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Short Communication

Relating High Insecticide Residues in Larval Breeding Habitats in Urban Residential Areas to the Selection of Pyrethroid Resistance in *Anopheles gambiae s.l.* (Diptera: Culicidae) in Akim Oda, Ghana

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Abstract

The main objective of this study was to assess insecticide contamination in *Anopheles* breeding habitats in urban residential areas and pyrethroid susceptibility status of mosquitoes found in the habitats. A larval survey was conducted in Akim Oda between July and October 2016. The larvae that were reared to adult were used for susceptibility test against four different pyrethroid insecticides (deltamethrin 0.05%, permethrin 0.75%, cyfluthrin 0.15%, and etofenprox 0.5%). Gas chromatography was used to analyze pesticide residues in water collected from the breeding habitats. High levels of permethrin and deltamethrin plus traces of several organochlorine and organophosphate insecticides were detected in the larval-breeding habitats. None of the four pyrethroid insecticides caused more than 10% mortality. *Anopheles coluzzii* Coetzee & Wilkerson dominated in the study area with high frequency of *kdr* mutation (93.5%). We report for the first time in Ghana, high levels of pyrethroid insecticides contamination in *Anopheles* breeding habitats in urban residential areas where there are no major agricultural activities. The contamination is suspected to be the major cause of pyrethroid resistance in the *Anopheles* species. Improper disposal of old insecticide-treated net and other domestic insecticides and the use of herbicides are suspected to be the source of insecticide contamination.

Key words: Ghana, herbicide, larvae, pyrethroid, resistance

Despite the global decline, malaria still remains a threat to public health in many endemic countries (GBD 2016). The major recommended strategy is the use of insecticide-treated net (ITN), which rely on insecticides especially pyrethroid insecticides. However, the efficacy of this important strategy is threatened by widespread of insecticide resistance in the vector species across Africa (Ranson et al. 2011). Over the years, resistance in *Anopheles gambiae* Giles (Diptera: Culicidae) to insecticides has been attributed to the intensive use of agricultural pesticides through the direct contamination of the pesticides into mosquito-breeding sites, which leads to resistance selection (Hien et al. 2017). Notwithstanding, the scaleup of ITN, excessive use of domestic insecticides, and pollution have also been implicated in contributing to the selection of resistance in malaria vectors (Riaz et al. 2009, Trape et al. 2011, Kudom et al. 2013). The major physiological mechanisms responsible for insecticide resistance are target-site mutations (*kdr* mutation in the case of pyrethroid insecticides), which occur when the insecticide no longer binds to its target, and detoxification enzyme-based resistance, which occurs when enhanced levels or modified activities of esterases, monooxygenases,

and glutathione S-transferases prevent the insecticide from reaching its site of action (Hemingway et al. 2004).

With the expansion of *Anopheles gambiae* Giles (Diptera: Culicidae) breeding to polluted waters (Kabula et al. 2011, Kudom 2015) coupled with the excessive use of domestic insecticides without proper disposal mechanisms particularly in urban areas in Ghana, it is possible that mosquito-breeding habitats in residential areas could also be contaminated with insecticide, which would eventually lead to resistance selection. This study was therefore conducted to assess insecticide contamination in *Anopheles* breeding habitats in urban residential areas and pyrethroid susceptibility status of the mosquito found in the habitats.

Materials and Methods

Study Area

This study was conducted in Akim Oda, the capital of Birim Central Municipal in the eastern Region of Ghana. The major income activity is agriculture but is restricted to the rural areas of the Municipality.

Akim Oda is in the forest zone with two rainfall seasons; April to June and September to November. Malaria is a public health problem in the Municipality as in other areas in Ghana. Total outpatient (OPD) malaria cases in the Municipality slightly rose from 86,896 in 2012 to about 97,957 in 2015. Similarly, children under 5 yr admitted to hospitals or other health facilities in the Municipality with malaria also rose from 1,741 in 2012 to about 1,838 in 2015. The major malaria control activity is the use of ITN and other antimosquito strategies such as the use of mosquito coil and aerosol spray.

Larval and Water Collection

Anopheles larvae were collected from a variety of habitats such as pools, puddles, and drainage channels in July, August, and October 2016. Mosquitoes were collected from a total of eight breeding habitats during the three collection periods. All the habitats were temporal and rain dependent and located in residential areas (Fig. 1). Water samples were also collected from each of the habitats in an amber jar. The larvae and the water samples were brought to the University of Cape Coast. The larvae were reared to adult and susceptibility of the adult conducted.

