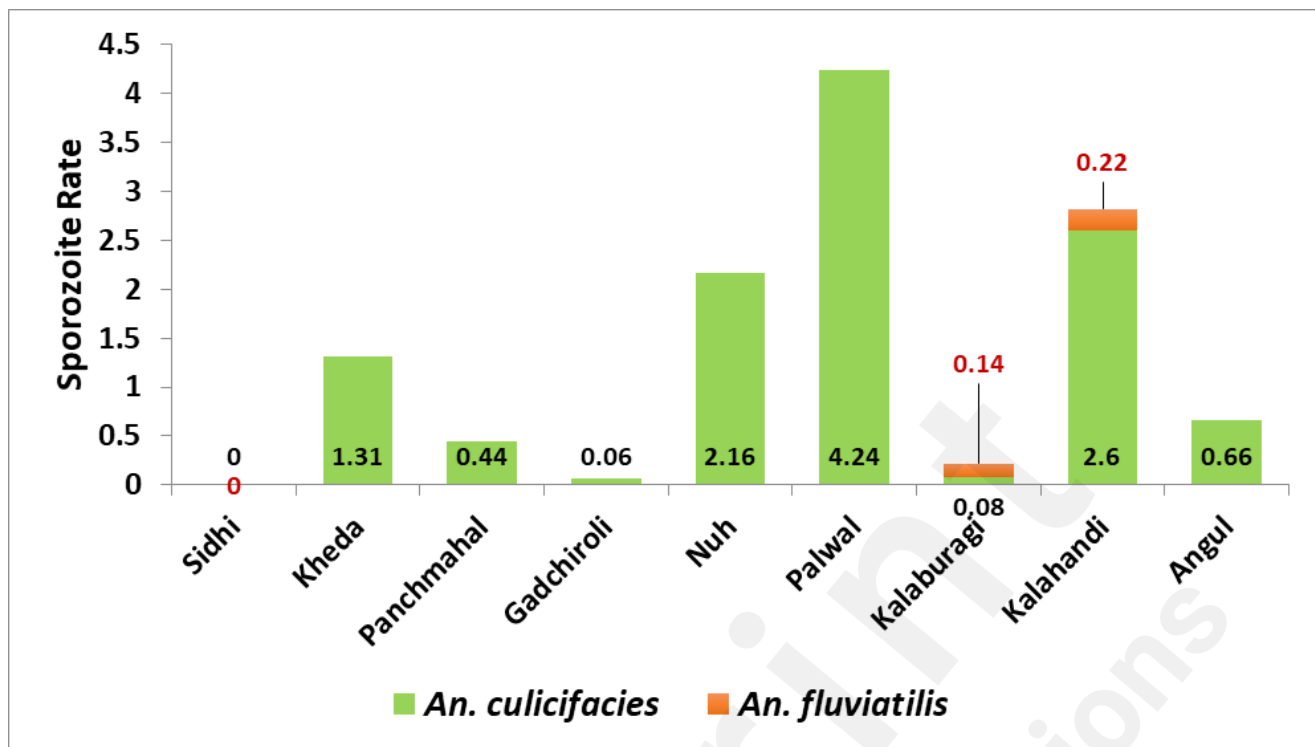


Figure 6: Sporozoite positivity rate (%) of *An. culicifacies* and *An. fluviatilis*

Haryana (Nuh and Palwal districts): In Nuh, 831 *An. culicifacies* were analyzed for *Plasmodium* infection and 18 were positive with a sporozoite positivity rate of 2.16%; higher positivity for *P. falciparum* (11) than *P. vivax* (7). Whereas in Palwal district, nine *An. culicifacies* were detected positive (7 *Pf* positive, 2 *Pv*) out of 212 screened and the sporozoite positivity rate was 4.24%.

Tripura (Dhalai and South Tripura districts) and Meghalaya (West Garo Hills and South Garo Hills districts): None of the primary (*An. minimus* and *An. baimaii*) or secondary vectors (*An. nivipes*, *An. philippinensis* and *An. annularis*) was found infected with malaria parasites.

Odisha (Angul and Kalahandi districts): The sporozoite rate of *An. fluviatilis* was 0.22% in Kalahandi. The sporozoite rate of *An. culicifacies* was 2.6% and 0.66%, in Kalahandi and Angul districts, respectively

Entomological inoculation rate (EIR)

Entomological inoculation rate of *An. culicifacies* in Kheda and Panchmahal districts of Gujarat was 3.09 and 0.47, respectively followed by 0.25 in Kalaburagi district of Karnataka. Entomological inoculation rate could not be calculated for other sites.

Vector susceptibility to insecticides

Susceptibility of *An. culicifacies*, *An. stephensi*, *An. fluviatilis*, *An. minimus* and *An. baimaii* collected from

the different study sites was tested against DDT, malathion and synthetic pyrethroids, viz. deltamethrin, cyfluthrin, and alphacypermethrin approved by the national malaria control programme. The susceptibility/resistance status is pictorially depicted in Figures 7 and 8.

