Evaluation of Insecticides Susceptibility and Malaria Vector Potential of *Anopheles annularis* s.l. and *Anopheles vagus* in Assam, India

Sunil Dhiman *, Kavita Yadav, Bipul Rabha, Diganta Goswami, S. Hazarika, Varun Tyagi
Division of Medical Entomology, Defence Research Laboratory, Tezpur, Assam, 784001, India

* sunildhiman81@gmail.com

**Abstract**

During the recent past, development of DDT resistance and reduction to pyrethroid susceptibility among the malaria vectors has posed a serious challenge in many Southeast Asian countries including India. Current study presents the insecticide susceptibility and knockdown data of field collected *Anopheles annularis* sensu lato and *An. vagus* mosquito species from endemic areas of Assam in northeast India. *Anopheles annularis* s.l. and *An. vagus* adult females were collected from four randomly selected sentinel sites in Orang primary health centre (OPHC) and Balipara primary health centre (BPHC) areas, and used for testing susceptibility to DDT, malathion, deltamethrin and lambda-cyhalothrin. After insecticide susceptibility tests, mosquitoes were subjected to VectorTest™ assay kits to detect the presence of malaria sporozoite in the mosquitoes. *An. annularis* s.l. was completely susceptible to deltamethrin, lambda-cyhalothrin and malathion. After insecticide susceptibility tests, mosquitoes were subjected to VectorTest™ assay kits to detect the presence of malaria sporozoite in the mosquitoes. *An. annularis* s.l. was completely susceptible to deltamethrin, lambda-cyhalothrin and malathion in both the study areas. *An. vagus* was highly susceptible to deltamethrin in both the areas, but exhibited reduced susceptibility to lambda-cyhalothrin in both the areas. *An. vagus* adult females were collected from four randomly selected sentinel sites in Orang primary health centre (OPHC) and Balipara primary health centre (BPHC) areas, and used for testing susceptibility to DDT, malathion, deltamethrin and lambda-cyhalothrin. After insecticide susceptibility tests, mosquitoes were subjected to VectorTest™ assay kits to detect the presence of malaria sporozoite in the mosquitoes. *An. annularis* s.l. was completely susceptible to deltamethrin, lambda-cyhalothrin and malathion in both the study areas. *An. vagus* was highly susceptible to deltamethrin in both the areas, but exhibited reduced susceptibility to lambda-cyhalothrin in BPHC. Both the species were resistant to DDT and showed very high KDT$_{50}$ and KDT$_{99}$ values for DDT. Probit model used to calculate the KDT$_{50}$ and KDT$_{99}$ values did not display normal distribution of percent knock-down with time for malathion in both the mosquito species in OPHC ($\chi^2 = 25.3; p = 0.0$), and also for deltamethrin in OPHC area ($\chi^2 = 15.4; p = 0.004$). Minimum infection rate (MIR) of *Plasmodium* sporozoite for *An. vagus* was 0.56 in OPHC and 0.13 in BPHC, while for *An. annularis* MIR was found to be 0.22 in OPHC. Resistance management strategies should be identified to delay the expansion of resistance. Testing of field caught *Anopheles* vectors from different endemic areas for the presence of malaria sporozoite may be useful to ensure their role in malaria transmission.
Introduction

In the recent years, scaling-up of long-lasting insecticidal nets (LLINs) and to some extent indoor residual spraying (IRS) using insecticides has been a pivotal element in mosquito control strategies. However, rapid emergence and geographical spread of insecticide resistance among malaria vectors has threatens the intervention programmes in many endemic Afro-Asian countries. Only four insecticide classes, which share two modes of action have been approved by the World Health Organization (WHO) for use in mosquito control programmes [1]. Since there are limited number of insecticide groups available, the options to switch over to comparatively more effective insecticide in control operations are restricted. Considering the importance of insecticides in malaria control, regular monitoring of insecticides susceptibility among Anopheles vectors is essential, primarily in the regions where malaria in endemic and subsistently remains a burden to the ethnic communities [2–4].

In India, synthetic pyrethroids have been widely used in LLINs, while DDT is used for IRS in many malaria endemic regions including northeastern states of India. However, few recent studies have indicated considerable level of resistance among some well established malaria vectors against synthetic pyrethroids and DDT in different states of India [5–8]. The northeast region of India is geographically isolated and shares international frontiers on three sides with malaria affected countries like Bangladesh, Bhutan and Myanmar. In this region, although Anopheles minimus and An. dirus are considered as major malaria vectors [5, 9, 10], but recently the abundance of these two mosquito species has decreased [11], while An. annularis and An. vags became increasingly important due to their high density during the peak malaria season and possible role in malaria transmission. Although both these species are primarily zoophilic, exophilic and exophagic, but also found feeding on human blood and maintaining malaria transmission in the plain areas of northeast India and adjoining Bangladesh [6, 9, 12, 13].

Anopheles annularis Van der Wulp, 1884 is widespread in many Asian countries and recently emerged as an important malaria vector in India and neighbouring countries [6, 10, 14, 15]. An. vags Doenitz, 1902 is extensively recorded in malaria endemic areas of Indian sub-continent and plays important role in malaria transmission in Bangladesh, Laos and Cambodia [12, 13, 16, 17]. Previous studies have shown resistance to DDT and reduced susceptibility to deltamethrin in An. annularis [6], but none of the study has recorded the insecticide resistance status of An. vags in northeast India. Although An. vags is a well known malaria vector in many countries now, but in India, no study has demonstrated its potential role in malaria transmission.