Insecticide Susceptibility Bioassay

Susceptibility of the female adults to four pyrethroid insecticides (deltamethrin 0.05%, permethrin 0.75%, cyfluthrin 0.15%, and etofenprox 0.5%) was assessed following the protocol described by WHO (2013). A test with 2- to 4-d-old, non–blood-fed, female mosquitoes in batches of 10 to 25 mosquitoes was exposed for 1 h to each of the insecticide-impregnated papers. The test was replicated three to five times for each of the insecticides. A test with a paper treated with silicone oil was run in parallel to serve as a control. Knockdown and final mortality were recorded at 1 and 24 h, respectively, after the exposure. Mosquitoes that were alive after 24 h were termed resistant and killed in a freezer, placed singly in a labeled 1.5-ml eppendorf tube. Those that were dead after 24 h were termed susceptible and were also put singly in similar tubes and labeled appropriately and stored in a refrigerator (–20℃) for later molecular bioassays. The temperature and humidity in the test room were 27.1℃ and 100%, respectively. Mosquitoes that were termed resistant were used for the molecular analysis. Species were identified according to morphological characteristics followed by polymerase chain reaction (PCR) assay according to Scott et al. (1993). Further PCR analysis to determine *Anopheles coluzzii* Coetzee & Wilkerson (Diptera: Culicidae) and *An. gambiae* Giles were also done using the SINE PCR protocol (Santolamazza et al. 2008). The same individuals were then tested for L1014F *kdr* mutations using the primers and protocol described by Martinez-Torres et al. (1998).

Pesticide Extraction

The pesticide in the water samples was extracted according to USEPA method 3510 (Edgell and Wesselman 1989). A 100 ml of the sample was measured and transferred into a 500-ml separatory funnel. About 50 ml of methanol and 50 ml of chloroform were each measured and added to the sample in the separatory funnel (i.e., [1:1 v/v] methanol, chloroform). The mixture was carefully shaken, and the phases were allowed to separate. The chloroform layer which was nonpolar and contained the organic pollutant was collected. The upper layer, that is, the methanol or the aqueous layer was discarded. The extraction was repeated with two 50-ml portions of methanol and chloroform. The chloroform layer was dried over anhydrous sodium sulfate, and the dried samples were then concentrated to 5 ml using rotary evaporator. This procedure was repeated for each sample. After the extraction, each sample was cleaned up
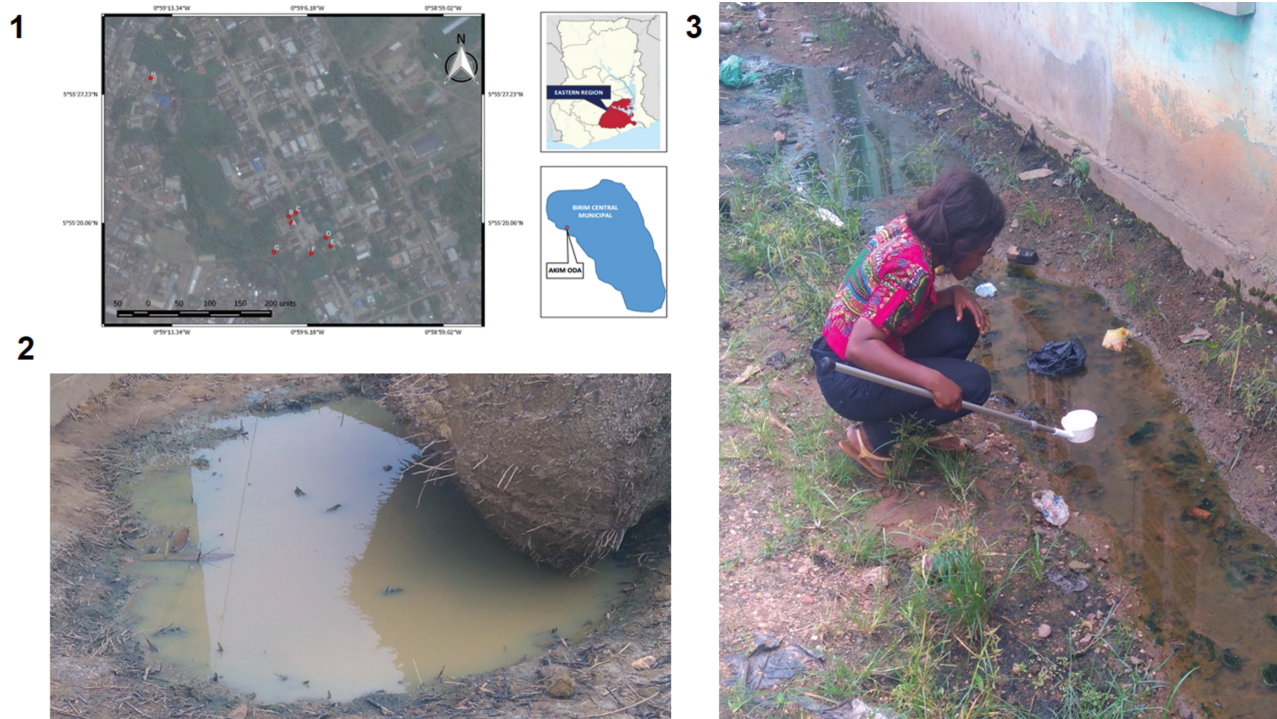