DDT (4%): *Anopheles culicifacies* was tested against DDT in all districts except Kheda and Panchmahal districts in Gujarat state, and this species was resistant to DDT in all the tested districts (<39% mortality) (Figure 7). *Anopheles baimaii* and *An. minimus* were susceptible to DDT in the study districts of Meghalaya and Tripura. *Anopheles stephensi* was resistant to DDT in both the districts of Haryana, and *An. fluviatilis* showed possible resistance to DDT in the two districts of Odisha (Figure 7).

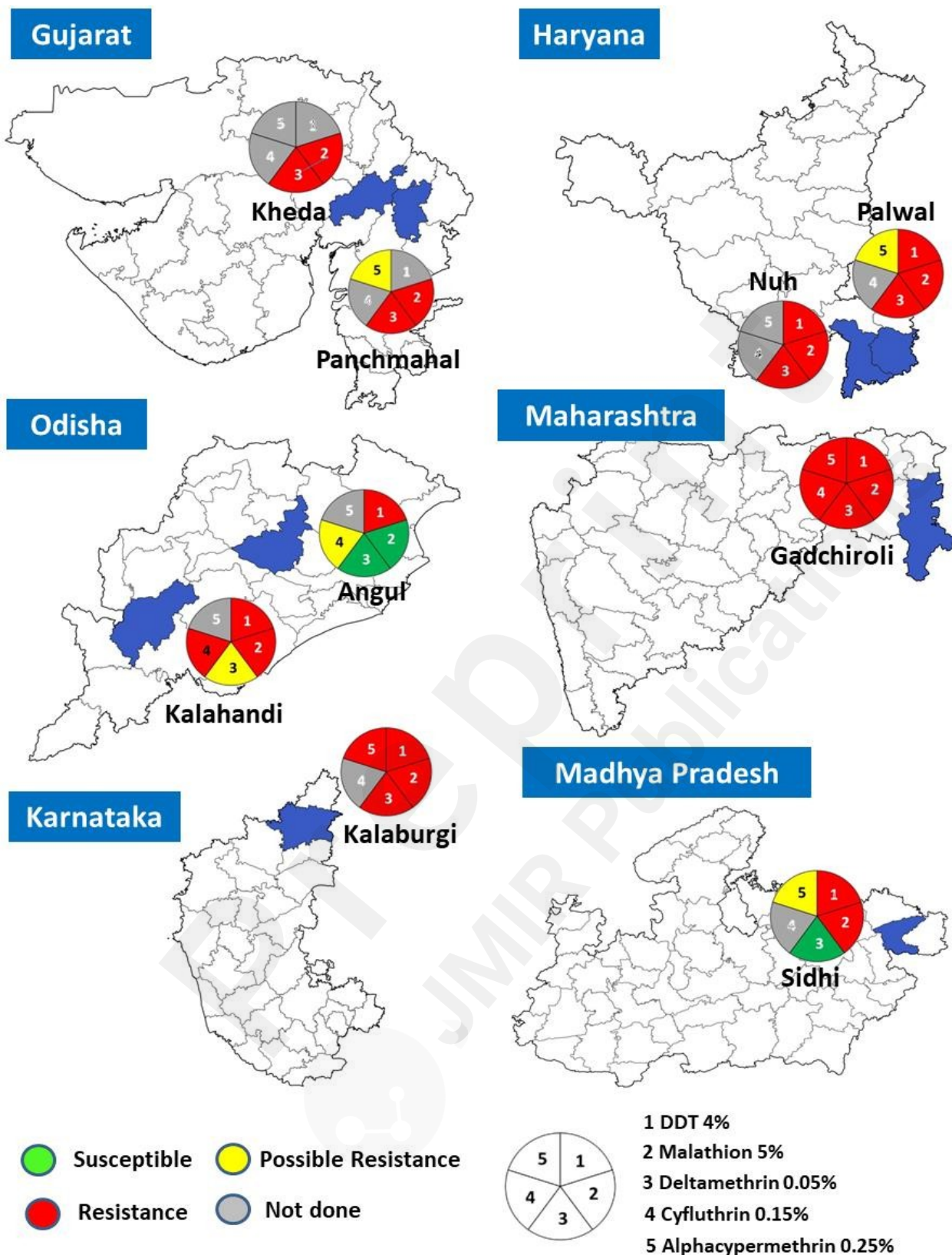


Figure 7: Insecticide susceptibility status of *An. culicifacies* in India (* ≥ 98 -100% mortality: susceptible; 90-97% mortality: possible resistance; <90% mortality: resistant)

Malathion (5%): *Anopheles culicifacies* was resistant to malathion (49-78% mortality) in all districts except Angul in Odisha, where it was susceptible. *Anopheles baimaii* was susceptible to malathion in all study

districts of the northeast except Dhalai, where test could not be performed. *Anopheles stephensi* was resistant to malathion in both the districts of Haryana, whereas *An. fluviatilis* was susceptible to malathion in the two districts of Odisha (Figure 8).

Deltamethrin (0.05%): *Anopheles culicifacies* was resistant to the synthetic pyrethroids, deltamethrin, in Maharashtra, Gujarat, Haryana, and Karnataka study sites but susceptible in Sidhi (Madhya Pradesh) and Angul (Odisha) districts and showed possible resistance (92.5% mortality) in Kalahandi district of Odisha. *Anopheles stephensi* was resistant to deltamethrin in both the districts of Haryana, whereas *An. fluviatilis* was susceptible to this insecticide in the two districts of Odisha (Figure 8).

