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Bionomics and Diversity Pattern of Malaria Mosquito *Anopheles minimus* in Keonjhar District of Odisha

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Abstract

Studies of sibling species, bionomics, distribution, role in malaria transmission, and identification of anopheline vector are significantly important from malaria control point of view. Control of malaria depends on certain aspects such as, response to insecticides, vectorial competence, host preference and resting behaviour. So vector bionomics have been a major area of research over many years. *An. minimus* was identified as a vector of malaria in east central region of Odisha. Keonjhar district of Odisha State, India is afflicted with high incidence of malaria since many decades. The present study was carried out for bionomics and diversity pattern of malaria mosquito *Anopheles minimus* in Keonjhar district of Odisha. *An. minimus* were collected from different ecotopes of Keonjhar district with hand catch method from both indoor and out door resting habitats, identified morphologically and members of the *Anopheles minimus* species complex was further identified at molecular form by polymer chain reaction assay (PCR). *Plasmodium falciparum* infection was determined following the dissection of the ovaries. Immatures were also collected from nearest breeding habitat such as perennial stream, well, pond and rice field. Stream is the most preferable breeding habitat of this species followed by pond, well and paddy field. The indoor per man hour density (PMDI) of *An. minimus* was found to be 6.09. The man hour density was found to increase from summer (March to June) to rainy (July to October). The peak prevalence of this species was noticed during the month of August. The adult specimens prefer to rest inside the room after blood feeding. The species is highly anthropophilic, with an overall human blood index (HBI) of 0.91.

Keywords: Bionomics; *Anopheles minimus*; Keonjhar; Sibling Species.

Introduction

Malaria is an acute parasitic illness caused by *Plasmodium falciparum* or *Plasmodium vivax* in India. It is highly endemic in many parts of India and most of the cases are from the state of Odisha [1]. Having 3.3% of 1,311 million population of the country, Odisha contributes 42.4% of the total malaria cases and 31.8 % of the total malaria deaths of the country during 2016 [2]. Of the total 30 districts of Odisha, Keonjhar is seriously affected by malaria for many decades [3,4,5,6] and malaria control has become a formidable task in this region. Several *Anopheles* species transmit malaria in India and disease epidemiology is complex due to varied biology of different species [7,8]. So the study of biology of anopheline vector is important in the epidemiology of malaria transmission and vector control operation. Most of the anophelines that are implicated in the transmission of malaria have been identified as species complex commonly known as sibling species having distinct gene pools and differ in biological characteristics. So far 30 species complexes have been described in different regions of the world [2]. Recent developments in vector biology have revealed that the vectorial capacity and competence of each sibling species is different, including their behavioral characteristics, breeding habitats, host specificity and susceptibility to malarial parasites and insecticides [8,9].

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Differences in the vectorial capacities and distribution patterns among sibling species are responsible for the wide variation in the endemicity of malaria in an area. Failure to recognize sibling species of anopheline taxa results in failure to distinguish between a vector and a nonvector which may adversely affect vector control efforts, if the vector is not correctly identified [10]. In India, *An. minimus* is regarded as one of the most predominant malaria vector species which are distributed in eastern to north eastern regions down to Odisha State. During the pre DDT era, this species was widely prevalent in sub-Himalayan foothills of Uttar Pradesh to eastern and northeastern region of India [9,10 11]. But after the introduction of DDT in National Malaria Eradication Programme (NMEP) in 1960s, this species was believed to disappear from Terai of Uttarakhand (formerly Uttar Pradesh), eastern Odisha, northeastern states and Nepal [12,13]. However, after a period of nearly 45 years of disappearance, this species reemerged in Keonjhar distrct of Odisha [14,15, 16]. It has also once again been proven as the major vector species in the foothill valley areas of eastern and northeast India [17,18,19]. Since the malaria epidemiology in Keonjhar district shows high in recent years, a study was undertaken to understand the biology and identification of sibling species of *An. minimus* in this district.

