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Insecticide Susceptibility status of *An. gambiae s.l.* (Culicidae: Giles) from selected in-land and coastal agricultural areas of Ghana

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Abstract
Malaria, a mosquito-borne disease is preventable and treatable, provided the right steps are taken to control it; it is regarded as today's biggest killer of children in Africa. In Ghana, *A. gambiae s.l.* plays an important role in the transmission of this disease. This study investigates the insecticide resistance status of *A. gambiae s.l.* collected from two different agricultural areas: Coastal and In-land in Ghana. Samples of *Anopheles* larvae were collected and reared to adults, after which susceptibility tests were performed on F1 female *A. gambiae* s.l. using standard WHO insecticide testing procedures by exposing the mosquitoes to Bendiocarb 0.1%, DDT 4.0%, Alphacypermethrin 0.5% and Pirimiphos methyl 5.0% at diagnostic doses [1]. Knock down and mortality counts at different time intervals were further carried out on the samples.
Both populations of *An. gambiae* s.l. from Inland and coastal areas were resistant to all tested insecticides. However, the Coastal, Korle-Bu population was more resistant to Alphacypermethrin (12.2% mortality) and DDT (71.1% mortality) than the coastal, Okyereko population, 50% and 10% for the same insecticides respectively. Mortality (245hr) was higher in Korle-Bu than in Okyereko. With the exception of Pirimiphos methyl and Bendiocarb, resistance of *A. gambiae* s.l. from the inland areas to all the insecticides was significantly higher than the coastal areas although vegetable growing is practiced in both areas. Information on the resistance mechanisms and species composition underlying the pattern of resistance in both areas is recommended.

Keywords: *Anopheles gambiae* s.l., insecticide resistance, inland, coastal, Ghana

1. Introduction
Malaria is endemic and perennial in all parts of Ghana and there are seasonal variations that are more prominent in the northern part of the country [1, 2]. The entire population of Ghana is at risk of malaria, children less than the age of five years and pregnant women are more prone to the severity of illness due to this disease (because of lowered, weak or compromised immunity). Between 3.1 and 3.5 million annual cases of clinical malaria are reported in public health facilities, of which 900,000 cases are in children under five years and 3,000-4,000 of them result in inpatient deaths (Ministry of Health, Ghana, unpublished report, 2003) [2].
Malaria prevention encompasses a variety of measures that may protect people against infection or the development of this disease in individuals. The World Health Organization supports insecticide based vector control activities as effective protective measures against malaria [3]. Individuals or households can practice a myriad of personal protective measures such as the use of protective clothing, repellents, bed nets or insecticides or environmental management to control transmission. Attempts are being made towards the production of malaria vaccines [3].
A. gambiae species complex and *A. funestus* are the major malaria vectors in Ghana [4]. These species usually rest indoors and bite late in the night and are mostly found in the rural and peri-urban areas [5]. The outdoor biting behavior of these mosquitoes is a common phenomenon in the northern savannah of Ghana [6]. *A. gambiae* complex is anthropophilic and utilizes different habitats that are indirectly or directly created by humans and their activities such as buildings [7], temporal pools, hoof prints of livestock and permanent water bodies created by construction works, rice fields and irrigation canals [8].

As part of efforts to control malaria in Africa, vector control activities are being undertaken in many countries. However, in 1993 resistance of African malaria vectors to pyrethroids was detected [9-10]. Available evidence over the years reveals that resistant genes to pyrethroid insecticides have been spreading rapidly and are now widespread in African malaria vectors, including *A. gambiae* s.l. [9]. The spread of insecticide resistance poses a challenge to the continued efficacy of current malaria control tools and is worsened by the indiscriminate use of insecticides in controlling both agricultural pests and vectors that threaten public health [9]. Suspected resistance was reported in Obuasi (Ashanti Region) and Kassena-Nankana (Upper East Region) districts of Ghana [11-13]. Results of susceptibility tests published for Obuasi in 2006 have shown much lower mortality rates than the 2010 results given for pyrethroids and carbamates [14]. One way to test the efficacy of insecticides is to determine the susceptibility status of the vector population in the area to new insecticides [15].

Bioassay studies on populations of *An. gambiae* complex from south-western Ghana showed resistance to both DDT and permethrin [16]. Another study in localities from coastal to far northern Ghana showed the occurrence and distribution of kdr mutation in *A. gambiae* complex but insecticide bioassays were not carried out [17]. Consequently, other studies have been conducted on resistance of *A. gambiae s.l.* to some insecticides in Ghana [18], but excluding some of the insecticides tested in this research. There is need for an environmentally benign and highly potent insecticide with long-lasting persistence; therefore, organophosphates and carbamates have been considered as alternatives [19].