Cyfluthrin (0.15%): Susceptibility tests against cyfluthrin, another synthetic pyrethroid (SP), were conducted in Odisha and Maharashtra only. *An. culicifacies* was resistant to cyfluthrin in Gadchiroli district of Maharashtra and Kalahandi district of Odisha, whereas 'possible resistance' was observed in the Angul district of Odisha. *Anopheles fluviatilis* was susceptible to cyfluthrin in both the districts of Odisha.

Alpha-cypermethrin (0.25%): Susceptibility to alpha-cypermethrin, yet another synthetic pyrethroid, was tested only for *An. culicifacies* against this synthetic pyrethroid, the mortality of *An. culicifacies* was 95 - 96.7% in Panchmahal, Palwal, and Sidhi districts, indicating a possible resistance, whereas in Gadchiroli (88.1% mortality) and Kalaburagi (16% mortality) districts, it was resistant.

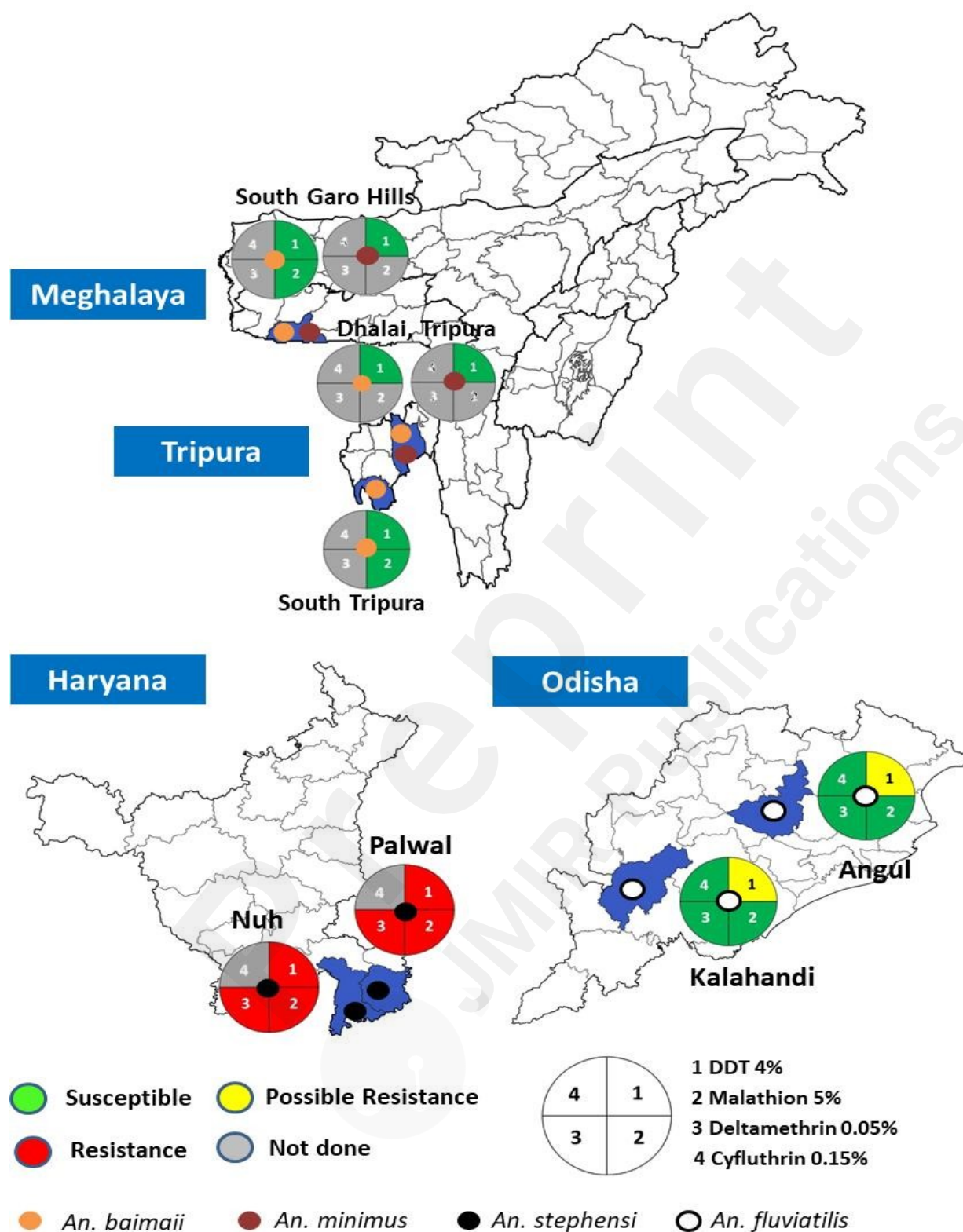


Figure 8: Insecticide susceptibility status of *An. stephensi*, *An. fluviatilis*, *An. minimus* and *An. baimaii* in India (* $\geq 98-100\%$ mortality: susceptible; $90-97\%$ mortality: possible resistance; $< 90\%$ mortality: resistant)

Discussion

The current study was carried out in 13 districts from eight different states of India, representing most of the eco-epidemiological zones including tribal, hilly, plain, forested region of the country. This is the first nationwide study covering almost all parts of the country, east, west, north, south, central and northeast India. Updated knowledge on bionomics is essential to strengthen the vector control activities and to measure the impact of such control measures [22]. Thus, the data generated during the current study are critical and more relevant to the country's malaria elimination goal. Regular surveillance of malaria vectors is essential to detect any change in their distribution, behavior and insecticide susceptibility status to reach the malaria elimination target. The results from the current study could augment to the understanding of these issues.