Fig. 1. A satellite map of Akim Oda showing the eight points, A–H (1) and pictures (2–3) where larvae and water were collected; in each of the points, larvae (used for susceptibility test when reared to adults) and water (used for the pesticide residue analysis) were collected at the same time (letters A–H represent the location of the breeding sites).

with a solid-phase extraction cartridge packed with a C18 adsorbent, which was conditioned with methanol. The final extracts were taken to a reference laboratory (Ghana Standard Authority) where a Varian CP-3800 Gas Chromatography was used to analyze each of the samples for pyrethroid, organophosphate, and organochlorine insecticides.

Results

A total of 27 insecticides from three different classes; pyrethroids, organochlorine, and organophosphate were identified in seven out of the eight breeding habitats (Tables 1 and 2). While pyrethroid insecticides were found in high levels, organophosphate and organochlorine insecticides were very low or within acceptable limits. The

Table 1. Pyrethroid identified in water collected from the larval-breeding habitats of *Anopheles gambiae* from Akim Oda

| Pyrethroid | Concn (µg/liter) in breeding habitat | | | RTL[a] (µg/liter) |
	A	B	H	
Deltamethrin	0.370	0.050	0.280	0.029
Fenvalerate	0.020	0.003	0.003	0.009
Cypermethrin	0.007	0.010	0.020	0.013
Lambda-Cyhalothrin	0.010	0.017	0.033	0.003
Permethrin	1.283	ND	0.543	0.018
Cyfluthrin	0.027	0.010	0.013	0.010

A: Pyrethroid insecticides, B: organophosphate and organochlorine insecticides. RTL = regulatory threshold level for surface water. [a]RTL values were obtained from Stehle and Schulz (2015). ND = not detected; letters A, B, and H represent the breeding sites where water and larvae were collected.

breeding habitats were dominated by *An. coluzzii* (86.5%, 32/37) alongside with few *An. gambiae* (13.5%, 5/37). The vector population was highly resistant to the four pyrethroid insecticides used for the susceptibility test (Table 3). A total of 303 female *Anopheles* mosquitoes exposed to the pyrethroid insecticides caused only about 6.93% mortality with no significant difference between the mean mortalities of the four insecticides ($F = 0.47$, df = 3, $P = 0.71$). Furthermore, the L1014F *kdr* mutation was also detected at an elevated frequency (homozygote mutation 64.5% [20/31], heterozygote mutation 29% [9/31], and the wild-type allele 6.5% [2/31]).

Discussion

The level of pyrethroid resistance and high frequency of *kdr* mutation in the vector population from this study is suspected to come from the insecticide contamination in the larval-breeding habitats. This result is consistent with what has been observed in agricultural areas where insecticides are used intensively (Hien et al. 2017). Evidence of exertion of selection pressure by insecticides in the larval-breeding sites leading to the selection of resistance genes has been shown in both laboratory and field studies (Riaz et al. 2009, Diabate et al. 2002). For example, in Burkina Faso, higher frequency of *kdr* mutation was found in cotton growing areas with the intensive use of insecticide than in rural areas where only food crops were grown without insecticides (Diabate et al. 2002). Other factors such as the scaling up of ITN and intensive use of domestic insecticides including mosquito coils and aerosol spray for mosquito control could play an additional role in selecting for resistance in the study area (Trape et al. 2011, Kudom et al. 2013).

