Potential malaria vector Anopheles minimus (species A) still persisting in North East India

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Abstract
Vector borne infectious diseases affect two third of the world’s human population and cause millions of deaths every year. Among these, malaria remains one of the major killer in the Indian sub-continent and transmitted uninterruptedly by many efficient vectors and their sibling species. In North East India (NE), Anopheles minimus has been recognized as an important vector which transmits majority of malaria cases. This study primarily focuses on to recognize the presence and distribution of sibling species of An. minimus in certain endemic areas of NE India. Anopheles mosquitoes collected were identified using available morphological keys. The genomic DNA was extracted from the mosquitoes and used to perform species specific PCR (ss PCR) for molecular identification of major malaria vector An. minimus sibling species. Morphological identification suggested the presence of An. minimus s/s in low density in the study area. The specimen of An. minimus subjected to ss PCR confirmed the prevalence of only one sibling species namely An. minimus ‘A’ in Sialmari and Chandubi areas. Though in low density, but malaria vector An. minimus is still present in certain endemic areas of NE India. The ss PCR assay used presently suggested that An. minimus sibling species A is prevailing in the region. Presently used ss PCR assay was simpler, faster, cheaper and more readily interpretable than the earlier assays. This information could be useful in understanding of current prevalence and distribution of malaria vector An. minimus and its sibling species complex in NE region of India.

Keywords: malaria, mosquito vector, Anopheles minimus, species specific PCR, North East India

1. Introduction
Malaria is a life threatening disease caused by Plasmodium parasites and transmitted among humans by Anopheles mosquitoes. Despite comprehensive interventions, malaria cases have shown increase globally during the year 2018 (228 million; 95% confidence interval: 206-258 million) as compared to the year 2017 (219 million; 95% confidence interval: 203-262 million) and year 2016 (216 million; 95% CI: 196-263 million). Malaria attributable deaths showed decline in 2018 (0.41 million), but largely remained unchanged compared to 0.44 million in the year 2017 and 0.45 million in 2016 [1]. The WHO South East Asian region has shown incidence rate fall, however India continue to contribute about 58% of malaria episodes in the region, and reported approximately 0.41 million of confirmed malaria cases during 2018 [1]. Plasmodium falciparum dominates the malaria transmission in India and accounts for >60% of malaria cases followed by P. vivax (>35% cases) annually. These two malaria parasites are uninterruptedly transmitted by six major vectors namely, An. culicifacies, An. fluvitalis, An. stephensi, An. minimus, An. dirus and An. annularis in different parts of the country [1-3].

North East region of India (latitude - 21°58’ N to 29°30’N and longitude- 88°3’ E to 97°30’ E) shares international border with many endemic countries, such as Bhutan, China, Myanmar and Bangladesh that report considerable malaria cases every year. The region predominantly has humid sub-tropical climate and comprises of hills, wetlands, dense rain forests and forest fringes to support vector mosquito growth and proliferation throughout the year. Although the region inhabits 3.5% of India’s population but contributes 17.5% of total malaria deaths reported in the country. Several Anopheles species have been incriminated as malaria vector in the region during the recent years [4-6] and associated with the transmission of both P. falciparum and P. vivax in the endemic pockets, but historically An. dirus (monsoon species)
and An. minimus (perennial species) have been regarded as important malaria vectors involved in majority of malaria infections. Both these vectors incriminated in different independent investigations unequivocally were major vectors in the region few years ago. However during the recent years the density of An. minimus and An. dirus has declined considerably. Studies have suggested that both these vectors have been either disappeared [7, 8] or prevailing in very low numbers, which may not be sufficient to maintain perennial malaria transmission [4, 9]. Furthermore, secondary malaria vectors which had insignificant epidemiological importance earlier were incriminated harboring malaria parasites and maintaining uninterrupted malaria transmission in the region [4, 9]. Although many investigations [8, 9] could not collect An. minimus during the study, but its prevalence in low density below collectable limit mainly in ecologically suited forest fringed pockets cannot be overruled.