Mosquito collections confirmed the presence of *An. culicifacies* in all the study districts except in districts of Assam and Tripura states. The density of *An. culicifacies* obtained indoors by hand catch and pyrethrum spray sheet collection suggested this species could be endophilic in most of the districts. The proportions of gravid and semi gravid mosquitoes were lower (31-33%) in Madhya Pradesh indicating that *An. culicifacies* was not completely endophilic and some proportions might be resting outdoors also. It might be an indication of the change in the resting behavior of *An. culicifacies* in Madhya Pradesh but needs larger studies to reaffirm this plausible change. This change in resting behavior might be due to the use of LLINs inside the houses. Such change in resting behavior of *An. fluviatilis* has been reported from Odisha, where it was found predominantly in mixed dwellings with high anthropogenic nature [23]. Similarly, in Kenya and Tanzania, a change in resting behavior of *An. gambiae* s.s. and *An. funestus* was observed from endophilly to exophilly after the introduction of LLINs [24]. For *An. stephensi*, indoor resting density was higher than outdoors, indicating possibly an endophilic behaviour also.

Anopheles culicifacies, which is one of the major malaria vectors in India, exist as a complex of five sibling species. Up to ~70% of malaria in India is transmitted by *An. culicifacies* [3]. Therefore, implementation of effective insecticide-based vector control interventions is important in the context of malaria elimination. *Anopheles culicifacies* sibling species have distinct distribution with varying proportions of each species in different parts of the country [3] which has been observed in this study as well. The importance of knowing the distribution and proportions of the species is important as species A, C, D and E are vectors and species B is a poor or non-vector [25-28]. They also vary in the development of resistance to different insecticides [28-30]. The results of the study showed that species A was predominant in Nuh, Palwal, and Kalaburagi districts, while species C was predominant in Sidhi and Gadchiroli districts. Species B was present in very low densities in Haryana while in Gujarat and Karnataka states, its density was higher. The current study provides an overview of the up-to-date distribution of sibling species in different different eco-epidemiological settings of the country. Previously, distribution pattern showed the omnipresence of species B wherever *An. culicifacies* was encountered. Species A was predominant in the northern part of the country, and C was predominant in the western and eastern regions, while species D was found in sympatric association with species A and B in the north-western region [31].

Resistance to DDT in *An. culicifacies* was detected in all the study sites i.e. almost all over the country, while to malathion and deltamethrin this species has developed resistance in most of the study districts. Deltamethrin resistance in *An. culicifacies* was reported earlier from Gadchiroli district of Maharashtra [32-33] and Kalahandi and four more districts of Odisha [34] and in Nuh (Haryana), it showed possible resistance [35]. A multi-district study conducted in 2009 on the susceptibility of *An. culicifacies* to DDT, malathion, and deltamethrin in multiple districts of Madhya Pradesh reported its resistance to deltamethrin in Mandla and Dindori districts, possible resistance in Balaghat, Betul, Chhindwara and Jhabua, Sidhi and Shadol, districts, but susceptible in Guna district [36]. The national vector control program in India mostly relies on insecticide-based indoor residual spraying (IRS) and long lasting insecticidal nets (LLINs). Therefore, resistance development in malaria vectors to synthetic pyrethroids is of great concern due to its extensive use in IRS and LLIN.

In the 1950s, DDT was used for IRS for vector control. But due to selection pressure, *An. culicifacies* was found resistant to this insecticide in 1959 [37]. Later, malathion was introduced for vector control in 1969. But sooner, resistance to malathion was reported in Gujarat in 1973 [38]. Further, reports of resistance to malathion were received from other states such as Andhra Pradesh, Orissa, Madhya Pradesh, Chhattisgarh, and Jharkhand [39]. In 1996, synthetic pyrethroids were introduced for IRS in India due to their safe, excito repellent, and knockdown properties. Six years after its first use, the first report of deltamethrin resistance in *An. culicifacies* came from Gujarat in 2002 [40]. Triple resistance (to DDT, malathion and deltamethrin) in *An. culicifacies* was reported from 31 districts in India [41] The results of the current study conform to the earlier findings reported.

Another malaria vector *An. stephensi* was resistant to deltamethrin, DDT and malathion in Nuh and Palwal districts of Haryana. At present, as a strategy for vector control in India, IRS is not used for the control of *An. stephensi*, except in Rajasthan, where it has been reported to be the primary vector of malaria [41] However, this species was reported to be double resistant, i.e. to DDT and malathion in seven districts and malathion and deltamethrin in one district [41]. In the current study, probably for the first time in the country, triple resistance (to DDT, malathion and deltamethrin) in *An. stephensi* was reported.