The objective of the present study was to investigate the insecticide susceptibility of An. annularis s.l. and An. vags in malaria endemic Udalguri district and Sonitpur district of Assam in northeast India. Since organochloride (OC), synthetic pyrethroids (SP) and organophosphate (OP) insecticides are used in malaria control intervention in the region, we have used DDT, deltamethrin, lambda-cyhalothrin and malathion as test insecticides in this study. Furthermore, VectorTest™ malaria sporozoite antigen panel assay has been used for the detection of Plasmodium circumsporozoite antigens in both the mosquitoes species.

Materials and Methods

Study area

Current study was conducted during March 2013 to August 2013 (pre-monsoon and monsoon season) in malaria endemic Udalguri and Sonitpur districts of Assam state of northeast India. In Udalguri district, four sentinel sites each were randomly selected in Orang primary health...
centre (OPHC) (26° 33’–26° 56’ N to 92° 07’–92° 22’ E), while in Sonitpur district, same number of sentinel sites were chosen in Balipara primary health centre area (BPHC) (26° 41’–27° 02’ N to 92° 38’–92° 59’ E) (Fig 1). The study districts are dominated by socio-economically backward ethnic tribes engaged mainly in tea based agriculture [4]. The climate is humid with an average annual rainfall of about 2,000 mm and temperature varying between 13.5°C to 35.0°C. Study area has many small rivers, duck rearing ponds, spread of tea gardens, vast paddy fields and forests, which provide sufficient breeding habitat for mosquitoes. Both the primary health centres report high incidence of malaria annually [4,18–20]. During the study year OPHC area reported malaria parasite slide positivity rate (SPR) of 2.40 and annual parasitic index (API) of 3.76, whereas BPHC area reported SPR and API of 2.12 and 0.24 respectively. Insecticides use has been intensive with several rounds of spray per growing season due to severe damage of tea and rice by insect pests. Synthetic pyrethroids are most commonly used in agriculture, whereas the use of organophosphate and carbamate based insecticides is comparatively less common. No specific permissions were required for conducting this activity in both the study areas. We have made the collection of mosquitoes only and none of the study in this research involved the collection and use of rare/endangered/protected animal species.

Mosquito collection, identification and resistance bioassay
Adult indoor resting mosquitoes were collected from the human houses during 0500–600 hours using suction tube and torch light. Mosquitoes were identified morphologically using standard keys used for the identification of medically important Anopheles mosquitoes. The study areas were subjected to a round of indoor residual spray of DDT in February 2013. Healthy and unfed adult females of An. annularis s.l. and An. vagus were exposed to World Health Organisation (WHO) insecticide pre-impregnated papers of DDT (4%), deltamethrin (0.05%), lambda-cyhalothrin (0.05%) and malathion (5%) obtained from University Sains Malaysia, Malaysia in WHO insecticide susceptibility evaluation test kits [21,22]. The control tests were performed using pre-impregnated paper with silicone oil (deltamethrin and lambda-cyhalothrin control), risella oil (DDT control) and olive oil (malathion control) along with each set of insecticide bioassay. Each time 10–15 mosquitoes were used in the test for 1 hour and cumulative knock-down was recorded after an interval of 10 minutes [6]. The mosquitoes were then transferred into the holding tube and fed on 5% sucrose solution. Mortality was recorded after a 24 hour holding period and the resistance status was defined according to WHO guidelines, which state that 98–100% mortality indicates susceptibility, 90–98% indicates the possibility of resistance that needs to be confirmed and <90% indicates resistance [23]. After the completion of each test, mosquitoes were re-identified to avoid any error and stored in labeled eppendorf tubes for malaria sporozoite antigen detection assay using VectorTest™ assay kit according to the standard manufacturer’s instruction.

Malaria sporozoite antigen assay
The adult female An. annularis s.l. and An. vagus were subjected to VectorTest™ assay kits (Vector Test System Inc., CA) to detect the presence of malaria sporozoite in the mosquitoes. For each test a pool of 20–25 mosquitoes of a species was put into the grinding tube provided with the assay kit and homogenised in grinding solution using plastic pestle. The tests were performed according to the standard manufacturer’s instruction provided with the assay kit. The VectorTest™ malaria sporozoite antigen assay is a highly specific and rapid immunochromatographic test for qualitative determination of circumsporozoite antigens of P. falciparum, P. vivax 210 and P. vivax 247 malaria parasite species in infected mosquitoes [24,25].
Data analysis

The mortality obtained in the mosquito species was corrected using Schneider-Orelli’s formula [26]. Knock-down time (KDT$_{50}$ and KDT$_{99}$) along with slope and 95% confidence interval (CI) were determined using Ldp Line computer programme. Chi-square ($\chi^2$) test was used to analyse the fitment of probit, while liner regression was used to evaluate if data deviate from linearity.
Results