At present, information regarding the current resistance status of malaria vectors to the selected insecticides from four different insecticide classes in the irrigation zones of the Greater Accra and Central Regions of Ghana is unknown. In order to achieve the above-stated objective, this research aims at determining the current resistance status of malaria vectors of Ghana (predominantly *A. gambiae s.l.*) by testing vectors from In-land and Coastal regions of Agricultural origin in the Greater Accra and Central regions.

2. Materials and Methods

Anopheles larvae samples were collected from In-land and Coastal sites, raised to adults, after which susceptibility tests were performed on the females using four different insecticides (Bendiocarb-0.10%, DDT-4.00%, Alphacypermethrin-0.50% and pirimiphosmethyl-5.00%) at diagnostic doses [3]. Knock down resistance (Kdr) was further carried out on the samples.

2.1 Study areas

The study was conducted in Korle-Bu (5 º 32' N, 0 º 13' W), a coastal community and Okyereko (5 º 25'N, 0 º 35' W), an inland community. These communities are areas located in the Southern part of Ghana (Fig. 1). The two areas (located within and nearby Accra) are vegetable growing zones and the inhabitants are predominantly farmers. Korle-Bu is located in the coastal savanna ecological zone, which is characterized by dry climatic conditions [4]. It has two rainfall peaks, the first occurring from April to June and the second from September to October [20]. The region receives rainfall between 740 mm and 890 mm each year. The lowest mean monthly temperature (about 26 ºC) is recorded during August and the highest (about 30 ºC) between March and April. The relative humidity throughout the year ranges between 65% and 75%. Some streams and ponds usually dry up in the dry season (There are two main seasons: Dry and wet). Okyereko on the other hand, is a village located about 50 km west of the coast in Accra, (5 º 25'N, 0 º 35' W), (Fig. 1). It has coastal savanna vegetation; annual average rainfall of 750 mm. The irrigation project at Okyereko has an area of about 1,685 km^2. The tributary of River Ayensu feeds the reservoir. Two canals on the left and right banks of the tributary convey water to the irrigation area for agricultural works.

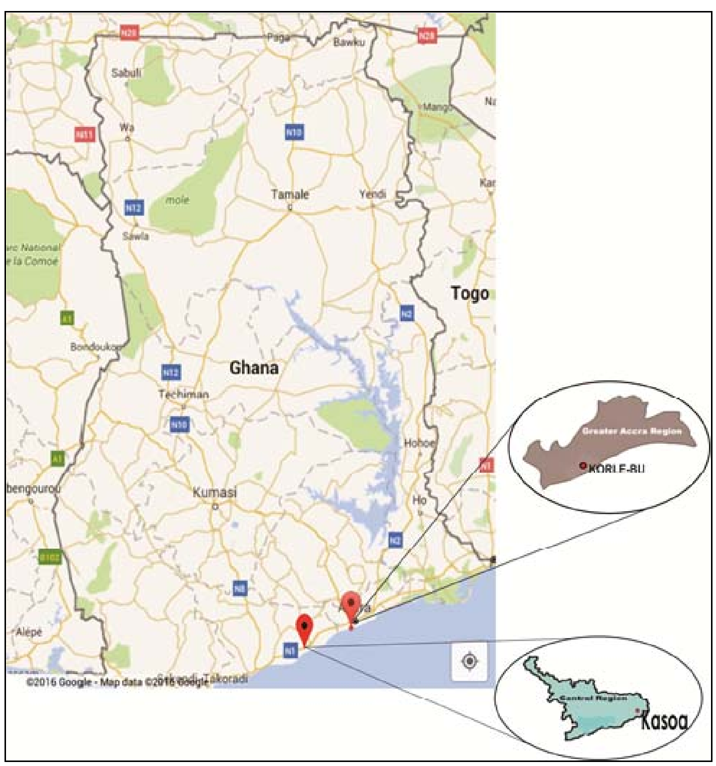

Fig 1: Map showing the study areas in Ghana

2.2 Sampling Methods

This study involved a cross-sectional survey which was carried out from September, 2014 to November, 2015. Mosquito larvae were collected using a dipper with a wooden handle, from diverse habitats (within the study sites), including shallow pools of water, temporal collections of water after rains, construction and sand winning sites, gutters, vegetable farming sites, rice farms and the edges of slow running streams. Most habitats had floating vegetation. The larvae were transported and reared to adulthood at the insectary of the Noguchi Memorial Institute for Medical Research, Legon-Ghana for susceptibility testing.