An. minimus s.l. (Funestus group) comprises of An. minimus s.s. (formerly known as An. minimus ‘A’), An. harrisi (formerly An. minimus ‘C’) and An. yaeyamaensis (formerly An. minimus ‘E’) [10-12]. An. minimus ‘E’ is restricted to Ryukyu Archipelago of Japan and not involved in malaria transmission [13], whereas species ‘A’ and ‘C’ are predominantly distributed throughout South East Asia region and responsible for malaria transmission. In North East region An. minimus ‘A’ has been incriminated as malaria vector and found associated in malaria transmission in many states of the region [22]. Although many molecular techniques have been developed to segregate sibling species, Van Bortel et al. [14] adopted an alternative method for the molecular identification of sibling species. This included PCR amplification of the rDNA internal transcriber spacer 2 (ITS2) region followed by BsiZI restriction enzyme digestion to distinguish An. aconitus, An. jeyporiensis, An. minimus ‘A’ and ‘C’, An. pampani, An. varuna and subsequently An. Culicifacies [14, 15]. Proper and accurate identification of any vector involved in malaria transmission, its distribution, behavior, vector competency and relative abundance is essential for successful control management. However, these are not easy to explain owing to difficulties in morphologically distinguishing different species from one another and from the other closely related groups. For instance, in central Vietnam, members of both An. dirus and An. minimus species complexes were earlier considered primary vectors. However, an earlier study [16] has revealed the fact that those mosquitoes which were identified as An. minimus earlier were actually An. varuna (a member of the Funestus Group that also includes the Minimus Complex). An. varuna, being an extremely zoophilic species in the study region cannot be regarded as a vector. This incorrect identification led to the misuse of rare and valuable resources and thus creating hindrances in vector management strategies. In order to mitigate the difficulty of morphological identification, varieties of molecular techniques have been developed for distinguishing An. minimus s.l. and other closely related species [10, 14, 17, 18]. The present study was aimed at investigating the presence of potential malaria vector An. minimus in the region and to identify its sibling species in northeastern region of India.

Methods

Study area and collection of Anopheles mosquitoes: Field collection of mosquitoes was performed during March 2016 to September 2016 (pre-monsoon and monsoon season). The adult Anopheles mosquitoes were collected from certain ecologically suited sentinel locations in Nameri (26.93° N - 92.88° E), Sialmari (26.71° N - 93.09° E), Jongakholi (25.98° N - 91.24° E), Chandubi (25.88° N - 91.43° E) areas of Assam (Fig. 1). These areas have vast forest land, scattered tea meadows and forest fringed inhabited areas with many streams, rivers and small irrigation canals with grassy margins, thereby providing conducive breeding habitat for An. minimus as well as for other Anopheles mosquitoes.

Fig 1: Collection sites: Anopheles minimus collection locations in Assam, North East India (map base taken from Google map; not to be scaled)

The indoor resting mosquitoes were located by torch light and collected by using mouth aspirators during early morning time (0500 – 0700 hr); whereas 6V battery operated CDC developed light traps were installed between 1800 hr to 0800 hr inside the human houses (total 4 trap nights/4 man hours at each site). The mosquitoes were morphologically identified using standard keys [19-21]. Since the study was primarily aimed at An. minimus mosquitoes, we separated mosquitoes that broadly look similar to An. minimus. These included An. jeyporiensis, An. minimus, An. aconitus and An. varuna and broadly categorized into An. minimus group in the present study. These mosquitoes were first used to identify An. minimus s.l. and subsequently the identified An. minimus s.l. specimens were processed for sibling species identification...
using species specific PCR assays. In the present study

**DNA Isolation:** The DNA extraction was performed as described previously [22]. Briefly, each adult female mosquito was homogenized by using polypropylene micro pestle (Tarson, India) in 2 ml micro centrifuge tube (Tarson, India) having filled with 100 µl lysis buffer containing 0.1 M Tris–HCl, 0.05 M EDTA, 0.2 M Sucrose, 0.05 % SDS, 0.1 M NaCl. The homogenate was immediately kept on ice for 10 min followed by heat treatment at 65 ºC for 30 min. Subsequently, 30 µl 5M potassium acetate was added and immediately transferred to ice for one hour followed by centrifugation at 13,000 rpm for 15 minutes at 10 ºC. A double volume of absolute chilled ethanol was added to the supernatant. The tube was left undisturbed for precipitation of DNA pallet was allowed to air dry and finally dissolved in 50 µl TE buffer for use as DNA template in PCR assays.

**An. minimus** species specific PCR assay: The assay

**Results**

**Mosquito collection**

In the present study different *Anopheles* species were collected from the sentinel study sites using mouth aspirator and CDC light traps (Table 2). Among the collected mosquitoes, two mosquito species namely, *An. annularis* and *An. vagus* were predominant and recorded in high number in all the collection sites. In Nameri, *An. vagus* was recorded in highest number both in trap collection (35.8%) ($\chi^2$ = 51.6; p < 0.0001) and indoor resting collection ($\chi^2$ = 16.7; p = 0.01) followed by *An. annularis* (26.9%). However in Jongakhuli, *An. annularis* was predominant in both CDC trap collection (72.7%; $\chi^2$ = 96.3; p < 0.0001) and indoor resting collections ($\chi^2$ = 43.6; p < 0.0001).