Materials and Methods

Study Area

The study was conducted during January 2007 to December 2009 in six randomly selected villages of Banspal Community Health Center (CHC) in Keonjhar district of Odisha, India. The study area is traversed by Singhbhum hill range with latitude 20°11" to 20°10"N and longitude 85°11" to 86°22" E and lies 573.4 to 601.2 m above sea level. The average annual rainfall is 132.9 mm with monthly rainfall ranging from 0 to 392.6 mm. The climate is characterised by a hot summer (March to June), a rainy (July to September) and a cool season (October to February).The minimum temperature ranges from 8°C in December to 32°C in May and maximum being 19°C in December to 42°C in May. The average relative humidity ranges from 30.6% in March to 85.45% in October. The villages under study are covered by deep forest and hilly streams. Malaria has been endemic in this district and the majority (>95 per cent) of cases are caused due to *P. falciparum*. During 2001 to 2008, malaria incidence in the district has shown an increasing trend, with annual parasite incidence (API) ranging from13.9 to 17.07 [2]. Since 1958 to 2000, DDT was used for the indoor residual spraying. From 2001 onwards, synthetic pyrethroids (lambda cylohalothrin or alpha cypermethrin) are being used for indoor residual spraying in place of DDT in 11 of the 13 PHCs of the district under the Enhanced Malaria Control Programme (EMCP) [4].

Immature Collection

The immature survey was carried out in all the available breeding habitats within one km radius of the study villages from January 2008 to December 2008. Habitats representing each type, i.e., streams, rivers, ponds, paddy fields, wells and borrow pits from each village were randomly selected and surveyed fortnightly to monitor the vector breeding and the immature of the vector species were collected. Sampling was undertaken using a standard dipper (10 cm in diameter and 300 ml capacity) in all habitats except wells and borrows pits. One dip was taken at every two meter (m) distance in streams, rivers and ponds along the edges. Two to five dips were taken from each borrow pit depending on the size. An iron bucket (20 cm top diameter, 14 cm bottom diameter and 20 cm height) was used to sample the immature in wells. Three to five samples were taken from each well, two to four from the sides and one from the centre. Successive samples were taken from wells at an interval of three to four minutes so as to allow the larvae to come to the surface and to redistribute themselves following the disturbance caused by the preceeding sampling. As rice is cultivated only during khariff season (June to December) in all the six index villages, larval survey in paddy fields was carried out from June 2008 to December 2008.A total of 18 rice plots, three from each ecotope, covering an area of approximately 4.5 hectares, was selected. A total of 100 dips each from stream and 50 dips each from paddy fields were taken in each village.

Collection of Adult Mosquitoes

Indoor resting collection was carried out with an oral aspirator during morning hours (06.00-08.00 hours) in nine fixed catching stations (six human dwellings and three cattle sheds) randomly selected in each village at monthly intervals. Outdoor resting collection was also done in 12 pit shelters spending five minutes in each village at monthly intervals. Mosquitoes were identified, and grouped according to their abdominal conditions using the keys of Christophers [20]. The ovaries of mosquitoes were dissected out to determine the parity using ovariolar dilatation method [21]. After dissection, the body parts

of individual specimen of *An. minimus* were kept in eppendorf tubes, dried for three h at 90^{0}C, and brought to the laboratory for identification of sibling species using the molecular methods [22]. Blood meals of the fully fed *An. minimus* females were analyzed using the agar-gel diffusion method [23] and the source of feeding.

Statistical Analysis

Correlation analysis and its significance by using t-test was carried out to see the relationship between rainfall and density of *An. minimus*.

Results

Immature Survey

A total of 2,341 anopheline immatures were collected during the entire study period out of which 1,808 anopheline mosquitoes emerged. During the entire study period, a total of 782 immatures were collected from the surveys in the streams and a total of 555 anopheline mosquitoes emerged out of which 249 (69.4%) were *An. minimus* species and106 (39.6 %) were other anophelines. Similarly, out of 540 collected immatures from the pond, a total of 424 anopheline mosquitoes emerged of which 41 (11.4%) were *An. minimus* species and 383 (88.6%) were other anophelines. From paddy field, a total of 534 immatures were collected out of which a total of 417 anopheline mosquitoes emerged and among them 65 (18.1%) were *An. minimus* species and 352 (81.9%) were of other anophelines. A total of 321 immature were collected from the surveys in the river and a total of 224 anopheline mosquitoes emerged out of which 4 (1.1%) were *An. minimus* species and 220 (98.9 %) were of other anophelines. No *An. minimus* emerged from the immatures collected from the well and borrow pit (Table 1).