Insecticide resistance bioassay

A total of 1,566 *An. annularis* s.l. and 1,998 *An. vagus* mosquitoes were tested in the present study to determine the susceptibility against insecticides (Tables 1 and 2). As per WHO guidelines [23], the mortality ranging between 98–100% indicates susceptibility, 90–97% as tolerant for which further investigation is needed, and <90% is considered resistant where pre-emptive action is required to manage the resistance against insecticides used for malaria vector control. Based on these recommendations, *An. annularis* s.l. was completely susceptible to deltamethrin, lambda-cyhalothrin and malathion in both the study PHCs as the corrected mortality observed was 100% (95% CI- 97.5–100.0), 99.3% (95% CI- 96.3–99.9) and 98.7% (95% CI- 95.3–99.6) respectively in OPHC (Table 1), while 98.1% (95% CI- 95.8–99.2), 98.6% (95% CI- 94.9–99.6) and 98.9% (95% CI- 96.1–99.7) respectively for the three insecticides in BPHC area (Table 2). *An. annularis* s.l. from both the study areas showed complete resistance to DDT and the corrected mortality recorded was below 75.2% (95% CI- 68.2–82.1). *An. vagus* mosquitoes were highly susceptible to deltamethrin but exhibited considerably reduced susceptibility to lambda-cyhalothrin in both the areas. The mortality of *An. vagus* to DDT was recorded below 83.3% (95% CI- 95.3–99.6) in the present study which indicated a high level of resistance to DDT. Against malathion, *An. vagus* mosquitoes were susceptible in OPHC (corrected mortality- 99.3%; 95% CI- 96.3–99.9), while suspected to be resistant in BPHC as the corrected mortality was found to be 97.6% (95% CI- 95.4–98.8) (Tables 1 and 2).

Knock-down effect

The knock-down effect of four insecticides determined against *An. vagus* and *An. annularis* s.l. in OPHC and BPHC over an exposure time period of one hour has been shown in Tables 1 and 2, whereas the percent knock-down achieved in both the locations has been depicted in Fig 2 (S1 Table) and Fig 3 (S2 Table) respectively. In *An. annularis* s.l., the KDT50 ranged from 24.7 to 25.3 minutes, while KDT99 ranged from 129.0 to 144.0 minutes during the study. Among all the tested insecticides, least knock-down percent of both the mosquito species (range—35.3–41.3%) was recorded for DDT, whereas highest knock-down percent ranging from 86.8 to 98.1 was observed for deltamethrin within one hour of exposure time. Both the mosquito species displayed very high KDT50 and KDT99 values for DDT in both the study locations. The KDT50 values of malathion and lambda-cyhalothrin ranged from 37.7 to 51.2 and 24.5 to 31.8 minutes respectively. Presently the probit model used to calculate the KDT50 and KDT99 values displayed normal distribution of percent knock-down with time for all the insecticides except malathion for both the mosquito species in OPHC (p<0.05) and *An. vagus* in BPHC ($\chi^2 = 25.3; p = 0.0$), and also for deltamethrin exposure to *An. vagus* in BPHC area ($\chi^2 = 15.4; p = 0.004$).

Malaria sporozoite antigen assay

In the present study, a total of 59 pools (N = 1,340) of female *An. vagus* and 39 pools (N = 780) of *An. annularis* s.l were tested for the presence of *Plasmodium* sporozoite antigen (S3 Table). For *An. vagus*, 3 pools were found positive in OPHC, whereas 1 pool was positive in BPHC area. Minimum infection rate (MIR) of *Plasmodium* sporozoite for *An. vagus* was found to be 0.56 in OPHC and 0.13 in BPHC. On the other hand only 1 pool was found positive for *An. annularis* in OPHC with a MIR of 0.22 and pool positive rate of 4.35 (Table 3). All the tested mosquito pools found positive for *Plasmodium* sporozoites corresponded to malaria parasite *Plasmodium falciparum*. 
Table 1. Toxicity and knock-down time of *An. annularis* and *An. vagus* in Orang primary health centre (OPHC) area.

| Insecticide (N) | Mosquito species | %KD<sub>1h</sub> (N) | KDT<sub>50</sub> (95% CI) | KDT<sub>95</sub> (95% CI) | Slope ±SD | χ² (p) | r | CM<sub>24 h</sub> (95% CI) |
|-----------------|------------------|----------------------|---------------------------|--------------------------|-----------|-------|---|--------------------------|
| Deltamethrin (150) | *An. annularis* | 90.7 (136) | 24.7 (22.9–26.5) | 129.0 (108.6–160.4) | 3.2±0.2 | 1.9 (0.7) | 1 | 100 (97.5–100.0) |
| DDT (150) | | 41.3 (62) | 99.2 (71.7–181.4) | 14960.1 (3210.2–319454.1) | 1.1±0.2 | 1.1 (0.9) | 1 | 75.2 (68.6–82.1) |
| Malathion (150) | | 62 (93) | 51.2 (43.0–73.3) | 384.7 (341.7–1583.1) | 2.7±0.2 | 14.7 (0.005) | 0.9 | 99.3 (96.3–99.9) |
| L-cyhalothrin (150) | | 90 (135) | 24.5 (22.8–26.1) | 116.7 (99.6–142.2) | 3.4±0.2 | 1.8 (0.8) | 1 | 98.7 (95.3–99.6) |
| Deltamethrin (150) | *An. vagus* | 96 (144) | 22.1 (20.6–23.7) | 102.4 (88.4–122.8) | 3.5±0.2 | 8.0 (0.08) | 0.9 | 83.3 (76.6–88.5) |
| DDT (150) | | 35.3 (53) | 159.5 (100.2–421.3) | 33625.9 (5099.1–2039497.9) | 1.0±0.2 | 4.1 (0.3) | 0.9 | 99.3 (96.3–99.9) |
| Malathion (150) | | 68 (102) | 41.2 (34.9–50.9) | 252.5 (203.4–566.9) | 2.9±0.2 | 11.7 (0.02) | 1 | 99.3 (96.3–99.9) |
| L-cyhalothrin (150) | | 90 (135) | 25.2 (23.6–26.8) | 109.1 (94.2–131.0) | 3.7±0.2 | 2.6 (0.6) | 1 | 97.3 (93.3–98.9) |