The source of insecticide contamination in the larval-breeding habitats in this study could possibly come from domestic activities since the breeding habitats were found in residential areas without any important agricultural activities. Although there were a number

Table 2. Organochlorine and organophosphate identified in water collected from the larval-breeding habitats of *Anopheles gambiae* from Akim Oda

| | Concn (µg/liter) in breeding habitat | | | | | | | RTL |
	A	B	C	D	E	F	H	
Dieldrin	ND	ND	ND	ND	ND	ND	0.003	0.030[a]
Endrin	0.010	ND	ND	ND	ND	ND	ND	0.061[a]
Heptachlor	0.983	0.180	0.167	0.043	0.647	ND	0.707	0.030[a]
Aldrin	0.050	ND	ND	ND	0.010	ND	0.033	0.030[a]
p,p,DDE	ND	ND	ND	ND	ND	ND	0.020	0.020[a]
P,P,DDT	0.003	ND	ND	ND	ND	ND	0.020	0.060[a]
P,P,DDD	0.127	0.030	0.053	ND	ND	ND	0.033	0.060[a]
delta-HCH	0.060	ND	ND	ND	ND	ND	0.030	0.010[a]
gamma-HCH	0.030	ND	ND	ND	ND	ND	0.023	0.010[a]
Endosulfan	0.063	0.020	0.037	ND	ND	ND	0.107	0.020[a]
Methoxychlor	ND	ND	ND	ND	ND	ND	0.190	20.000[a]
gamma-chlord	0.163	ND	ND	ND	0.010	ND	0.103	–
Diazinon	ND	ND	ND	ND	ND	0.007	0.030	0.080[a]
Parathion-et	ND	ND	ND	ND	ND	0.013	ND	0.013[a]
Chlorpyrifos	0.033	ND	ND	0.100	0.267	0.023	ND	0.0035[a]
Dimethoate	1.300	ND	ND	ND	ND	0.000	1.933	6.200[b]
Profenofos	0.240	0.003	0.007	ND	ND	0.107	0.060	0.300[a]
Fonofos	ND	ND	0.017	ND	ND	0.007	ND	10.000[c]
Ethoprophos	ND	ND	ND	ND	ND	0.020	ND	1.000[a]
Methamidophos	ND	ND	ND	ND	ND	0.050	ND	–

A: Pyrethroid insecticides, B: organophosphate and organochlorine insecticides. RTL = regulatory threshold level for surface water; RTL values were obtained from [a]Hamilton et al. 2003, [b]CCME 2008, [c]US EPA 1987; ND = not detected; letters A, B, C, D, E, F, and H represents the breeding sites where water and larvae were collected.

of backyard gardens, insecticides are hardly used in such farms. The use of insecticide/pesticide and how it is disposed of is not much regulated in Ghana. Hence, it is very difficult to know with certainty the history of pesticide use, particularly in urban areas. Used insecticide

Table 3. Susceptibility status of *Anopheles coluzzii* from Akim Oda to four different pyrethroid insecticides

Insecticide[a]	% Mortality (N)[b]	Mean % mortality ± SEM (N)	Collection date[c]
Deltamethrin (0.05%)	16.7 (36)		21 July 2016
	7.1 (28)	6.6 ± 3.4 (84)	10 Aug. 2016
	0 (20)		06 Oct. 2016
Cyfluthrin (0.15%)	14.3 (35)		21 July 2016
	0 (21)		10 Aug. 2016
	0 (20)	4.0 ± 2.6 (76)	06 Oct. 2016
Permethrin (0.75%)	12.5 (40)		21 July 2016
	0 (17)	6.3 ± 6.2 (57)	10 Aug. 2016
Etofenprox (0.5%)	7 (43)		21 July 2016
	0 (23)		10 Aug. 2016
	0 (20)	2.0 ± 1.3 (86)	06 Oct. 2016
Control (PY)	0 (43)		21 July 2016
	5.2 (19)		10 Aug. 2016
	8.3 (24)	3.3 ± 5.9 (86)	06 Oct. 2016

[a]Insecticide-treated papers including the control (PY) were purchased from WHO (Vector Control Research Unit, University of Malaysia, Malaysia).

[b]Batches of 10–25 were exposed for 1 h to each of the insecticides while knockdown and final mortality were recorded at 1 and 24 h post-exposure, respectively; SEM, standard error of the mean.