Collection of Adults

A total of 2,166 female anophelines belonging to 14 species were collected by hand catches from indoor resting collection in study area (Fig. 1) during January 2007 to December 2009 out of which 27% were *Anopheles minimus*, 20.3% were *An. fluviatilis* and 12.7% were *An. culicifacies*. Among the other collected anopheline species, the most prevalent was *An. vagus* (n = 173, 8 %) followed by *An. subpictus* (n =152, 7.0%), *An. varuna* (n = 120, 5.5%), *An. jeyporiensis* (n = 104, 4.8%), *An. jamesi* (n = 81, 3.7%), *An. annularis* (n = 74, 3.4%), *An. palidus* (n = 31, 1.4%), *An. maculatus* (n = 25, 1.2%), *An. splendidus* (n = 25, 1.2%) and *An. aconitus* (n = 14, 0.6%). The PMD of all anopheline species collected from indoor resting collection is enlisted (Table 2). The indoor per man hour density (PMDI) of *An. minimus* was found to be 6.09. Not a single specimen of *An. minimus* was collected from cattle sheds. Hence, it may be concluded that *An. minimus* is endophilic and prefers to rest on human dwelling. The month wise PMD of *An. minimus* collected from indoor resting collection varied from a low in July (1.33) to a peak in the month of November (13.5) to October (14.5) (Fig. 2). When the relationship between the quantum of rain fall and indoor resting density of *An. minimus* was compared, a non significant negative correlation (r=-0.962; P>0.05) was found. Though the indoor resting density showed a positive correlation with the average temperature (r=0.554, P>0.05) and humidity (r=0.365, P>0.05), but it was not significant. Analysis of abdominal condition of day time indoor resting females shows that 55.1% were in semigravid condition followed by 38.7% full fed, 4.2% gravid and 2.1% unfed. As the proportion of semigravid was found high, it indicates that *An. minimus* rest indoors after feeding and when the eggs fully develop, they leave indoor. As many as 335 female *An. minimus* obtained from various types of collection were dissected out for physiological age grading based on follicular relics. Of the 335 *An. minimus* dissected females, 64.1 per cent (n=215) were parous (having one or more dilatation). The highest proportion parous (0.68) was recorded during winter season, followed by summer (0.64) and rainy (0.57) seasons.

A total of 200 blood meal samples of *An. minimus* species were tested and the result showed that 91.7% indicated positive reaction with human anti sera and 8.3 % of the sample did not show any reaction. This result suggests that the population of *An. minimus* is highly anthropophilic, with an overall human blood index (HBI) of 0.91. A total of 535 *An. minimus* were dissected out for finding out the plasmodial infection in gut and gland out of which 0.93% (n=5) were found positive for sporozoite. No gut infection was noticed in *An. minimus*. A total of 100 specimens of *An. minimus* collected by different methods were identified morphologically and taken up for identification of ITS2 region in PCR assay. Amplification of ITS2 from all the specimens generated a product of equal to 520 base pair (bp) length which is similar to the finding of Garros et al [22] (Fig. 3). When this amplified DNA was mixed with species specific primers [24] and amplification was carried out, the species diagnostic assay produced band nearer on 184 bp region. Comparing the amplified product with previous study [22,24,25,6], the species was identified as *An. minimus.*

Table 1: Number of anopheline immature collected and number of *Anopheles minimus* emerged from different breeding habitats

Sl. No	Breeding habitat	No. of Dips Taken	No. of Dips + VE for Anopheline Immature	Per Dip Positve	Total Immature collected	Per Dip Immature Density	No of anopheline mosquito emerged	No of *Anopheles minimus* emerged	Percentage of *Anopheles minimus* emerged
1	Stream	1984	424	0.21	782	0.39	555	249	44.9
2	River	1900	386	0.20	321	0.17	224	4	1.8
3	Ponds	2518	497	0.20	540	0.21	424	41	9.7
4	Wells	843	123	0.15	123	0.15	151	0	0.0
5	Paddy fields	1821	412	0.23	534	0.29	417	65	15.6
6	Borrow pits	201	41	0.20	41	0.20	37	0	0.0
7	Total	9267	1883		2341		1808		

Table 2: Number of anopheline collected and man hour density in indoor and outdoor resting habitats