During the study, *An. minimus* mosquitoes were collected in very low number. In Sialmari collection area, we found 4 *Anopheles* mosquitoes in bad condition and the morphological features of these specimens were deteriorated. Therefore we could not identify them correctly; however these were identified to *An. minimus* species 'A' only to identify species 'A', species 'C' or hybrid of 'A' and 'C' from the collected samples.

**Table 1:** Detail of primers used for differentiating *An. minimus* ‘A’ and ‘C’ within *An. minimus* sibling species complex

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Primer</th>
<th>Sequence</th>
<th>PCR Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Universal forward</td>
<td>ATCACTCCTGTACGTGATC</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>An. minimus</em> ‘A’</td>
<td>GGCGCCTGTAGTTAGTTG</td>
<td>184 bp</td>
</tr>
<tr>
<td>3</td>
<td><em>An. minimus</em> ‘C’</td>
<td>GGTGTCCACTCATTACCGG</td>
<td>509 bp</td>
</tr>
</tbody>
</table>

**Table 2:** Morphological identified *Anopheles* species, collected in CDC Trap and hand catch during the study

<table>
<thead>
<tr>
<th>S. No</th>
<th>Location</th>
<th><em>Anopheles</em> species</th>
<th>CDC trap collection (%)</th>
<th>$\chi^2$ (p)</th>
<th>Indoor resting collection (%)</th>
<th>$\chi^2$ (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nameri</td>
<td><em>An. jeyporiensis</em></td>
<td>3 (4.5)</td>
<td>51.6 (&lt;0.0001); df=6</td>
<td>1 (2.6)</td>
<td>16.7 (0.01); df=6</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. aconitus</em></td>
<td>2 (3.0)</td>
<td></td>
<td>2 (5.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. varuna</em></td>
<td>4 (6.0)</td>
<td></td>
<td>5 (12.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. vagus</em></td>
<td>24 (35.8)</td>
<td></td>
<td>12 (30.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. annularis</em></td>
<td>18 (26.9)</td>
<td></td>
<td>7 (17.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. philippinensis</em></td>
<td>10 (14.9)</td>
<td></td>
<td>7 (17.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. crawfordi</em></td>
<td>6 (9.0)</td>
<td></td>
<td>5 (12.8)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Sialmari</td>
<td><em>An. jeyporiensis</em></td>
<td>7 (13.2)</td>
<td>25.3 (0.0001); df=5</td>
<td>2 (3.4)</td>
<td>28.2 (&lt;0.0001); df=5</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. minimus/ An. varuna</em></td>
<td>4 (7.5)</td>
<td></td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. aconitus</em></td>
<td>4 (7.5)</td>
<td></td>
<td>4 (10.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. varuna</em></td>
<td>6 (11.3)</td>
<td></td>
<td>6 (16.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. vagus</em></td>
<td>18 (34.0)</td>
<td></td>
<td>11 (29.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. annularis</em></td>
<td>14 (26.4)</td>
<td></td>
<td>14 (37.8)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Jongakholi</td>
<td><em>An. aconitus</em></td>
<td>1 (1.8)</td>
<td>96.3 (&lt;0.0001); df=3</td>
<td>2 (5.4)</td>
<td>43.6 (&lt;0.0001); df=3</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. vagus</em></td>
<td>12 (21.8)</td>
<td></td>
<td>7 (18.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. annularis</em></td>
<td>40 (72.7)</td>
<td></td>
<td>24 (64.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. crawfordi</em></td>
<td>2 (3.6)</td>
<td></td>
<td>4 (10.8)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Chandubi</td>
<td><em>An. varuna</em></td>
<td>6 (14.3)</td>
<td>10.5 (0.01); df=3</td>
<td>5 (13.2)</td>
<td>9.19 (0.03); df=3</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. vagus</em></td>
<td>18 (42.9)</td>
<td></td>
<td>16 (42.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>An. annularis</em></td>
<td>10 (23.8)</td>
<td></td>
<td>9 (23.7)</td>
<td></td>
</tr>
</tbody>
</table>
Identification of Anopheles minimus sibling species

Species specific PCR for Anopheles minimus sibling species diagnosis used genomic DNA of field collected An. minimus s.l. and amplified separately with primer specific for species ‘A’, species ‘C’ and both ‘A’ and ‘C’ for hybrid detection along with 5.8S forward primer for each reaction. First reaction with primers set ‘A’ yielded 184 base pair band that are specific to An. minimus species ‘A’ while PCR reactions for species ‘C’ and ‘A/C’ hybrid did not produce any band (Fig. 2).