Anopheles fluviatilis was susceptible to malathion and synthetic pyrethroids, but showed possible resistance to DDT in Kalahandi and Angul districts of Odisha. This vector species was susceptible to deltamethrin and malathion in most parts of the country except in Gadchiroli (Maharashtra) where a possible resistance was reported [33]. In Mayurbhanj district of Odisha, a possible resistance to malathion was reported in *An. fluviatilis*. It was found susceptible to DDT in Odisha but resistant in Jharkhand and Chhattisgarh [41-42]. In the current study, *An. minimus* and *An. baimaii*, the two primary malaria vectors in the north-east, were susceptible against DDT and malathion. Another study by Raghavendra et al. (2017) reported that these two vector species were susceptible to DDT, malathion and deltamethrin, whereas, in the eastern state of Odisha, there was only one report of

resistance in *An. minimus* to DDT [41].

The WHO has suggested three main strategies, viz. rotation, mosaics, and mixtures, for the insecticide resistance management (IRM) in malaria vectors [43]. The main objective of the IRM was to prevent the emergence of resistance in susceptible populations, delaying insecticide resistance evolution, or reversing it to a level compatible with the efficient use of insecticides for vector control [44]. Information on insecticide resistance among malaria vectors and the underlying mechanism(s) of resistance in different ecological settings are necessary for formulating rational strategies for insecticide use and resistance management [45]. Therefore, further research studies on frequency and intensity of insecticide resistance and its underlying mechanisms among malaria vectors are crucial to suggest an appropriate resistance management strategy. Use of synergist piperonyl butoxide (PBO) based LLINs effectively reduced the insecticide-resistant (synthetic pyrethroid)-vector populations [46]. The ICMR-Vector Control Research Center had evaluated the efficacy of a PBO-alphacypermethrin incorporated long-lasting insecticidal net in experimental huts in Odisha and found better than the reference net in terms of mosquito mortality in the huts and cone bioassays [47]. Another possibility of managing the insecticide-resistant populations would be the use of attractive toxic sugar baits supplemented with LLIN use, although it is still under development and evaluation stage [48-50]. All these suggested measures are of great epidemiological importance for India.

We screened more than 8000 anopheline mosquitoes for *Plasmodium* parasite infection but only two species, *An. culicifacies* and *An. fluviatilis* were found positive. One of the earlier studies carried out in the Nuh district in 2015-2016 reported a sporozoite positivity rate of 0.26% [51], lower than that reported in the current study (2.1%). The observed variability in the sporozoite rate might be due to the differences in sample size, usage of LLINs and malaria endemicity of the study sites. Moreover, the high positivity rate of *An. culicifacies* in Nuh and Palwal districts might be due to the predominant proportion of sibling species A (>99%) in the area, which is an established malaria vector in malaria-endemic regions of India [51]. In an earlier study conducted in eight southern districts of Odisha, sporozoite positive *An. culicifacies* was reported only from the Kandhamal district (1.5% infectivity rate), and none of the mosquito pools was positive in the rest of the districts, including Kalahandi [52]. In the case of *An. stephensi*, none was found positive for *Plasmodium* infection in Nuh and Palwal districts; might be due to the abundance of *An. stephensi* mysorensis form, which, being primarily a zoophagic in nature, is not a competent vector [3]. An HBI of 0-0.01 observed in this study confirms the zoophilic behavior of *An. stephensi* mysorensis form in the area.

Limitations

The current study was countrywide surveillance of malaria vectors but we could collect all primary vectors of malaria in India except *An. sundaicus*, which is the only vector in Andaman and Nicobar Islands, India. This is the major limitation of our study. Another limitation of the current study was the unavailability of data on intensity bioassays for determination of intensity of insecticide resistance among main malaria vectors. The outcome of the intensity assays could suggest on continuation of existing insecticide vector control or change of insecticide. The third limitation of the study was lack of information on mosquito larval breeding habitats. This information might provide the more comprehensive picture of anopheline breeding habitats present in the selected study sites. The last limitation of the study was lack of data on parity status of vector mosquitoes which is a robust indicator of mosquito age.

Conclusions

The current study highlights all essential aspects of vector bionomics, such as seasonal prevalence, resting and biting behavior, insecticide resistance status, composition of sibling species and malaria transmission potential. The study clearly indicates a growing resistance development in *An. culicifacies* against synthetic pyrethroids in the country. These findings necessitate continuous monitoring of insecticide resistance for effective planning, implementation, and evaluation of malaria vector control strategies. Furthermore, as sibling species can vary in the rate of development of insecticide resistance, monitoring should be done at the sibling species level rather than at the sensu lato level. Change from endophilic nature of *An. culicifacies* to exophily observed in parts of India necessitates continuous monitoring for such behavioral changes in the vector species in the light of extensive use of LLINs in the country. The local and focal behavior of malaria is due to variation in biological characters of vector species, cultural aspects of human population, and environmental factors of a region. Therefore, further research is crucial on vector behavior to substantiate certain findings that have a bearing on malaria transmission to strengthen the control measures.

Acknowledgements

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Data Availability

The data sets generated during or analyzed in this study may be made available from the corresponding author on reasonable request.