Sl. No	Species	Indoor resting habitat			Outdoor resting habitat		
		Total Number collected	Total Man hours Spent	PM D	Total Number collected	Total Man hours Spent	PMD
1	*An. aconitus*	14	96	0.15	0	45	0.00
2	*An. annularis*	74	96	0.77	0	45	0.00
3	*An. culicifaies*	276	96	2.88	0	45	0.00
4	*An. fluviatilis*	439	96	4.57	3	45	0.07
5	*An. jamesii*	81	96	0.84	0	45	0.00
6	*An. jeyporiensis*	104	96	1.08	0	45	0.00
7	*An. maculatus*	25	96	0.26	0	45	0.00
8	*An. minimus*	585	96	6.09	0	45	0.00
9	*An. nigerrimus*	67	96	0.70	0	45	0.00
10	*An. palidus*	31	96	0.32	0	45	0.00
11	*An. splendidus*	25	96	0.26	0	45	0.00
12	*An. subpictus*	173	96	1.80	2	45	0.04
13	*An. vagus*	152	96	1.58	6	45	0.13
14	*An. varuna*	120	96	1.25	5	45	0.11
15	Total	2166			16		

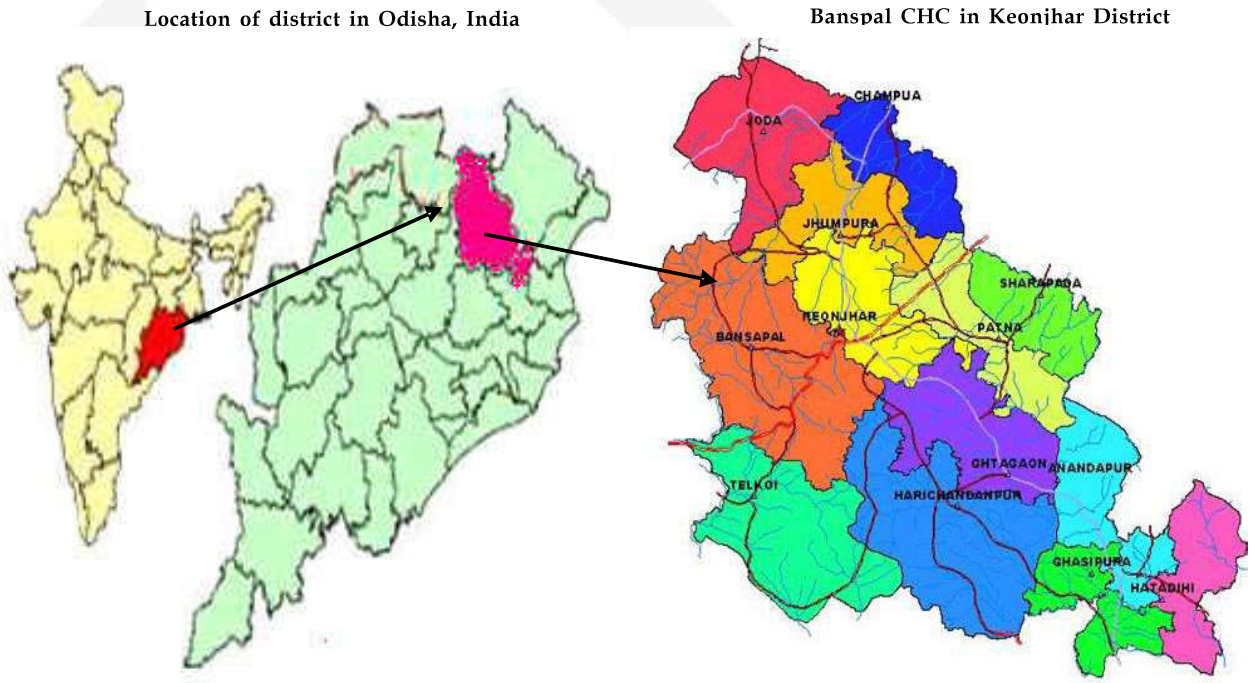

Fig. 1: Map of study sites

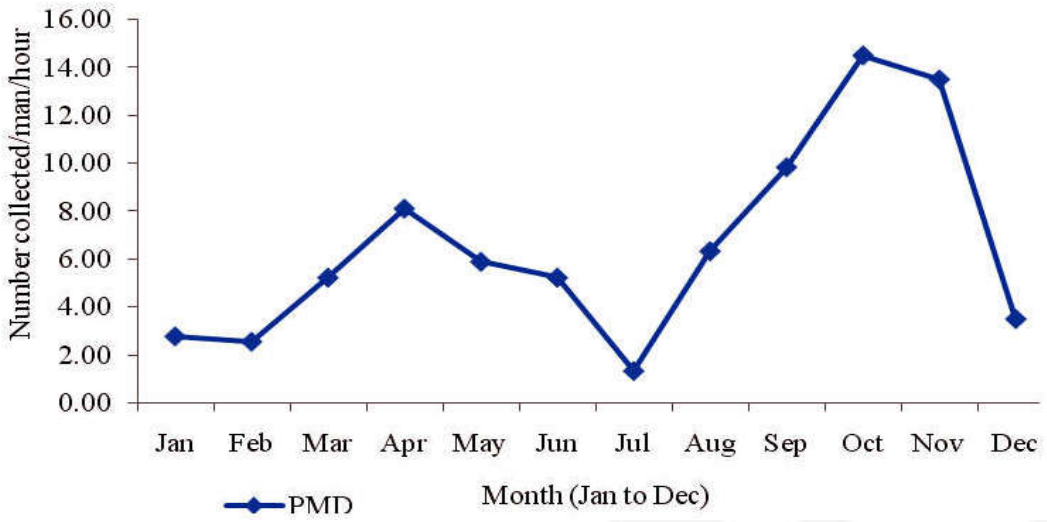

Fig. 2: Per man hour density (PMD) of *An. minimus* in different months

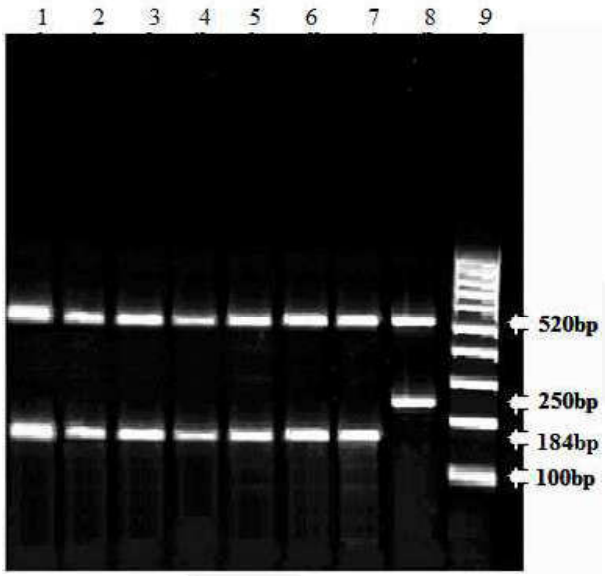

Fig. 3: Species specific PCR assay of ITS2 region of *An. minimus*; lane 1 to 7- *An. minimus* A, lane 8-*An. varuna*, lane9 -100bp DNA ladder

Discussion

The study was undertaken in Keonjhar district of Odisha which is hyperendemic for *falciparum* malaria being transmitted by *An. minimus* and *An. fluviatilis* [4].Though during the pre-DDT era this species was incriminated as vectors of malaria all along the foothills of the Himalayas extending from the Terai region of Uttar Pradesh to Assam and the neighboring eastern region, during post-DDT era this species has been considered as a major malaria vector only in the areas of north-eastern region of the country [4]. Though different levels of malaria control were achieved in many parts of India, no appreciable results have been achieved in Keonjhar district and till date malaria continues to be one of the major public health