KDT—knock-down time in minutes; CM—corrected mortality in percent; N—number, CI—confidence interval, SD—standard deviation, r—correlation coefficient.

doi:10.1371/journal.pone.0151786.t001

Discussion

Presently, WHO insecticide bioassays were performed on *An. annularis* s.l. and *An. vagus* mosquitoes to assess their susceptibility to DDT, deltamethrin, malathion and lambda-cyhalothrin in two endemic districts of Assam in northeast India. Different level of susceptibility to the tested insecticides has been observed in the study. WHO recommends the use of 2–3 days old female mosquitoes for insecticide bioassay, however currently field collected mosquitoes representing natural age-structured populations were tested to determine the resistance status. Hence there was a mix of mosquitoes of different age, which probably produced higher mortality than expected by using young mosquitoes. Previous studies have reported that as compared to the young mosquitoes, the level of detoxifying enzymes, namely GST and monooxygenase often decreases with age, leading to an increase in the insecticide susceptibility level [17, 27, 28].

Table 2. Toxicity and knock-down time of *An. annularis* and *An. vagus* in Balipara primary health centre (BPHC) area.

| Insecticide (N) | Mosquito species | %KD<sub>1h</sub> (N) | KDT<sub>50</sub> (95% CI) | KDT<sub>95</sub> (95% CI) | Slope±SD | χ² (p) | r | CM<sub>24 h</sub> (95% CI) |
|-----------------|------------------|----------------------|---------------------------|--------------------------|-----------|-------|---|--------------------------|
| Deltamethrin (272) | *An. annularis* | 86.8 (236) | 25.3 (23.9–26.7) | 144.0 (124.8–170.9) | 3.1±0.2 | 4.0 (0.9) | 1 | 98.1 (95.8–99.2) |
| DDT (373) | | 37.3 (139) | 91.4 (79.8–109.3) | 1357.3 (841.5–2592.5) | 2.0±0.2 | 3.1 (0.5) | 1 | 65.1 (61.3–70.8) |
| Malathion (139) | | 79.9 (111) | 37.7 (35.4–40.3) | 170.8 (139.6–222.6) | 3.5±0.3 | 6.6 (0.2) | 1 | 98.6 (94.9–99.6) |
| L-cyhalothrin (182) | | 80.8 (147) | 31.8 (29.6–34.1) | 231.3 (182.8–313.1) | 2.7±0.2 | 4.7 (0.3) | 1 | 98.9 (96.1–99.7) |
| Deltamethrin (424) | *An. vagus* | 98.1 (416) | 20.5 (18.1–22.7) | 85.8 (76.4–105.9) | 3.7±0.1 | 15.4 (0.004) | 1 | 99.1 (97.6–99.6) |
| DDT (326) | | 36.5 (119) | 105.8 (87.6–136.8) | 2831.6 (1444.8–7437.6) | 1.6±0.1 | 3.6 (0.5) | 1 | 70.0 (65.1–74.9) |
| Malathion (335) | | 90.7 (304) | 38.6 (34.8–42.5) | 91.9 (86.1–115.6) | 6.2±0.3 | 25.3 (0.0) | 1 | 97.6 (95.4–98.8) |
| L-cyhalothrin (313) | | 83.1 (260) | 31.1 (29.8–32.5) | 147.2 (129.7–171.1) | 3.4±0.2 | 0.3 (1.0) | 1 | 88.6 (84.9–91.9) |

KDT—knock-down time in minutes; CM—corrected mortality in percent; N—number, CI—confidence interval, SD—standard deviation, r—correlation coefficient.

doi:10.1371/journal.pone.0151786.t002
The results demonstrated that both the mosquito species displayed a high level of biological resistance to DDT as the corrected mortality ranged from 83.3% (95% CI = 76.6–88.5) to as low as 65.1% (95% CI = 61.3–70.8) during the study. Although DDT is extensively used in public health programmes, but resistance to DDT is widespread among many efficient mosquito vectors in different parts of India [6–8, 29–31]. The KDT$_{50}$ and KDT$_{99}$ values were also found to be very high and never recorded below 91.4 minutes (95% CI = 79.8–109.3), suggesting that the tested mosquitoes were not much knock-down sensitive to DDT. However, relatively low value of KDT has been recorded in the regions where mosquitoes are susceptible to DDT, whereas high KDT values have been recorded from the regions which reported high level of DDT resistance [6, 29, 32]. DDT is most accepted insecticide in India and its use for many decades now has resulted in high selection pressure and widespread of insecticide resistance. Although $kdr$ mutations have been reported to confer resistance to DDT, but a recent study has again raised this concern by suggesting that about 30% of phenotypically resistant mosquitoes did not present $kdr$ mutations [33].