Discussion

An. minimus has been recognized as an efficient malaria vector in North East region of India since long and held responsible for perennial transmission of Plasmodium parasite, as evidenced by high sporozoite rates recorded in different seasons [23]. In the region, An. minimus s.l. is endophilic and prefers to rest hiding on darker areas of walls, ceilings, cloths folding and temporary structures inside the human dwellings after taking blood meal. Studies have shown that An. minimus has very high affinity for human blood and reported anthropophilic index of > 90% in North East region of India [4, 6, 24]. Considering this, the mosquito collections in the present study were made inside the human houses with an objective to collect An. minimus mosquitoes. This species is still sensitive to various insecticidal formulations used in intervention programmes; hence it is argued that An. minimus may be exhibiting behavioral changes by preferring to bite and rest outdoors than indoors in order to avoid direct contact with the indoor sprayed insecticides. Although investigations have reported significant declining trend in An. minimus density [4] to virtually nil level in different states of North East region of India, however none of the study so far has evidenced that density has not declined but behavioral plasticity in An. minimus has led it to feed human blood and rest outdoor.

In the recent years there has been considerable change in the ecology in the region involving deforestation, increasing spread of cultivated lands and reduction in the breeding habitat which has influenced the vector composition drastically [25, 26]. An. minimus females are highly selective for egg laying places and lay eggs in grassy margins of unpolluted rivers and streams. Therefore water bodies lacking such supported ecology for An. minimus breeding may sometime account for absence of larvae in water and adults in the nearby places.

In the present study, we could not collect An. minimus mosquitoes in high number but still showed the An. minimus are still present in the region. Species specific PCR assay used was able to identify species ‘A’ of An. minimus complex. Different PCR based methods have been used successfully in identification of mosquito complexes accurately [18, 22, 27-31]. These methods are simple, precise and more accurately interpreted, hence could be used in segregating the closely related sibling species from each other. Furthermore, the multiplex PCR assay used presently was also able to identify sibling species ‘A’ of An. minimus among all the mosquito species used for the assay in the study. The PCR assay used produced unambiguous variation in PCR products of various An. minimus group mosquitoes in South East Asian countries, thus making this assay suitable and reliable for accurate identification of this mosquito complex [18].

Studies have reported that An. minimus was once widely prevalent and involved in disease transmission in Himalayan foothills of northern to eastern region of India [8, 23, 24]. However, later thought to have been disappeared as studies did not report this species from Sub-Himalayan foothill regions [9]. However, this species appeared in eastern region after more than four decades and involved in malaria transmission [32, 33]. Similarly, malaria vectors that were disappeared completely and believed to be eliminated under
the influence of control interventions or ecological changes were found to have been re-emerged later and incriminated as vector involved in malaria outbreaks [33].

Many studies conducted during past few years have reported malaria cases in North East region of India in the absence of An. minimus, while establishing that other anopheline mosquitoes that played insignificant role in malaria transmission previously, have taken over as important vectors and involved in continuous transmission of Plasmodium species throughout the year [8, 9]. The present study conducted in forest fringed foothills areas that provide suitable breeding and proliferation ecology for An. minimus s.l. confirms that this important vector has not disappeared completely but limited its prevalence into certain favorable areas. Present study has identified An. minimus species ‘A’ which is a well-known vector in the region. The findings further suggest that An. minimus may be thriving under selection pressure as numbers of insecticidal products are in place to target this important malaria vector. However it may re-surge in high density once these large scale interventions targeting this mosquito are withdrawn considering that it is disappeared completely.

Conclusion

Present study has attempted to establish that An. minimus ‘A’ is existing in the North Eastern region of India but limited to certain ecologically suitable pockets in forest fringed areas. The environmental conditions still favour the prevalence of this potential vector which is probably striving under selection by changing ecology as well as increasing insecticidal pressure. Therefore complete disappearance of this mosquito in the study region can be overruled. Study also emphasizes that it is not appropriate to conclude An. minimus group identification without using PCR like sensitive methods, as it may give confusing results uninvited for vector control programmes.

Acknowledgements

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Reference