problems. Recently, *An. minimus* species was rerecorded in Singhbhum hills of east-central India [4] with a sporozoite rate of 1.4% [5]. Knowledge on species composition and human biting habits of anopheline mosquitoes in malarious areas is essential for designing appropriate vector control programs and also in understanding the epidemiology of malaria. Although several studies have been conducted on various aspects of bionomics of *An. minimus* in West Bengal and north eastern states [27, 28, 29, 30], no adequate and systematic information is available on this aspect for the reappearance of *An. minimus* in Keonjhar for which the study was undertaken.

According to Rao, *An. minimus* generally rests in houses and cattle shed during day time [15]. During the present study, *An. minimus* was collected only from human dwellings and not from cattle sheds, indicating its preference to rest in human dwellings. In Assam also, *An. minimus* was reported to rest predominately in human dwellings [19].Contrary to this finding, a study conducted in Darrang district of Assam [31] showed that the ratio of *An. minimus* resting in human dwellings to cattle sheds was 5.9: 15.4 [31]. The reason for the absence of *An. minimus* in the present study was because of the open type of cattle sheds without walls. The abdominal condition of day time resting females provide an additional evidence of their resting behavior [32]. In the present study, it was observed that among the indoor resting females of *An. minimus,* the proportion of semi gravid was markedly lesser than fully feds and the

proportion of gravid was lower than semi gravid, which indicates that a large proportion leave indoors before completion of their gonotrophic cycle. This leads to believe that the population of *An. minimus* in the study area tends to be more exophilic for resting.