There was complete susceptibility to deltamethrin as the corrected mortalities recorded were above 98.1% for both the mosquito species in both the study areas. However a reduced sensitivity was observed for lambda-cyhalothrin in An. vagus as the corrected mortality observed ranged from 88.6 (95% CI = 84.9–91.9) to 97.3% (95% CI = 93.3–98.9). The KDT$_{50}$ and KDT$_{99}$ values for deltamethrin and lambda-cyhalothrin were comparable for both the
species, but interestingly these values for lambda-cyhalothrin in *An. vagus* were found to be 1.5 (KDT_{50}) and 1.7 (KDT_{99}) fold high than deltamethrin in BPHC area.

Synthetic pyrethroids including deltamethrin and lambda-cyhalothrin are widely used in various public health programmes to control mosquitoes in many countries. However, in the recent years efficacy of these insecticides against potential malaria vectors has been found to reduced in endemic areas [2, 3, 6, 17, 34]. The present study area has vast paddy fields and large scale vegetable cultivation throughout the year, and the pyrethroids are widely used in the control of agricultural pests. Furthermore, the pyrethroid based long lasting insecticidal nets

![Graph A](image1.png)

**Fig 3.** Knock-down rate for different insecticides during 1 hour of exposure in Balipara primary health centre (BPHC) area. *An. vagus* (A), *An. annularis* (B). DM- deltamethrin, MA- malathion, LC- lambda-cyhalothrin.

doi:10.1371/journal.pone.0151786.g003

| Species       | Location | Pool (n) | N    | Positive | MIR   | PPR  |
|---------------|----------|----------|------|----------|-------|------|
| *An. vagus*   | Orang    | 27 (20)  | 540  | 3        | 0.56  | 11.11|
|               | Balipara | 32 (25)  | 800  | 1        | 0.13  | 3.13 |
| *An. annularis*| Orang    | 23 (20)  | 460  | 1        | 0.22  | 4.35 |
|               | Balipara | 16 (20)  | 320  | 0        | 0.00  | 0.00 |
| **Total**     |          | 98       | 2120 | 5        | 0.24  | 5.10 |

Table 3. *Plasmodium* sporozoite detection using VectorTest™ panel assay.

N—total number tested; n—number in each pool; MIR—minimum infection rate; PPR—pool positive rate

doi:10.1371/journal.pone.0151786.t003
(LLINs) have been considered as the cornerstone of malaria control programmes and distributed free of cost by the government agencies in the recent years. All these activities have increased the selection in malaria vectors to this class of insecticides. A significant level of resistance to pyrethroid was found associated with the agriculture intensity in Africa, indicating that resistance level increases with the increase in agriculture spread [2, 35]. Presently, the tested mosquito were knock-down sensitive to pyrethroid as the KDT50 an KDT99 values were considerably lower but found to be higher than achieved for An. annularis previously [6]. In a study conducted in Mekong region, An. vagus was found to be highly knock-down resistant to deltamethrin and revealed the presence of a L1014S kdr mutation [17].

Present results revealed complete susceptibility to malathion except for An. vagus in BPHC area where the mortality recorded was 97.6% (95% CI = 95.4–98.8) indicating reduced susceptibility which warrants regular monitoring. Although malathion is extensively used in the control of vector mosquitoes in different endemic regions of India but no study has clearly indicated the development of resistance to malathion [29]. Malathion is mostly used in fogging to control dengue vectors during the epidemics and not in the control of malaria vectors, therefore the chances of exposing Anopheles mosquitoes to malathion are limited except some accidental exposure.

Mutations in the voltage gate sodium channel gene have been shown as important mechanism conferring high level of cross resistance to DDT and synthetic pyrethroids. Currently no evidence of cross resistance to DDT and pyrethroids has been observed, however the study has underlined the existence of DDT resistance in malaria vectors and possible decline in sensitivity to the synthetic pyrethroids, but does not suggests the mechanism which could be attributed to the problem of resistance. Studies have very well documented the role of target-site mutations in insecticide resistance, however these were not found solely responsible for resistance and some detoxifying genes acting in concert with these mutations in voltage gated sodium channels were reported to confer extreme levels of resistance [28, 35–39]. A recent research demonstrated that glutathione S-transferase gene GSTe2 was the most over-expressed detoxification gene in DDT and permethrin resistant Anopheles funestus mosquitoes [40], whereas another study [41] claimed that mutation L1014F was more efficient in conferring resistance to DDT as compared to pyrethroids, which might be a reason that the mosquitoes in the present study displayed high level of resistance to DDT. Furthermore, the studies have also suggested that kdr may act with certain unidentified co-factors to create resistance phenotype [42] or the resistance could be a multigenic phenomenon [43], thereby unable to fully explain the resistance mechanism. A study conducted in Bihar state of India to assess the utility of DDT based indoor residual spray found that sand flies were susceptible to deltamethrin but high level of resistance was observed to DDT [44].