2.3 Laboratory rearing of mosquitoes

At the insectary of Noguchi Memorial Institute for Medical Research (NMIMR), water with pre-adult mosquitoes (larvae or pupae) were poured into plastic trays (5cm x 27cm x 36cm) to a depth of approximately 2cm and each tray was labeled to indicate the site and date of collection. The trays containing mosquito larvae were kept at (27-31) ºC and 62% relative humidity with a 12:12h light and dark cycle. Every two days, about 200mg of ground Vitakraft (Mix Bremen Germany, Impression 3) premium color flake-mix was used to feed the larvae. The development of larvae was monitored regularly and all those that had pupated were collected into shallow plastic cups /small beakers using Pasteur pipettes, and then placed in appropriately labeled cages for adult emergence. All emerged mosquitoes were fed on 10% sugar solution imbibed in balls of cotton wool. Only 2-5 days old sugar fed female adult mosquitoes were picked from the cages and used for bioassays. Those pupae that escaped our sight, remained in the trays and have emerged were trapped in the cover net and carefully introduced into the main cage by simply opening one end of the tray and allowing them to enter the cage. The adult mosquitoes were identified into species using the criteria of Gilles and deMeillon, (1968) [3] and *An. gambiae* s.l. were sorted into cages.

2.4 WHO Insecticide Susceptibility tests

Four (4) replicates of 20 to 25 aspirated unfed F1 female mosquitoes were used for susceptibility tests (including 2 controls). Four insecticides i.e. Alphacypermethrin 0.5%, Bendiocarb 0.1%, Pirimiphos methyl 5% and D.D.T. 4% were tested against the mosquitoes at diagnostic doses using WHO procedure [10]. The WHO criteria for determining resistance status of mosquitoes was applied, i.e. 98–100% mortality indicates susceptibility, 90% mortality suggests resistance and 90–97% mortality requires confirmation of resistance.

Abbott's formula was used to correct for mortality whenever control mortality was between 5 and 20% [10]. Probit analysis of SPSS was used to determine the KD_{50} and KD_{95} times.

2.5 Ethical consideration

Strict adherence was paid to ethical parameters in the study areas. The study protocol was approved by TUMS and NMIMR ethical Committees and all other ethical issues including confidentiality of research participants were adhered to at all stages of the protocol implementation.

3. Results

A total of 973 female *Anopheles* mosquitoes were used for the bioassays. Based on WHO criteria, the *Anopheles* mosquitoes were resistant to all insecticides at both study sites except Okyereko where suspected resistance was recorded for pirimiphos methyl with a mortality of 90.5% (Table 2, Fig. 2) [3]. Levels of resistance of *An. gambiae* s.l. to the selected insecticides varied between the in-land and coastal areas. Korle-Bu (Coastal area) recorded 52.5% (Standard Error=1.2) 24 hour mortality to Alphacypermethrin whilst 88.9% (Standard Error =0.3) was recorded for Okyereko (In-land area). The In-land area had a comparatively higher mortality to Alphacypermethrin, indicating a relatively lower resistance to this insecticide. Of interest is DDT which is highly resistant in many areas of Ghana. The level of resistance was higher in Korle-Bu (12.2% Mortality, Standard Error=0.9) in comparison with the coastal area, Okyereko- the in-land site (Fig. 2). Mortality against Bendiocarb at both study sites was same (12.3% Mortality, Standard Error=0.9). There was a significant difference (P‹0.01) in 24 hr mortalities for Alphacypermethrin, Bendiocarb and DDT from both study sites, with Okyereko (In-land area) recording higher values of mortality (Fig. 2). However, mortality for Pirimiphos methyl (83.3%) was lower in Okyereko as compared with Korle-Bu (90.5%), the Coastal area. Error bars represent 95% confidence interval (CI)

)land-Mean knocked down rate for the 4 insectcides at both localities (Coastal and In-1Table