However, during the entire study period, not a single *An. minimus* was collected from outdoors. In the present study, since the villages are surrounded by forest, the potential resting shelters outdoors are vast. Probably, this could be the reason for not collecting a good number of *An. minimus* outdoors. However, it is necessary to focus intensively on outdoor collection especially by digging more ideal pit shelters and by fixing light-traps so as to confirm the exophilic behaviour of this vector species. Recently, endophily of *An. minimus* has been reported in Assam by some researchers [33, 15]. In Sonpur [19] and Tamulpur Primary Health Centre (PHC) [34] of Assam, most of the *An. minimus* collected from houses were either fully fed, semi gravid or gravid in almost equal proportion indicating its endophilic behaviour. Therefore, it was suggested that *An. minimus* population in Tamulpur PHC, though endophilic, exhibited some degree of exophily and these findings need further confirmation. Outside India, endophily of *An. minimus* was reported in China [35] and Burma [36]. An important finding of this study was that the vector species rest mainly on walls of the houses and a small number was collected from roofs, hanging clothes and other articles and under beds. Dev (1996) observed in Assam that *An. minimus* prefer to rest on walls, hanging clothes and other articles and under beds [19]. In the present study, the reason for less finding these vector species on roofs, eaves and hanging objects inside human dwellings could be attributed to the structure of the huts in the studied area. There was a gap of two to three feet between the two side walls and roof which makes the house more lighted immediately after day breaks. Because of this, mosquitoes avoid resting on light exposed portions such as roof, eaves and hanging objects in houses and preferred to rest on walls at a height of three to four feet which is relatively darker. The density of this vector species does not correlate with rainfall in the study area and their abundance throughout the year might be due to the presence of perennial streams, and the preferential breeding habitat of this vector species [15]. The increase in density during post rainy months could be due to enhanced breeding in paddy fields [15]. In Assam, peak abundance of *An. minimus* was noticed during rainy season [19]. The results of breeding habitat analysis showed that stream and pond were the perennial breeding habitats and paddy field was the seasonal breeding habitat. The intensity of breeding of *An. minimus* was higher in streams followed by paddy fields and ponds. In Assam, it is also reported that *An. minimus* predominantly breed in streams [9, 31].

A study in Kanchanaburi village, Thailand showed that forest cleared for sugar cane cultivation, created widespread breeding ground for breeding *An. minimus* and the species preferred to breed in sunshine [37]. *An. minimus* was found to be the predominant human sucking mosquito with a high parous rate and exhibiting endophagy throughout the year of the present study. The sporozoite rate of *An. minimus* was found to be high and no gut infection was noticed in any of the *An. minimus* species dissected in the present study. In most of the *P. faciparum* predominant district of Assam, this species *An. minimus* also show high rate of sporozoite infection [38]. In spite of the undisputed vectorial importance of *An. minimus* in malaria transmission in east central India, there is a paucity of information on their species identification. From the 100 samples tested in the present study, no intraspecific variation was detected in the sequence of the ITS2 region in mosquitoes of *An. minimus* and all were of species A. This sibling species has been incriminated as a malaria vector in Assam [39] and also in the study area earlier [4,5,40].

Conclusion

Surveys conducted in the present study area in 1980s and 1990s did not record the presence of *An. minimus*. However, 45 years after the lunching of malaria eradication programme, recently this species has been reported to in the present study area [4]. As malaria is the major public health problem and the trend of malaria cases is in increasing order for the last five years. Information generated in the study, particularly the indoor resting pattern, breeding behavior and feeding behaviour would be useful for following aspects.

1. Integration of vector control measures and improvement of preventative assess before transmission season.
2. Specific designing of Information, Education and Communication (IEC) and behaviour change communication (BCC) activities to changing behaviour of local tribal people for accepting Indoor Residual Spray (IRS) and insecticide Treated Mosquito Net (ITMN) to control the man vector contact and alerting them how malaria causes a serious problem to incur huge expenditure towards treatment without preferring to vector control measures.

3. To dispel various misconceptions and myths regarding malaria transmission and disseminate information that mosquitoes are the sole agent of transmitting malaria causing parasites.
4. Regarding ecotopes, top hill and foot hill villages have to be considered with top priority for controlling the malaria as the density of *An. minimus* was higher in this ecotope.

Further investigation on the impact of deforestation, ecological parameters such as soil type, altitude, rainfall and temperature on the breeding and resting habitats of *An. minimus* in this study area and a control area is suggested.

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