Susceptibility to common insecticides and detoxifying enzyme activities in Anopheles sundaicus (sensu lato) after cessation of indoor residual spraying of insecticides in the Jaffna Peninsula and its surroundings in northern Sri Lanka

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Abstract

Background: Sri Lanka has been malaria-free since 2013 but re-introduction of malaria transmission by infected overseas travelers is possible due to a prevalence of potent malaria vectors. Knowledge of the insecticide resistance status among Anopheles vectors is important if vector control has to be reintroduced in the island. The present study investigated the insecticide susceptibility levels and resistance mechanisms of Anopheles sundaicus (sensu lato) (previously classified as Anopheles subpictus species B) an important malaria vector in the Jaffna Peninsula and its surroundings in northern Sri Lanka after indoor residual spraying of insecticides was terminated in 2013.

Results: Species-specific PCR assays identified An. sundaicus (s.l.) in four locations in the Jaffna and adjacent Kilinochchi districts. Bioassays confirmed that An. sundaicus (s.l.) collected in Kilinochchi were completely susceptible to 0.05% deltamethrin and 5% malathion and resistant to 4% dichlorodiphenyltrichloroethane (DDT), whereas those from Jaffna were relatively susceptible to all three insecticides. Kilinochchi populations of An. sundaicus (s.l.) showed significantly higher glutathione S-transferase activity than population from Jaffna. However, Jaffna An. sundaicus (s.l.) had significantly higher Propoxur-resistant acetylcholinesterase activity. Activities of non-specific esterases and monooxygenases were not significantly elevated in An. sundaicus (s.l.) collected in both districts.

Conclusions: The susceptibility to malathion and deltamethrin in An. sundaicus (s.l.) suggests that they can be still used for controlling this potential malaria vector in the Jaffna Peninsula and adjacent areas. Continuing country-wide studies on other malaria vectors and their insecticide susceptibilities are important in this regard.

Keywords: Anopheles sundaicus complex, Jaffna Peninsula, Insecticide-detoxifying enzymes, Insecticide resistance bioassays, Malaria, Mosquito vectors, Sri Lanka
Background
Malaria had been endemic in Sri Lanka for centuries until indigenous transmission was eliminated from the island in 2013 [1]. However, many cases of malaria-infected travelers arriving from endemic countries are reported every year and therefore the potential for resuming indigenous transmission remains high due to the prevalence of many anopheline vectors in the island [1]. This challenge is exacerbated by the recent spread from India of the efficient urban malaria vector Anopheles stephensi to Sri Lanka [2, 3]. The Jaffna Peninsula in the Jaffna District and areas surrounding the Jaffna lagoon in the Kilinochchi District in northern Sri Lanka are coastal areas that were badly affected by malaria during the three decades of armed conflict that ended in 2009. The Anti-Malaria Campaign (AMC) in the north, specifically in the Kilinochchi District, faced logistic problems and a shortage of resources for its malaria control activities during the war.

Anopheles culicifacies species E was the major malaria vector in Sri Lanka while An. annularis, An. subpictus (s.l) and An. sundaicus (s.l) functioned as important secondary vectors together with other minor vectors before the elimination of malaria [4–10]. Anopheles subpictus exists as a species complex with members showing different bio-ecological traits relevant to malaria transmission [11, 12]. However, molecular genetic characterization of the An. subpictus complex after 2010 showed that mosquitoes previously identified morphologically as sibling species A, C and D belonged to a single group termed species A, while sibling species B belonged to the An. sundaicus complex [9, 10], a major vector of malaria in coastal zones of many Southeast Asian countries [13]. In Sri Lanka too An. sundaicus (s.l) is mainly found in coastal zones [9, 10], which include the 1130 km² Jaffna Peninsula and areas in the Jaffna and Kilinochchi districts that surround the Jaffna Lagoon [14].

Kilinochchi and Jaffna districts in the Northern Province were among the previously malaria endemic administrative districts. The civil war of 1983–2009 in north and east of Sri Lanka limited studies on malaria vectors to a few in the Jaffna District. These studies suggested that morphologically characterized An. subpictus (s.l) was the predominant anopheline species in Jaffna [15, 16] with a higher sporozoite rate than An. culicifacies [17]. The An. sundaicus (s.l) identified at that time exclusively by morphology as An. subpictus species B, was the predominant anopheline species collected in 2008 from locations in the Jaffna District [15]. It was also found in 2008 that An. subpictus species B [now regarded as An. sundaicus (s.l)] as well as An. subpictus species C and D (now regarded as An. subpictus A) were susceptible to 5% malathion but highly resistant to 4% dichlorodiphenyltrichloroethane (DDT) [15].