Conflicts of Interest

None

Abbreviations

CS: Cattle Sheds

EIR: Entomological Inoculation Rate

HBI: Human Blood Index

HLD: Human Landing Density

HD: Human Dwellings

IRS: Indoor Residual Spraying

ICMR: Indian Council of Medical Research

LLIN: Long Lasting Insecticidal Nets

MHD: Man Hour Density

PSC: Pyrethrum Spray Collection

WHO: World Health Organization

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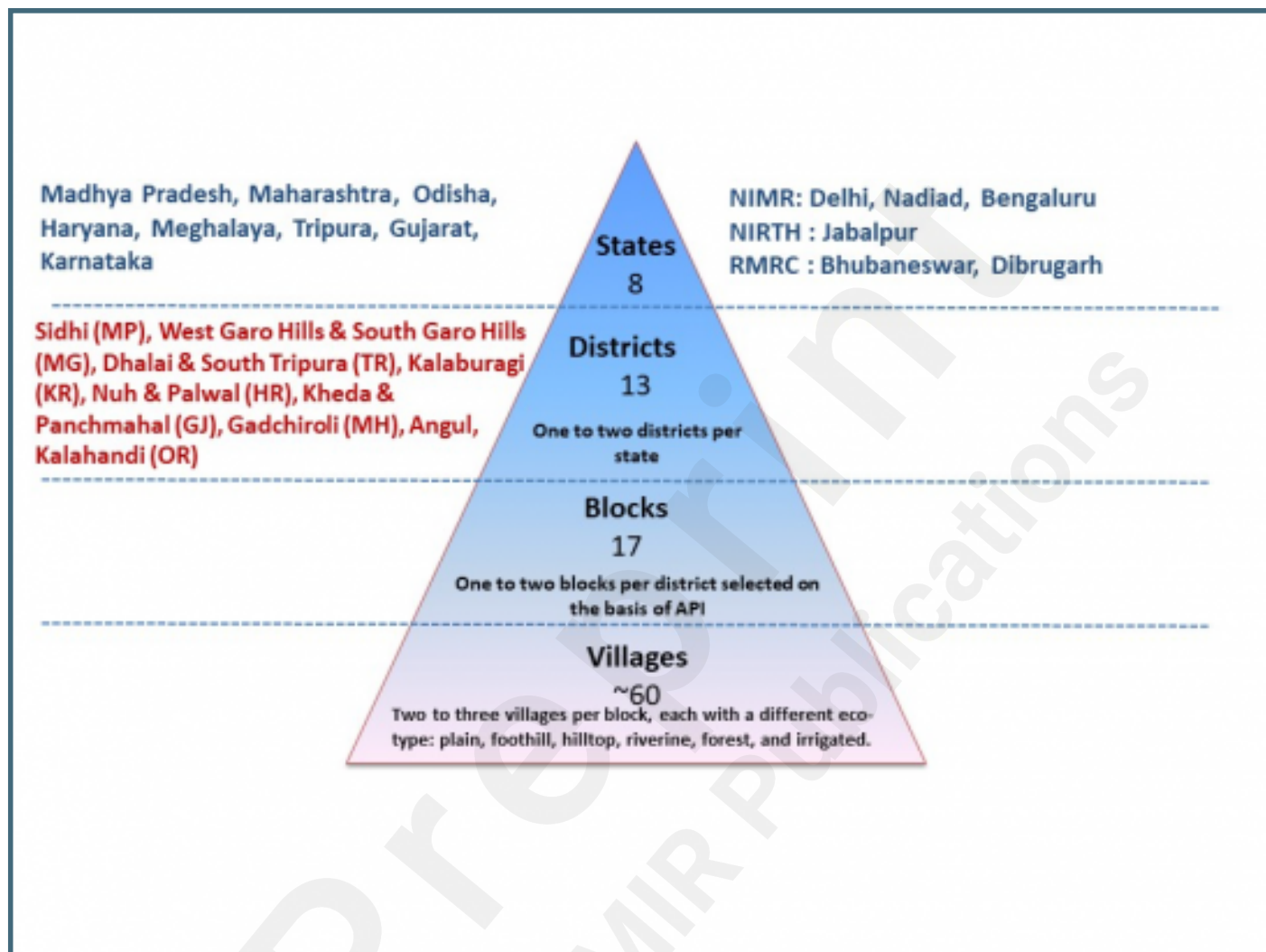
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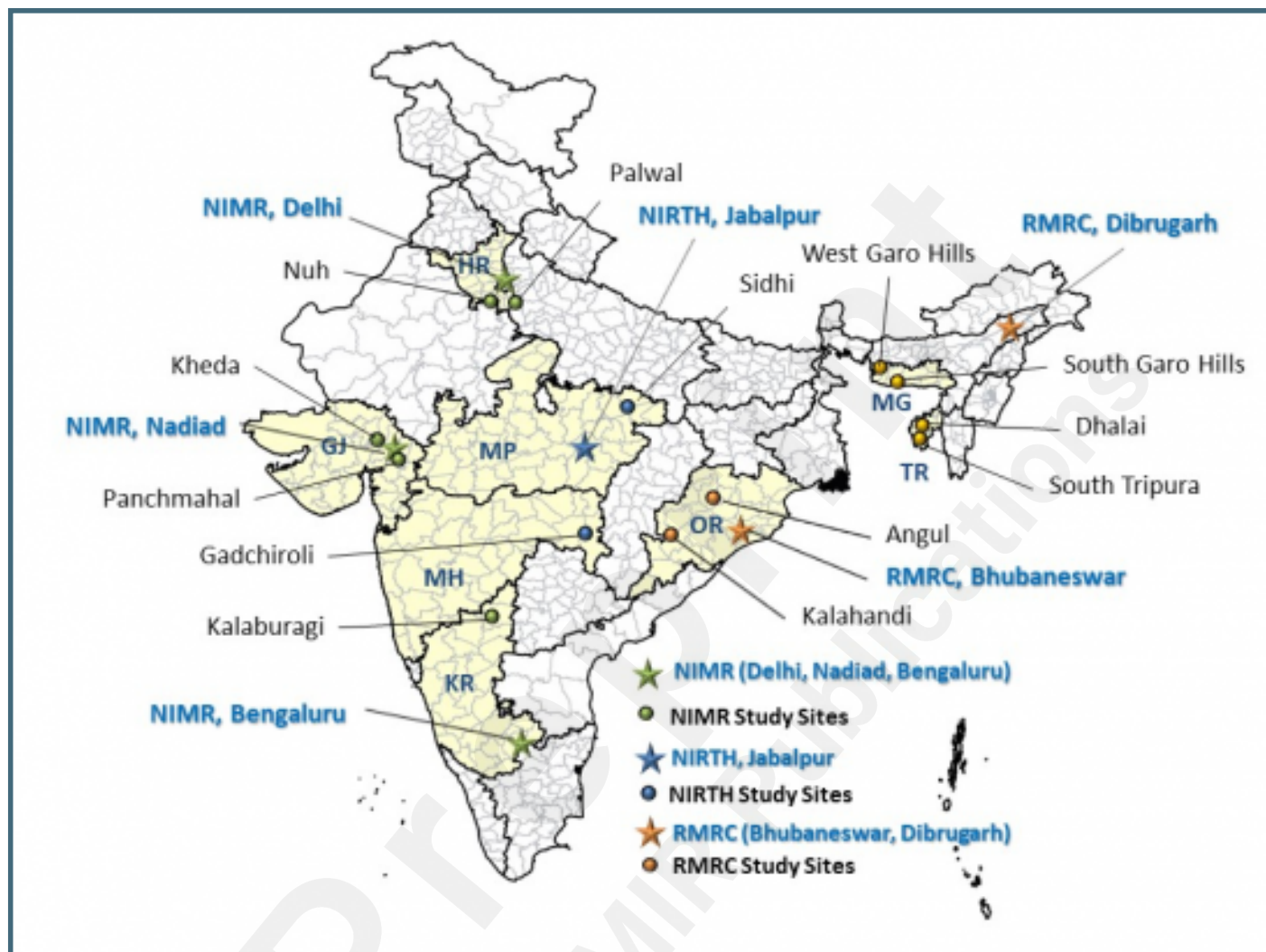
Supplementary Files