Locality	Insecticide	Total mosquitoes tested	Total KD (30 mins)	Total KD (60 mins)	Mean KD ± SE (30 mins)	Mean KD ± SE (60 mins)
Korle-Bu(Coastal)	Alphacypermethrin	80	80	80	100.0±0.0	100.0±4.3
	Primphos-methyl	84	0	11	0.0±0.0	13.1±0.8
	Bendiocarb	81	13	20	16.0±0.5	24.7±4.1
	DDT	82	1	1	1.2±0.3	1.2±0.3
Okyereko(In-land)	Alphacypermethrin	81	81	81	100.0±0.0	100.0±0.0
	Primphos-methyl	78	2	17	2.6±0.3	21.8±0.7
	Bendiocarb	81	0	21	0.0±0.0	25.0±4.3
	DDT	76	3	4	3.9±0.3	53.0±0.5

Table 2: Suceptibility level of *An. gambiae* s.l. exposed to four insecticide impregnated papers using WHO's recommended method, conducted in two agricultural areas (Coastal and In-land), Ghana, West Africa, 2015-2016

Locality	Insecticide	Treated group			Untreated group	
		Total mosquitoes tested	Total dead	Mortality rate (%) ± SE	Total mosquitoes tested	Mortality rate (%)
Korle-Bu	Alphacypermethrin	80	42	52.5 ± 1.2	40	0.0
	Pirimphos-methyl	84	76	90.5 ± 0.4	40	0.0
	Bendiocarb	81	10	12.3 ± 0.3	40	0.0
	DDT	82	10	12.2 ± 0.9	40	0.0
Okyereko	Alphacypermethrin	81	72	88.9 ± 0.3	40	0.0

	Primphos-methyl	78	65	83.3 ± 1.7	40	0.0
	Bendiocarb	81	10	12.3 ± 0.3	40	0.0
	DDT	76	54	71.1 ± 2.9	40	0.0

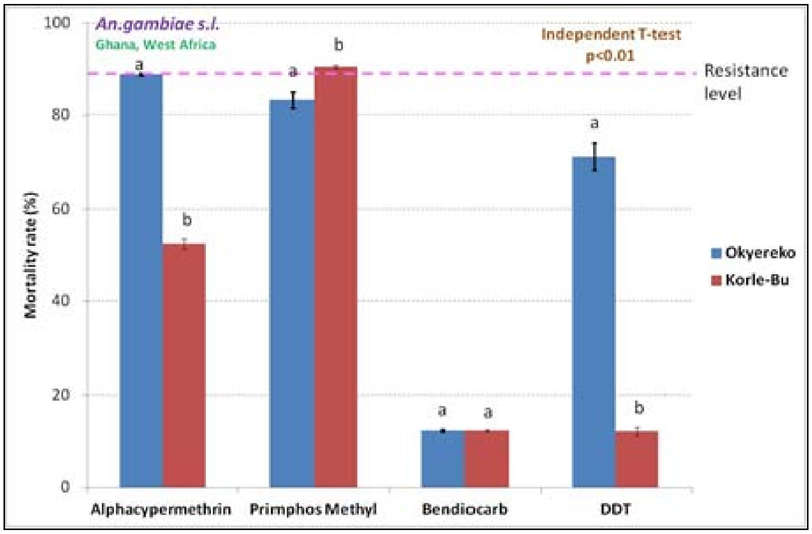

Fig 2: Comparison of suceptibility level of *An. gambiae* s.l. exposed to four insecticides using WHO's recommended method in two agricultural areas, Ghana, West Africa, 2015-2016

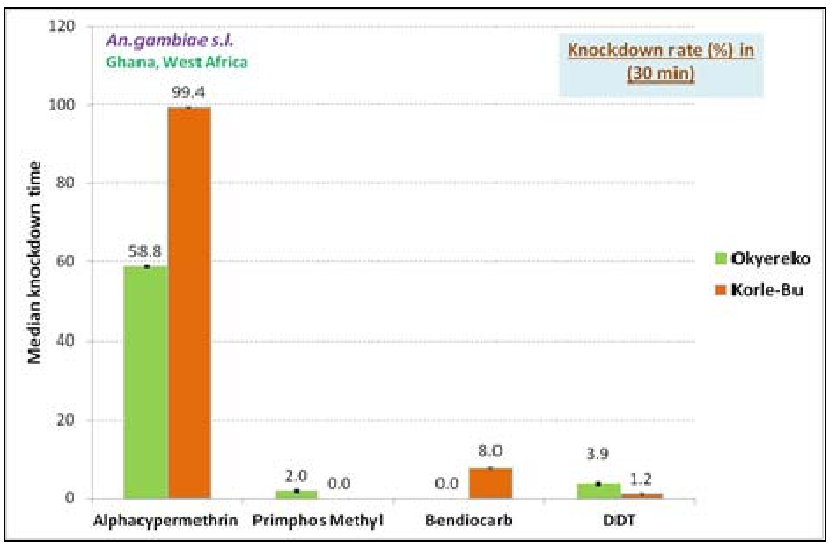

Fig 3: Comparison of median knockdown time (30 min) of *An. gambiae* s.l. exposed to four insecticides using WHO's susceptibility kit in two agricultural communities, Ghana, West Africa, 2015-2016