We previously reported insecticide susceptibility and resistance mechanisms in members of the An. subpictus complex, including sibling species B/An. sundaicus (s.l), collected from sites in the North Western and Eastern provinces of Sri Lanka (Fig. 1) [18], but the study excluded the Northern Province because of the ongoing civil war. We have now extended this study to

Fig. 1 Locations of study sites. a Location of Sri Lanka in relation to India. b Administrative boundaries of Northern Province (NP), Eastern Province (EP) and North Western Province (NWP). c Locations of sample collection sites (1–4) in the districts of Jaffna and Kilinochchi.
An. sundaicus (s.l.) collected from sites in the Jaffna Peninsula or its vicinity in the districts of Jaffna and Kilinochchi in the Northern Province. Understanding the insecticide resistance status and its biochemical basis in malaria vectors is important for vector control should indigenous malaria transmission re-emerge in the country.

Methods

Study locations, sample collection and species identification

Adult anopheline mosquitoes were collected monthly for 15 months from December 2014 to February 2016 using cattle baited hut (CBH), cattle baited net (CBN) and indoor (IC) collection techniques [9, 10, 18] from three locations in the Kilinochchi District (Palai, Kandavalai and Iranaimadu) and one location (Kudathanai) in the Jaffna District (Fig. 1). These study sites were selected based on their previous malaria endemicity and coastal proximity. Each location had two or more mosquito collection points: 1. Kudathanai (3.4 km from the nearest coast) had four mosquito collection points (9°44′47.5332″N, 80°16′19.9956″E; 9°44′49.2828″N, 80°16′21.9288″E; 9°44′49.2432″N, 80°16′25.1328″E; and 9°44′44.9052″N, 80°16′24.532″E); 2. Palai (3 km from the nearest coast) had three mosquito collection points (9°36′26.838″N, 80°19′14.3892″E; 9°36′34.6032″N, 80°19′55.8228″E; and 9°36′20.4372″N, 80°19′51.0312″E); 3. Iranaimadu (16 km from the nearest coast) had two mosquito collection points (9°27′14.1444″N, 80°29′9.7944″E; 9°27′38.2212″N, 80°29′13.1964″E; and 9°27′12.618″N, 80°29′38.5332″E).

Larval collections (LC) were carried out at each location from stagnant water bodies, e.g. sand pools and ponds, with 350 ml dippers and then reared as described previously under contained conditions in the insectary of the Department of Zoology, University of Jaffna to reach adulthood [18]. Larvae were maintained under laboratory conditions (28 ± 2 °C, 12h photoperiod and ~70% relative humidity) in the same water from the habitats where they were collected in 1.5 l capacity plastic trays with powdered fish meal given twice a day as additional food.

The collected anopheline mosquitoes and emerging adults from LC were identified morphologically as previously described [10, 18, 19]. Morphologically-identified blood-fed An. subpictus (s.l.) adults were maintained in the insectary to obtain F1 progeny as described previously [20]. Adults emerged from LC and F1 progeny of blood-fed An. subpictus (s.l.) mosquitoes were transferred to adult mosquito cages and fed on sugar pledges. Other anopheline species identified in the collections were not processed for analysis. Three- to five-day-old An. subpictus (s.l.), the F1 progeny of field-collected adult females, as well as adults obtained from field-collected larvae were used for insecticide bioassays, biochemical assays and DNA-based identification.

Bioassays for insecticide susceptibility

The standard World Health Organization (WHO) procedures were followed to determine the insecticide susceptibility status of adult mosquitoes [21]. Non-blood fed adult mosquitoes from the F1 progeny of field-collected blood-fed female mosquitoes and those developing from field-collected larvae in the Kilinochchi and Jaffna districts respectively, were pooled and separately tested for each district collection in duplicate assays with the WHO discriminating dosages of 0.05% deltamethrin, 5% malathion and 4% DDT using WHO bioassay test kits as previously described [18]. The WHO criteria were used to define a population as susceptible (>98% mortality), suspected for resistance (90–98% mortality) and resistant (<90% mortality) [22].

A total of 76 mosquitoes collected from the Jaffna (n = 37) and Kilinochchi (n = 39) districts identified morphologically were confirmed by PCR. This included 19 and 24 blood-fed females collected in the field at the sites in the Jaffna and Kilinochchi districts respectively that gave rise to F1 adult progeny used in the insecticide bioassay and enzyme assays in addition to adults that emerged from larval collections. A total of 397 and 305 adults derived from F1 progeny and field-collected larvae from Kilinochchi and Jaffna districts, respectively, were used for bioassays. These were made up of 66 and 90% of the F1 progeny of the adults that were used for species-specific PCR assays from Jaffna and Kilinochchi districts, respectively. Similarly, out of the 80 mosquitoes used for enzyme assays, 84 and 68% were from the F1 progeny of the blood-fed adults that were tested for species identification from sites in the Jaffna and Kilinochchi districts, respectively.