[c]Larvae were collected from eight breeding sites, A–H, and all the breeding sites were small, temporal, and rain dependent; 21/07/2016—H; 10/08/2016—A; 06/10/2016—B, C, D, E, F, G.

products such as old insecticide nets, pieces of mosquito coils, and containers of aerosol sprays are mostly disposed or left in the open environment. Chemicals from these items could leach into the soil and contaminate mosquito larval habitats. For instance, there is a widespread practice of the use of old insecticide-treated bed nets to fence backyard gardens in Ghana (Fig. 2). This was widely seen in the study area, and it could be the major source of pyrethroid contamination in the breeding sites. This may also explain the high levels of permethrin and deltamethrin insecticides in the breeding habitats since most of the ITN distributed by the Ghana Malaria Control Program are treated with these insecticides. The use of herbicides and other agrochemicals to control weeds could also be one of the sources of contamination to the breeding sites, particularly organophosphate insecticides. In one occasion during data collection, we witnessed glyphosate herbicide (Wynca 360SL) being used in the study site. Exposure of mosquito larvae to sublethal doses of herbicides and their derivatives have been shown to enhance the insect's tolerance to different insecticides (Riaz et al. 2009). It is for this reason that the low levels of organochlorine and organophosphate insecticides observed in the breeding habitats is worrying and also needs immediate attention. Already, *Ace1* (G119S)-resistant gene responsible for organophosphate and carbamate resistance has been detected in *Anopheles* population from the study area, albeit in low frequency (Essandoh et al. 2013). Hence, continuous exposure to the sublethal doses could increase the frequency of the resistance genes in the vector population.

Previous studies in Ghana have found insecticide contamination in soil, drinking water, surface water, fishes, and mosquito-breeding habitats (Achonduh et al. 2008, Essumang 2015, Gbeddy et al. 2015, Fosu-Mensah et al. 2016). However all of them were associated with agricultural activities or large water bodies that receive runoff from several sources including agricultural areas. With regard to mosquitoes, Achonduh et al. (2008) detected cypermethrin and

Fig. 2. Pictures showing the practice of using old insecticide-treated nets to fence backyard gardens in some urban residential areas in Ghana (a and b: Cape Coast, c and d: Akim Oda). Each of the fences is made up of a different brand of nets having different insecticides and different manufacturing dates.

other organophosphate insecticides in *Anopheles*-breeding habitats in urban agricultural area in Accra while Essumang (2015) also detected allethrin, deltamethrin, and DDT and its derivatives in lagoons and other bodies suspected to be breeding mosquitoes, but no entomological work was conducted to confirm or identify the presence of mosquitoes or the species found in that water bodies. To the best of our knowledge, this is the first report identifying high levels of pyrethroid insecticides contamination in *Anopheles*-breeding habitats in urban residential areas where there are no major agricultural activities. The sample size of this study is limited and hence difficult to determine the extent of occurrence or importance of this phenomenon on the selection of pyrethroid resistance among vector mosquitoes in Ghana. Nevertheless, the results highlight another possible cause of selection of resistant mosquito vectors at the larval stages in residential areas. This phenomenon needs to be explored further to gain a deeper understanding of the extent of occurrence and its impact on insecticide resistance in mosquitoes in the country.

The contamination of chemicals to surface waters and other water bodies may not only threaten malaria control but the general aquatic biodiversity. Indeed, studies have shown that pesticide concentration within the regulatory accepted thresholds reduces species richness at the taxonomic family level by about 30% of freshwater invertebrates (Beketov et al. 2013, Stehle and Shulz 2015). Therefore, pesticide levels above the regulatory threshold could have a serious implication on aquatic biodiversity.

The dominance of *An. coluzzii* in the study site was unexpected. From the model described by de Souza et al. (2010) coupled with prevailing environmental conditions (forest zone, high precipitation, rain-dependent temporal breeding sites) in Akim Oda, *An. gambiae s.s* was rather expected to dominate the study area. Nevertheless, the dominance of *An. coluzzii* in urban polluted waters is consistent with the findings from different urban areas in Ghana (Kabula et al. 2011, Kudom 2015). It appears that the tolerance of *An. coluzzii* to ions (White et al. 2013) is favoring its distribution in relatively polluted breeding habitats especially in urban polluted habitats and semi- or permanent-breeding habitats.

In summary, the phenomenon of contamination of pesticides in *Anopheles*-breeding habitats causing the selection of resistant populations is well known but mostly found in agricultural areas where insecticides are used intensively. We report for the first time in Ghana, high levels of pyrethroid insecticides contamination in *Anopheles*-breeding habitats in urban residential areas where there are no major agricultural activities. The result from this study highlights the need to regulate and monitor the use and disposal of domestic insecticides and their products, especially, the disposal of old ITNs. Expanding this study to cover other urban areas may give a complete picture of the situation in Ghana.

Acknowledgments

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