Table 3: Mortality (%) and knockdown times (KDT in min) obtained with the four insecticide assays at the study sites, Ghana, 2015-16

	Alphacypermethrin				Bendiocarb				Pirimiphos methyl				D.D.T.			
Study site	**No. of tested**	**Mortalily±SD**	**KDT50**	**KDT95**	**No. of tested**	**Mortalily±SD**	**KDT50**	**KDT95**	**No. of tested**	**Mortalily±SD**	**KDT50**	**KDT95**	**No. of tested**	**Mortalily±SD**	**KDT50**	**KDT95**
Korle-Bu (coastal)	80	52.5±3.98	11.967	19.403	81	12.3±2.6	89.273	319.560	84	90.5±2.3	122.207	288.757	82	12.2±2.6	8880530.701	7.120E+10
Okyereko (inland)	81	88.9±2.5	74.750	173.076	81	12.3±2.6	74.750	173.076	78	83.3±3.01	148.296	584.657	76	71.1±3.7	393693.481	2934781704.622

4. Discussion

According to W.H.O's standard for susceptibility testing of mosquitoes [3], all insecticides were resistant to the tested insecticides (p‹0.01).The level of resistance (12.3% mortality, Standard Deviation= ± 0.3) to bendiocarb was the lowest and the same in both study areas (Fig.2, Table2). This result suggests that perhaps the pressure of bendiocarb application in both study areas was similar hence, the same level of resistance was observed. Anopheles mosquitoes at Okyereko had recorded significantly higher levels of mortality (p‹0.01) to Alphacypermethrin (88.9% mortality, Standard Deviation= ± 0.3) whilst those of Korle-Bu had 52.5% for mortality, with a Standard Deviation of ± 1.2. Of the two insecticides (Alphacypermethrin and DDT), the in-land community had higher values for mortality, suggesting lower resistance status of malaria vectors in such areas. It shows that there was less resistance to DDT and Alphacypermethrin at Okyereko (Fig. 2.Table2), suggesting a possible excessive use of such insecticides at Korle-Bu hence, the observed trend. A different trend was observed for pirimiphos methyl where

Korle-Bu had a slightly higher level of resistance (90.5% mortality, Standard Error= ± 0.4) against 83.3% mortality, Standard Error= ± 1.7 for Okyereko (Table 2). An interesting result was recorded for alphacypermerthrin where mosquitoes from both locations attained the same mean knocked down values at 30 minutes(100.0±0.0 SE), after which a sharp difference was observed from 30- 60 minutes (100.0 Mean kd±4.3SE for Korle-Bu-Coastal, 100.0Mean kd±0.0 SE for Okyereko-In-land area). This may suggest a sharp change in resistance status of mosquitoes after 30 minutes. The mosquitoes had similar reactions to alphacypermethrin from the initial expossure time for 30 minutes, after which they started developing different levels of resistance. It therefore means that this insceticide can function effectively for 30 minutes and is not capable of maintaining its efficacy for long. Also, the presence of different species of *Anopheles gambiae* complex might have contributed to the above results. Therefore, species composition could play a role in the resistance status of malaria vectors. Results of knock down time for 50 and 95 minutes are presented in table 3. It is recommended that future research be conducted on the proportion of members of *A. gambiae* complex in order to determine the role such species play in the resistance status of the general population of malaria vectors in the areas.

In Ghana, previous studies in the coastal area of Accra have indicated that *Anopheles* mosquitoes were resistant to the most tested insecticides including DDT, Dieldrin, Fenitrothion, Permethrin, Deltamethrin, Lambda-cyhalothrin, Cyfluthrin, Propoxur, Bendiocarb, and some synergists [15]. The present study on susceptibility status of *An. gambiae s.l.* from selected In-land and Coastal agricultural areas of Ghana, capturing Okyereko irrigation site is in line with a similar one conducted in this area, where more than 96% of the tested population were *An. coluzzii* (a species of *An. gambiae* s.l.) and Kdr mutation prevalence was 90% [22]. Regarding DDT, although we found 71.1% mortality in the tested population at Okyereko (In-land), they reported 0% in the same area. Our tests with pyrethroid insecticide, alphacypermethrin, resulted to 88.9% mortality, but Chabi *et al*., (2016) reported less than 40% mortality using permethrin and deltamethrin, two commonly used insecticides in IRS and LLINs [21].