Biochemical assays

Adult female mosquitoes emerging from F1 progeny and LC collections were subjected to biochemical assays using the microplate method described previously [18]. Eighty individuals from each district were subjected individually to total protein, carboxylesterase (EST), glutathione S-transferase (GST), monooxygenase (MO) and acetylcholinesterase (AChE) assays as described previously [18]. Specific activities of > 0.25 μmol/mg/min for EST, > 0.40 μmol/mg/min for GST, and > 0.35 equivalent units for MO were considered to be discriminating activity levels that can contribute to metabolic resistance in An. subpictus (s.l.) in Sri Lanka [18, 23]. According to WHO guidelines, remaining AChE activities after
Propoxur inhibition in > 70%, 30–70% and < 30% of the population were categorized as homozygous resistant (RR), heterozygous resistant (RS) and homozygous susceptible (SS), respectively [24].

**Allele-specific PCR assay (AS-PCR) to distinguish An. sundaicus from An. subpictus**

DNA from adult females that gave F1 progeny and individual mosquitoes emerging from field-collected larvae was extracted using Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The extracted DNA was used for diagnostic allele specific PCR as described previously [10]. The diagnostic size of the PCR product for An. subpictus species A was ~300 bp while that for An. sundaicus (s.l.) was ~400 bp as previously reported [10].

**Data analysis**

The two-tailed Student’s t-test for matched samples was performed to determine significant differences between the Kilinochchi and Jaffna mosquito populations in the susceptibility to insecticides and enzyme activities of GST, EST and MO. The Chi-square test was performed to assess significant differences in proportions of the three categories of AChE (SS, RS and RR) between mosquitoes collected in the two districts.

**Results**

During the 15 month study period a total of 980 and 752 adults were morphologically identified as An. sundaicus (s.l.) from collections in the Kilinochchi and Jaffna districts, respectively. Anopheles annularis, An. barbirostis, An. culicifacies (s.l.), An. jamsai and An. psedojamsai were present in the collections but not used in the studies. The collected An. subpictus (s.l.) from both districts collectively comprised 53, 25, 19 and 3% of mosquitoes collected by LC, CBN, CBH and IC, respectively.

Mosquitoes collected from Kilinochchi showed 100% mortality to both 0.05% Deltamethrin and 5% malathion and but only 31% mortality to 4% DDT indicating resistance to DDT. Although mosquitoes collected from Jaffna showed high mortality with deltamethrin (97%), malathion (96%) and DDT (91%), the results indicate the possibility of some resistance to all three insecticides according to the WHO criteria [21, 22]. The Kilinochchi population showed significantly higher resistance to DDT than Jaffna population, but there were no statistically significant differences in susceptibility to deltamethrin and malathion between the two districts (Table 1).

The enzyme activities and the percentage of mosquito populations that had enhanced enzyme activities are shown in Table 2. Although significantly different activities of EST ($t_{150}=3.76, P < 0.001$) and MO ($t_{150}=15.53, P < 0.001$) were observed between the Kilinochchi and Jaffna population, neither population alone or collectively had activities of the two enzymes above the discriminatory levels for resistance reported for Sri Lankan An. subpictus (s.l.) [23]. Significantly ($t_{146} = -16.98, P < 0.001$) elevated GST activities above the reported discriminatory levels were seen in all of the Kilinochchi population compared with only 30% of the Jaffna population. The results of AChE assays to detect the percentage remaining activity of AChE in the presence of Propoxur are also presented in Table 2. The Chi-square test revealed a significant ($\chi^2 = 13.41, P = 0.0012$) association between the districts and the remaining activity of AChE in the three WHO categories of resistance, with the Jaffna An. sundaicus (s.l.) mosquitoes showing a greaterAChE active site alteration than in Kilinochchi.

**Discussion**

Because the PCR assays (Fig. 2) revealed that all 76 tested specimens belonged to An. sundaicus (s.l.), and all An. subpictus species B-like mosquitoes recently independently identified through existing morphological criteria in coastal and inland northern Sri Lanka were shown genetically belong to the An. sundaicus complex [9, 10], it is reasonable to assume that the vast majority, if not all the mosquitoes tested in the insecticide bioassay and enzymatic assays, are An. sundaicus (s.l.) and not An. subpictus species A. To our knowledge, the present study is the first to investigate insecticide resistance and insecticide resistance mechanisms in An. sundaicus (s.l.) in the Northern Province of Sri Lanka.