In the present study, altogether five pools out of total 98 pools were detected positive using VectorTest™ for the presence of Plasmodium antigen suggesting that both these species play important role in malaria transmission. Four pools of An. vagus were found positive for Plasmodium falciparum revealing that An. vagus might be playing crucial role as malaria vector in the study area. During the past few years An. vagus has emerged as an important malaria vector, reported in large number in India and neighbouring countries [10,12] and incriminated as vector of malaria in India [45] and Bangladesh [12, 13]. An. annularis s.l. although found positive for Plasmodium in only one pool but regarded as important malaria vector in many endemic regions of India [6, 9]. In the present study primary malaria vectors An. minimus and An. dirus did not encounter, however recognised malaria vectors An. culicifacies (N = 68) and An. fluviatilis (N = 14) were recorded in low density and only one pool belonging to An. culicifacies s.l. in BPHC was found positive for Plasmodium falciparum sporozoite (MIR = 1.6). A recent investigation conducted in northeast India has revealed that 21.1% of the wild collected
An. annularis were fed on human blood, while 2.6% were found positive for Plasmodium falciparum malaria parasite [6]. VectorTest™ antigen panel assay has been found effective in monitoring the disease spread by detecting malaria parasite in the wild collected mosquitoes [24, 25]. The used assay is rapid, one step procedure and qualitatively identifies specific peptide epitopes of circumsporozoites of the types of Plasmodium sporozoites. The present results confirm the resistance to DDT and reduced susceptibility of pyrethroid insecticides which could gradually increase and spread into the other areas where complete susceptibility is reported at present. Mosquito control research and comprehensive vector tool development requires thorough analysis of such results and their consequences in a large area. Current study was carried out in two high malaria reporting and logistically accessible areas, however such studies using other malaria vectors should also be conducted in far flung and inaccessible areas which experience considerable toll of malaria related mortality and morbidity annually. Large number of mosquito specimen corresponding to well known and all possible malaria vector species are needed to be tested from different areas to get a clear insight about the role of each vector in malaria transmission in northeast India.

Conclusion

For the first time field collected An. vagus and An. annularis s.l. mosquitoes using such a large sample size were evaluated against different insecticides in northeast India and found completely resistant to DDT, while completely sensitive to deltamethrin. Lambda-cyhalothrin susceptibility was reduced in An. vagus. Further investigations are recommended to understand the mechanism underlying the phenotypical resistance to DDT and declining susceptibility to synthetic pyrethroid in order to guide judicious selection of suitable insecticides for vector control interventions. Resistance management strategies should be identified and considered to delay the expansion of insecticide resistance. Present results strongly advocate that both An. annularis and An. vagus may be playing more important role in malaria transmission than thought previously. Testing of Plasmodium parasite presence in the field caught potential Anopheles vectors prevailing in high density from different areas, in addition to the well established vectors, could be useful to highlight the role of these little known and practically ignored vectors in malaria transmission.

Supporting Information

S1 Table. knock-down of An. annularis and An. vagus in OPHC. (XLS)

S2 Table. Knock-down of An. annularis and An. vagus mosquitoes in BPHC. (XLS)

S3 Table. Data of Vectortest malaria sporozoite panel assay. (XLS)

Acknowledgments

Authors are thankful to Dr Vijay Veer, Ex-Director, Defence Research Laboratory, Tezpur for providing necessary help and technical advice during the entire study. We are grateful to the villagers of the study areas for allowing collection of mosquitoes in the houses during early morning hours. The help and support rendered by the Gaonburhas (village headman) of the study villages and local health workers is deeply acknowledged.
Author Contributions
Conceived and designed the experiments: SD KY DG BR. Performed the experiments: SD KY DG BR SH VT. Analyzed the data: SD KY. Contributed reagents/materials/analysis tools: SD SH. Wrote the paper: SD KY SH VT. Involved in the revision of manuscript during the entire review process: KY SD VT DG BR.