Study on the mechanisms of resistance to these insecticides will help us to apply relevant actions towards combating malaria vectors in Ghana. Previous studies on the mechanisms of resistance in *An. gambiae s.l.* showed that in the most resistant mosquito populations, resistance to permethrin was associated with reduced mortality, not only with respect to this insecticide but also towards deltamethrin [22]. Resistance mechanisms are proved in this species against Pyrethroids, Carbamates, Organochlorines and Organophosphates, respectively [13]. A recent study in Korle-Bu study site in Ghana showed Kdr resistance to permethrin [15]. Comparing our results with this study, the resistant rates of tested mosquitoes to both pyrethroids and carbamates had increased in Korle-Bu. In order to overcome the Kdr resistance in recent years, a new generation of LLINs impregnated with pyrethroids+synergists have been distributed in Ghana [23], but results of the studies as compared with the field efficacy of this generation of bednets and the previous ones varied. It means that the insecticide resistance in a population of mosquitoes can be induced by different modes of action.

Our findings show that *An. gambiae s.l*. as the main vector of malaria in Ghana was resistant to all insecticides tested. This finding is alarming because the insecticides tested were selected from four different groups, i.e. Organochlorines, Organophosphates, Carbamates and Pyrethroids. Other insecticides from the same groups have tested positive to resistance by previous researchers. So, we seem to have exhausted insecticide usage and there is no more effective weapon against these species in the study areas for indoor residual spraying (IRS). Meanwhile, this measure, alongside the distribution of long lasting insecticide impregnated bednets (LLINs) are the main vector control activities that are being employed to minimize vector-human contact in Ghana and other malaria endemic countries as well.

The main objectives of Ghana's National Malaria Control Monitoring and Evaluation Plan (2014-2020) as captured in the President's Malaria Initiative are to reinforce the health information systems and processes in order to provide timely, accurate, reliable, and valid data for programmatic planning, management, and decision-making (2016-Ghana-malaria-operational-plan) [3]. Ghana's malaria vector control program relies solely on insecticide-based interventions such as indoor house spraying with residual insecticides or insecticide treated bed nets; therefore, the country needs to base its decision-making process on sound Entomological surveillance and insecticide resistance monitoring. In view of that this exercise would contribute immensely to the effective planning of malaria control programs in the two regions (including the capital city of Accra), the rest of Ghana and all areas with A. gambiae s.l. populations in Africa and beyond and to better inform stakeholders about the need to adopt alternative measures for malaria control, other than the use of insecticides in general.

5. Conclusion

In conclusion, the results of this study show the main malaria vector of Ghana *(A. gambiae s.l.*) has become resistant to all available groups of insecticides, even though the levels of resistance were not the same in the study sites. This may be as a result of insecticide overuse (leading to high insecticide pressure) for plant protection against pest and insect attacks in order to maximize profit in agricultural activities and to feed the ever increasing population of the country hence, the increase in insecticide resistance of insect vectors, particularly the malaria vector, *A. gambiae* s.l. It is hereby recommended that researches be conducted towards the determination of mechanism(s) of resistance to different groups of insecticides since there were differences in levels of resistance. Also, authorities in charge of malaria control should consider other vector control strategies where Biological agents such as larvivorous fish are used, larviciding and personal protection as alternative measures of control. It was observed that many farmers utilize insecticides that have been banned years ago but are still illegally imported into the country. Even though such insecticides are used for agricultural purposes, their dosages and frequencies of use affect the resistance of malaria vectors. We also encourage sustainable training of community personnel for the correct use of LLINs.

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6.1 Declaration
The authors declare no conflict of interest

6.2 Sponsorship
This research was co-sponsored by Tehran University of Medical Sciences, International Campus - Islamic Republic of Iran and the Noguchi Memorial Institute for Medical Research, with kind support from Dr. Samuel Kweku Dadzie of the Department of Parasitology in Ghana. It was also approved in accordance with tenets of Helsinki Declaration with Ethical approval number: IR.TUMS.REC.1394.571.

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