The greater resistance to DDT of mosquitoes from Kilinochchi compared to Jaffna may be due the higher prevalence of elevated GST activity in Kilinochchi compared with Jaffna. DDT resistance in morphologically identified An. subpictus (s.l.) was first reported in 1969 and a reduction in resistance was detected after

### Table 1 Mortality in An. sundaicus (s.l.) exposed to three insecticides

| Insecticide | Mean % mortality ± SD (no. of mosquitoes tested) | t-value | P-value |
|-------------|-----------------------------------------------|---------|--------|
| Kilinochchi | Jaffna                                        |         |        |
| Deltamethrin (0.05%) | 100 (110) | 97.3 ± 3.8 (100) | $t_{10}=3.0$ | 0.09 |
| Malathion (5%) | 100 (136) | 96.4 ± 0.7 (105) | $t_{102} = -2.0$ | 0.18 |
| DDT (4%) | 30.9 ± 8.7 (151) | 91.2 ± 0.3 (100) | $t_{10}=3.1$ | 0.01 |

**Abbreviations:** S susceptible (≥ 98% mortality), R confirmed resistance (< 90% mortality), V possible resistance and verification needed (90–97% mortality) [22], SD standard deviation
cessation of DDT use for IRS and its replacement with malathion in the early 1970s [1, 25]. Later, due to the development of a GST-based resistance mechanism, which was suggested to be favored by high DDT application prior to malathion introduction, an increase in the DDT-resistant population was observed among An. subpictus complex [suspected to be a mixture of An. subpictus species A and An. sundaicus (s.l.)] after 1983 [26, 27]. No elevation of EST or MO was detectable in the two populations, suggesting that EST and MO do not contribute to DDT resistance in the two districts.

Anopheles sundaicus (s.l.) populations, except that of Northern Province, are reported to have developed resistance to pyrethroid insecticides in other parts of the country [23]. Perhaps the relatively limited previous use of pyrethroids for vector control in the two northern districts for IRS and the absence of elevated ES and MO might be the reason for the observed relative susceptibility to deltamethrin. The results suggest that the Jaffna An. sundaicus (s.l.) mosquitoes may show weak resistance to deltamethrin but confirming this and the investigating potential underlying mechanisms requires more extensive investigation. The higher percentage remaining activity of AChE seen in the Jaffna population may be due to a continuing and more intensive use of organophosphate and carbamates pesticides for agriculture in the Jaffna Peninsula. Further studies with more sampling sites from both districts are needed to establish this.

Indoor residual spraying (IRS) was, until recently, the principal method of malaria vector control in Sri Lanka. Sri Lanka has undertaken different insecticide regimes at different times over the last six decades for malaria control.
control [18, 23]. DDT introduced at the end of World War 2 for IRS was highly effective in controlling malaria until resistance developed in the 1960s and 1970s, causing it to be replaced by the organophosphate malathion in 1977. Pyrethroids have been used for IRS since 1994 on the whole island except for the Northern Province due to the development of resistance to malathion [1]. However, IRS has been scaled down or has ceased since 2013 in the island and is now only performed in the vicinity of the residences of persons identified to have contracted malaria abroad.

In northern Sri Lanka, malaria control activities were curtailed in the Jaffna and Kilinochchi districts during the civil war as the regional AMC faced logistical problems and a shortage of resources. Vector control activities in the Jaffna and Kilinochchi districts in the Northern Province were mainly restricted to IRS with malathion until 2002 when it was replaced by the pyrethroid deltamethrin [15].

High susceptibility to common insecticides shown by An. sundaicus (s.l.) populations from other parts of the country was attributed to its exophagic and exophilic nature [18]. However, collection of An. sundaicus (s.l.) in IC and CBH techniques during the present and an earlier study [15], along with high sporozoite rates [17], indicates some endophagic and endophilic behavior in northern Sri Lanka, for which IRS and insecticide-treated bed nets can be effective. Our previous study on the resistance and resistance mechanisms in Eastern and North Western provinces in coastal areas of mainland Sri Lanka (Fig. 1) suggested that An. sundaicus (s.l.) populations were resistant to DDT but relatively susceptible to malathion and pyrethroids [18], compatible with the present observations in Northern Province. Cessation of malathion use for IRS since 1994 on the whole island and in Northern Province in 2002, along with the careful use of pyrethroids for IRS and its cessation in 2006, may contribute to the continued relative susceptibility to the two insecticides. Development of resistance is associated with a fitness cost and mosquito populations can in time lose resistance in the absence of insecticide selection pressure [28, 29]. It is pertinent, however, that organophosphate and pyrethroid insecticides continue to be used for agricultural purposes and personal protection, respectively, in Sri Lanka [30], and this might eventually contribute to the development of resistance to the two classes of insecticides.

Conclusions
The results suggest that malathion and deltamethrin may still be effectively used to control Anopheles sundaicus (s.l.) in the Jaffna and Kilinochchi districts but indicate the need for continued monitoring of insecticide resistance in the two districts and elsewhere in the country.
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