Figures

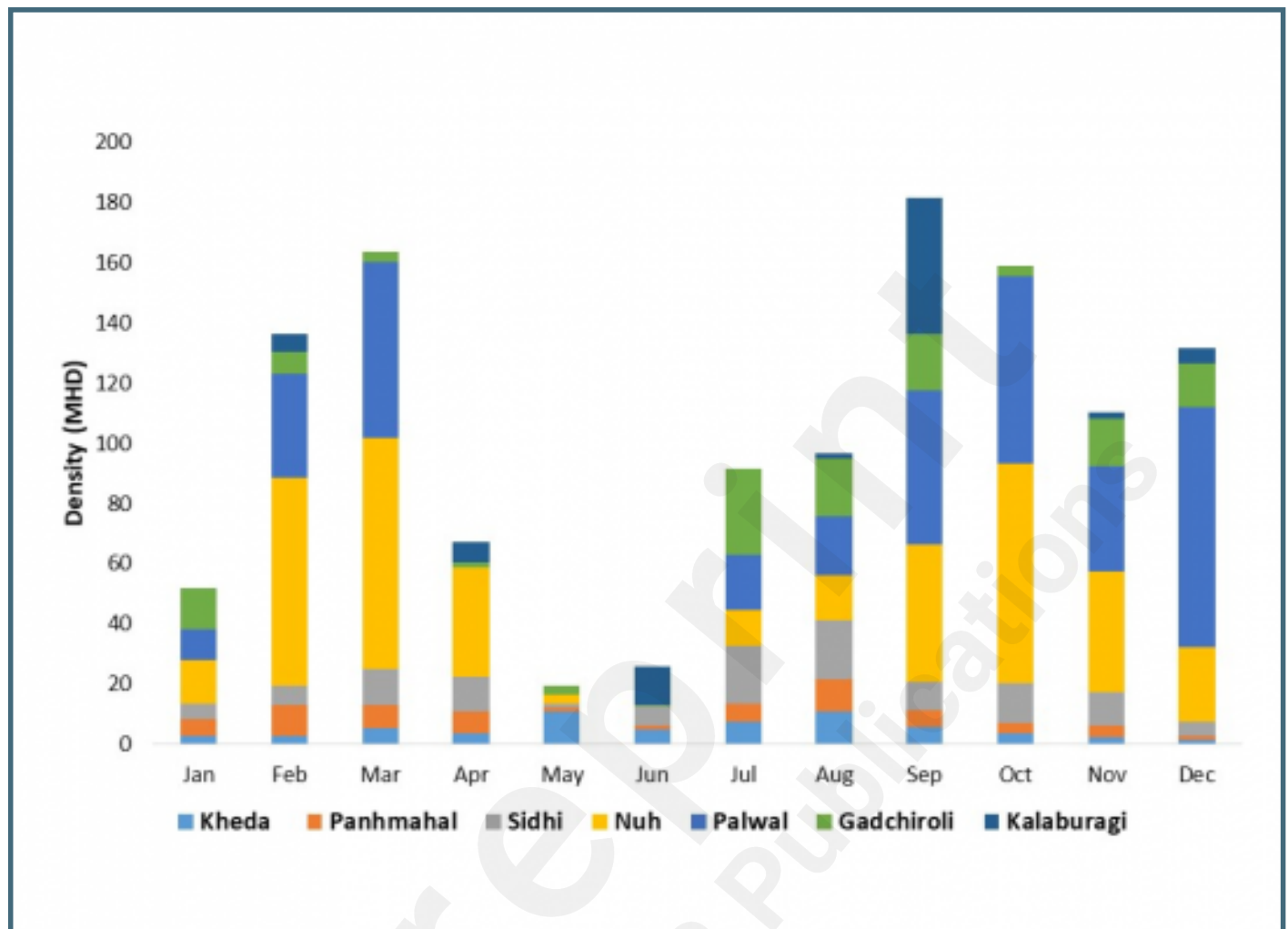
Criteria for study site selection. Abbreviation: GJ-Gujarat; HR-Haryana; KR-Karnataka; MG-Meghalaya; MP-Madhya Pradesh; MH-Maharashtra; OR=Odisha; TR-Tripura, NIMR-National Institute of Malaria Research; NIRTH-National Institute for Research on Tribal Health; RMRC-Regional Medical Research Center).



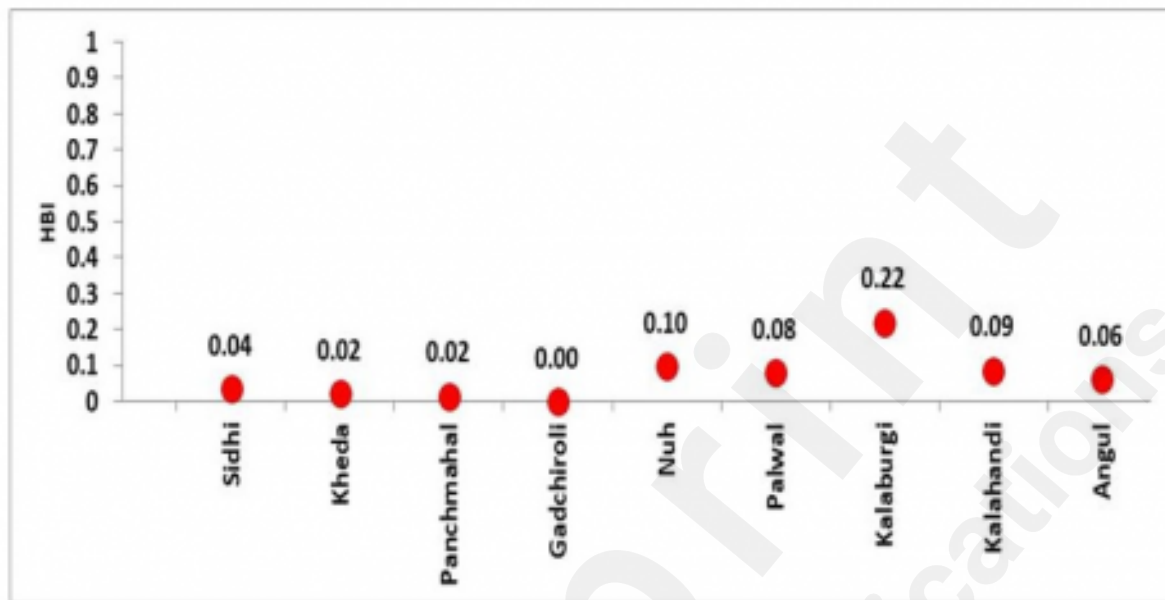
Location of participating institutes and study sites (Abbreviation: GJ-Gujarat; HR-Haryana; KR-Karnataka; MG-Meghalaya; MP-Madhya Pradesh; MH-Maharashtra; OR=Odisha; TR-Tripura; NIMR-National Institute of Malaria Research; NIRTH-National Institute for Research on Tribal Health; RMRC-Regional Medical Research Center).



Monthly density of *An. culicifacies* collected from selected districts of India.



Human blood index (HBI) of *An. culicifacies*.



Sibling species composition of *An. culicifacies*.



Sporozoite positivity rate (%) of *An. culicifacies* and *An. fluviatilis*.