References
1. Constant VA, Koudou BG, Jones CM, Weetman D, Ranson H. Multiple-insecticide resistance in Anopheles gambiae mosquitoes, Southern Cote d'Ivoire. Emerg Infec Dis. 2012; 18(9):1508–1511.
2. Agossa FR, Gnanguenon V, Anagonou R, Azondekon R, Aizoun N, Sovi A, et al. Impact of insecticide resistance on the effectiveness of pyrethroid-based malaria vectors control tools in Benin: Decreased toxicity and repellent effect. PLoS One. 2015; 10(12):e0145207. doi: 10.1371/journal.pone.0145207 PMID: 26674643
3. Ndiath MO, Mazenot C, Sokhna C, Trape JF. How the malaria vector Anopheles gambiae adapts to the use of insecticide-treated nets by African populations. PLoS One. 2014; 9(6):e97700. doi: 10.1371/journal.pone.0097700 PMID: 24892677
4. Yadav K, Dhiman S, Rabha B, Saikia PK, Veer V. Socio-economic determinants for malaria transmission risk in an endemic primary health centre in Assam, India. Infect Dis Pov. 2014; 3:19.
5. Dhiman S, Gopalakrishnan R, Goswami D, Baruah I, Singh L. Malaria epidemiology along Indo-Bangladesh border in Tripura state, India. South East Asian J Trop Med Pub Hlth. 2010; 41(6):1279–1289
6. Dhiman S, Rabha B, Goswami B, Das NG, Baruah I, Bhola RK, et al. Insecticide resistance and human blood meal preference of Anopheles annularis in Assam Meghalaya border, Northeast India. J Vector Borne Dis. 2014; 51:133–136.
7. Mishra AK, Chand SK, Barik TK, Dua VK, Raghavendra K. Insecticide resistance status in Anopheles culicifacies in Madhya Pradesh, central India. J Vector Borne Dis. 2012; 49:39–41. PMID: 22585243
8. Shetty V, Sanil D, Shetty NJ. Insecticide susceptibility status in three medically important species of mosquitoes, Anopheles stephensi, Aedes aegypti and Culex quinquefasciatus, from Bruhat Bengaluru Mahanagara Palike, Karnataka, India. Pest Manag Sci. 2013; 69(2):257–267. doi: 10.1002/ps.3383 PMID: 22926921
9. Dhiman S, Bhola RK, Goswami D, Rabha B, Kumar D, Baruah I, et al. Polymerase chain reaction detection of human host preference and Plasmodium parasite infections in field collected potential malaria vectors. Pathog Glob Health. 2012; 10(3):177–180.
10. Dev V, Sharma VP. The dominant mosquito vectors of human malaria in India. In: Sylvie Manguin, editor. Anopheles mosquitoes: New insights into malaria vectors. Rijeka, Croatia: In Tech. 2013; 239–271.
11. Yadav K, Dhiman S, Rabha B, Goswami D, Saikia PK, Veer V. Disappearance of An. minimus and An. dirus from certain malaria endemic areas of Assam, India. J Arthropod-Borne Dis. 2016; Article in press. Available at http://jad.tums.ac.ir/index.php/jad/article/view/391.
12. Alam MS, Khan MGM, Chaudhury N, Deloer S, Nazib F, Bangali AM, et al. Prevalence of anopheline species and their Plasmodium infection status in epidemic-prone border areas of Bangladesh. Malar J. 2010; 9:15. doi: 10.1186/1756-387x-9-15 PMID: 20074326
13. Bashar K, Tuno K. Seasonal abundance of Anopheles mosquitoes and their association with meteorological factors and malaria incidence in Bangladesh. Parasit Vectors. 2014; 7:442. doi: 10.1186/1756-3305-7-442 PMID: 25233890
14. Singh RK, Haq S, Kumar G, Dhiman RC. Bionomics and vectorial capacity of Anopheles annularis with special reference to India: a review. J Commun Dis. 2013; 45(1–2):1–16. PMID: 25141549
15. WHO. Anophele species complexes in South and South-east Asia. World Health Organization, SEARO Tec Pub. 2007; 57:17–19.
16. Rueda LM, Pecor JE, Harrison BA. Updated distribution records for Anopheles vagus (Diptera: Culicidae) in the Republic of Philippines, and considerations regarding its secondary vector roles in South-east Asia. Trop Biomed. 2011; 28(1):181–187. PMID: 21602785
17. Verhaeghen K, Van Bortel W, Trung H, Sochantha T, Keokenchanh K, Coosemans M. Knockdown resistance in Anopheles vagus, An. sinensis, An. paraliae and An. peditaeniatus populations of the Mekong region. Parasit Vectors. 2010; 3:59. doi: 10.1186/1756-3305-3-59 PMID: 20646327
18. Nath MJ, Bora AK, Yadav K, Talukdar PK, Dhiman S, Baruah I, et al. Prioritizing areas for malaria control using geographical information system in an endemic district of Assam, India. Public Health. 2013; 127:572–580. PMID: 23701814
19. Yadav K, Nath MJ, Talukdar PK, Saikia PK, Baruah I, Singh L. Malaria risk areas of Udalguri district of Assam, India: a GIS-based study. Int J Geogr Inf Sci. 2012; 26(1):123–131.

20. Kumar D, Dhiman S, Rabha B, Goswami D, Deka M, Singh L, et al. Genetic polymorphism and amino acid sequence variation in Plasmodium falciparum GLURP R2 repeat region in Assam, India, at an interval of five years. Malar J. 2014; 13:450. doi: 10.1186/1475-2875-13-450 PMID: 25416405

21. WHO. Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticide on treated surfaces. WHO/CDS/CPC/MAL/98.12. 1998; WHO press, Geneva, Switzerland.

22. WHO. Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets. WHO/CDS/NTD/WHOIES/GCDPP/3. 2006; WHO press, Geneva, Switzerland.

23. WHO. Test procedures for insecticide resistance monitoring in malaria vectors mosquitoes. Geneva, Switzerland: World Health Organization, WHO press 2013; 1–39.

24. Ryan JR, Dave K, Emmerich E, Garcia L, Yi L, Coleman RE, et al. Dipsticks for rapid detection of Plasmodium in vectoring Anopheles mosquitoes. Med Vet Entomol. 2001; 15:225–230. PMID: 11434560

25. Sattabongkot J, Kiattibut C, Kumpitak C, Ponlawat A, Ryan JR, Chan AST, et al. Evaluation of the VecTest malaria antigen panel assay for the detection of Plasmodium falciparum and P. vivax circumsporozoite protein in anopheline mosquitoes in Thailand. J Med Entomol. 2004; 41:209–214. PMID: 15061280

26. Puntener W. Manual for field trials in plant protection. Second edition. Agricultural Division, Ciba-Geigy Limited, 1981.

27. Chouaibou MS, Chabi J, Bingham GV, Knox TB, N'Dri L, Kesse NB, et al. Increase in susceptibility to insecticides with aging of wild Anopheles gambiae mosquitoes from Cote d'Ivoire. BMC Infect Dis. 2012; 12:214. doi: 10.1186/1471-2334-12-214 PMID: 22974492

28. Xu T, Zhong D, Tang L, Chang X, Fu F, Yan G, et al. Anopheles sinensis mosquito insecticide resistance: comparison of three mosquito sample collection and preparation methods and mosquito age in resistance measurements. Parasit Vectors. 2014; 7:54. doi: 10.1186/1756-3305-7-54 PMID: 24472598

29. Yadav K, Rabha B, Dhiman S, Veer V. Multi-insecticide susceptibility evaluation of dengue vectors Stegomya albopicta and St. aegypti in Assam, India. Parasit Vectors. 2015; 8:143. doi: 10.1186/s13071-015-0754-0 PMID: 25886449

30. Tikar SN, Mendki MJ, Sharma AK, Sukumaran D, Veer V, Prakash S, et al. Resistance status of the malaria vector mosquitoes, Anopheles stephensi and Anopheles subpictus towards adulticides and larvicides in arid and semi-arid areas of India. J Insect Sci. 2011; 11:85. doi: 10.1673/031.011.8501 PMID: 21870971

31. Raghavendra K, Verma V, Srivastava HC, Gunasekaran K, Sreedari U, Dash AP. Persistence of DDT, malathion & deltamethrin resistance in Anopheles culicifacies after their sequential withdrawal from indoor residual spraying in Surat district of Gujarat India. Indian J Med Res. 2010; 132:260–264. PMID: 20847371

32. Betson M, Jawara M, Awolola TS. Status of insecticide susceptibility in Anopheles gambiae s.l. from malaria surveillance sites in The Gambia. Malar J. 2009; 8:187. doi: 10.1186/1475-2875-8-187 PMID: 19656399

33. Ndiath MO, Sougoufara S, Gaye A, Mazenot C, Faye O, Sokhna C, et al. Resistance to DDT, malathion & deltamethrin resistance in Anopheles gambiae mosquitoes from Cote d'Ivoire. BMC Infect Dis. 2012; 12:214. doi: 10.1186/1471-2334-12-214 PMID: 22974492

34. Dhiman S, Veer V. Culminating anti-malaria efforts at long lasting insecticidal net?. J Inf Pub Hlth. 2014; 7:457–464.

35. Nkya TE, Akhouayri I, Poupardin R, Batengana B, Mosha F, Magesa S, et al. Insecticide resistance mechanisms associated with different environments in the malaria vector Anopheles gambiae s.l. in The Gambia. Malar J. 2014; 13:28 doi: 10.1186/1475-2875-13-28 PMID: 24469052

36. Ranson H, N’Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends Parasitol. 2011; 27 (2):91–98. doi: 10.1016/j.pt.2010.08.004 PMID: 20843745

37. Protopopoff N, Matowo J, Malima R, Kavishe R, Wright A, et al. High level of resistance in the mosquito Anopheles gambiae to pyrethroid insecticides and reduced susceptibility to bendiocarb in north-western Tanzania. Malar J. 2013; 12:149. doi: 10.1186/1475-2875-12-149 PMID: 23638757

38. Mitchell SN, Rigden DJ, Dowd AJ, Lu F, Wilding CS, Weetman D, et al. Metabolic and Target-Site Mechanisms Combine to Confer Strong DDT Resistance in Anopheles gambiae. PLoS One. 2014; 9 (3):e92662. doi: 10.1371/journal.pone.0092662 PMID: 24675797
39. Singh OP, Dykes CL, Sharma G, Das MK. L1014F-kdr mutation in Indian Anopheles subpictus (Diptera: culicidae) arising from two alternative transversions in the voltage-gated sodium channel and a single PIRA-PCR for their detection. J Med Entomol. 2015; 52(1):24–27. doi: 10.1093/jme/jtu013 PMID: 26336276

40. Riveron JM, Yunta C, Ibrahim SS, Djouaka R, Irving H, Menze BD, et al. A single mutation in the GSTe2 gene allows tracking of metabolically based insecticide resistance in a major malaria vector. Genome Biol. 2014; 15:R27 doi: 10.1186/gb-2014-15-2-r27 PMID: 24565444

41. Nwane P, Etang J, Chouaibou M, Toto JC, Kerah-Hinzoumbe C, Mimpfoundi R, et al. Trends in DDT and pyrethroid resistance in Anopheles gambiae s.s. populations from urban and agro-industrial settings in southern Cameroon. BMC Inf Dis. 2009; 9:163.

42. Bass C, Nikou D, Donnelly MJ, Williamson MS, Ranson H, Ball A, et al. Detection of knockdown resistance (kdr) mutations in Anopheles gambiae: a comparison of two new high throughput assays with existing methods. Malaria J. 2007; 6:111.

43. Brooke BD. Kdr: Can a single mutation produce an entire insecticide resistance phenotype? Trans Roy Soc Trop Med Hyg. 2008; 102:524–525. doi: 10.1016/j.trstmh.2008.01.001 PMID: 18295809

44. Coleman M, Foster GM, Deb R, Singh RP, Ismail HM, Shivam P, et al. DDT-based indoor residual spraying suboptimal for visceral leishmaniasis elimination in India. PNAS. 2015; 112(28):8573–8578. doi: 10.1073/pnas.1507782112 PMID: 26124110

45. Prakash A, Bhattacharyya DR, Mohapatra PK, Mahanta J. Role of the prevalent Anopheles species in the transmission of Plasmodium falciparum and P. vivax in Assam state, north-eastern India. Ann Trop Med Parasitol. 2004; 98(6):559–68. PMID: 15324463