Susceptibility Status of *Anopheles gambiae* s.l. (Diptera: Culicidae) from Cabbage Growing Areas Associated with Pyrethroid and Organophosphate Use in Accra, Ghana

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Abstract

Resistance in malaria vectors is likely to be caused by the massive use of insecticides in agriculture. *Anopheles gambiae* s.l. collected from breeding grounds in two cabbage growing areas within Accra were assessed for levels of resistance to 0.75% permethrin, 0.05% deltamethrin, 5% malathion and 4% DDT using standard WHO susceptibility test kits. Pyrethroid and organophosphate residue levels in soil and run-off water from these cabbage farms were determined and possible association between resistance and residue levels were established. Compared to the susceptible 'Kisumu' strain, both Korle-Bu and Airport populations were highly resistant to DDT and gave resistance levels which were over nine-fold for permethrin and over 2.5-fold for deltamethrin. Both wild and susceptible populations showed full susceptibility to malathion. The S and M forms of *A. gambiae* s.s. were found to occur in sympatry in the two study sites with a higher frequency of S form in the Airport area. Toxicity testing of extracts of soil and run-off water from these cabbage farms, using brine shrimp lethality tests, showed high level of toxicity, indicative of the presence of residues of insecticides. Differential fractionation of these extracts using solid phase extractor (SPE) suggests that the bulk of residues in these extracts may be pyrethroids and organophosphates. No correlation was observed between either residue levels or residual bioactivity in soil and run-off water, and resistance levels in *A. gambiae* s.l. populations, collected from breeding grounds within the farms under investigation. It is proposed that resistance in *A. gambiae* larvae in these breeding sites contaminated with agricultural insecticides may have occurred over time due to continuous exposure to sub-lethal doses.

Introduction

Resistance to insecticides has become a limiting factor in the use of these compounds in the control of many insect pests or vectors. Many examples have shown that resistance of malaria vectors to insecticides has most likely been due to massive use of insecticides in agriculture, especially in cotton and rice production (Lacey & Lacey, 1990; Chandre *et al*., 1999a; Diabeté *et al*., 2002). Moreover, more than 90% of all insecticides produced have been used for agricultural purposes, and this has created serious problems for mosquito control programmes (Roberts & Andre, 1994).

When pesticides are applied to protect crops from pests and diseases, only around 15% of the preparation hit the target. The rest is distributed in the soil and air (Varca, 2002). It is known that most of the applied pesticides are subject to many transport and conversion processes. Thus, they do not remain at their target site but often enter aquatic environments via soil percolation, air drift or surface run-off, affecting abundance and diversity of non-target species, and produce complex effects on the ecosystems, thus, altering trophic interactions (Rand *et al*., 1995).

In Ghana, cabbage cultivation may be considered a major contributor to pesticide contamination of the environment in the urban areas. Due to increasing demand for cabbage, especially in urban areas, cabbage growers in Ghana have resorted to monoculture and intensive cultivation of the crop. This practice has led to increased insect pest populations and manifestation, thus, affecting optimum yield. Additionally, with the all year round suitable environmental conditions for survival and development, pest outbreaks occur with enormous

damage (Afun *et al.,* 1992) on cabbage farms. The growers, in an attempt to protect their crop and investment, resort to the use of insecticides to control insect pests. For example, Brempong-Yeboah (1992) observed that cabbage growers in the Accra Plains used large quantities of insecticides (mostly organophosphates and pyrethroids) and concoctions against the diamondback moth. Increased frequency of application ranging from 2-3 days interval was also observed.

Although there are positive benefits from agriculture, the use of pesticides for agricultural purposes has a direct impact on human health through increased human exposure to agro-chemicals, or increased exposure to vectors of diseases, such as malaria (SIMA, 2003). An important negative impact is the role of agrochemicals run-offs in the evolution of insecticide resistance in *Anopheles* mosquitoes that transmit malaria parasites (SIMA, 2003). Work done in Burkina Faso by Diabaté *et al.* (2002) showed that agricultural use of insecticides was involved in the incidence of resistance to permethrin and DDT in field populations of mosquitoes. Klinkenberg & Boelee (2002) reported that urban agriculture, by way of open-space irrigated vegetable farms, may lead to the creation or extension of breeding sites for the vector of malaria, thereby, influencing transmission of malaria in Ghana.

Pools of water which serve as breeding sites may be run-offs from sprayed farms and may contain insecticide residues. Moreover, there have been reports of pyrethroid resistance in populations of *A. gambiae* s.s. in Accra (Adasi *et al*., 2000; Adeniran, 2002). The present study, carried out in Accra between September 2004 and March 2005, was aimed at investigating resistance in *A. gambiae s.l.* vectors of malaria in and near cabbage farms, determining insecticide residue levels and establishing possible association between resistance development and residue levels.

Materials and methods

Study area
The study area was within the Accra metropolitan area in Ghana. Accra is situated in the driest coastal plains of Ghana with a mean annual rainfall ranging between 740 mm and 890 mm. The mean monthly temperature is between 25 ℃ and 30 ℃, and the relative humidity ranges between 65% and 75% (Dickson & Benneh, 1988). Samples were collected from cabbage farms in Korle-Bu (05º 32' N, 00º 13' W) and Airport Residential Area (05º 35' N, 00º 11' W). Irrigated urban agriculture by way of open-space irrigated vegetable farming is practiced in these areas. The cabbage farms were selected on the basis of availability of mosquito breeding sites located within or near the farm. Mosquitoes were collected as larvae or pupae from breeding grounds inside cabbage farms and brought to the insectary for emergence of adults. A susceptible *A. gambiae* s.s. from Kisumu (Kenya) was provided by the Noguchi Memorial Institute for Medical Research and used as the reference strain.

Bioassays were carried out for wild and susceptible populations using standard WHO susceptibility test for 0.75% permethrin, 0.05% deltamethrin, 5% malathion and 4% DDT (WHO, 1998). DDT (4%) was used to check for cross-resistance in the same population. Non-blood fed female mosquitoes aged between 2–5 days old were used. Twenty-five adult female mosquitoes were exposed to insecticide-impregnated papers purchased in standard testing tubes from WHO, and the number of mosquitoes knocked-down was recorded for up to 60 min. at an interval of 10 min. All tests were undertaken at 25 ± 2 ℃ and 55–78% relative humidity, and a natural photoperiod was maintained. They were then transferred to clean holding tubes where 10% sucrose was provided on cotton wool, and mortality determined 24 h after exposure. Control mosquitoes were exposed to papers impregnated only with olive oil and each test was replicated four times.

Mosquitoes used in bioassays were identified to the species level using polymerase chain reaction, following the method of Scott *et al*. (1993). *A. gambiae* s.s. mosquitoes were further

identified as M or S form by polymerase chain reaction – restriction fragment length polymorphism, using the method of Fannello *et al*. (2002).

Isolation of insecticides residues
   Insecticide standards were selected based on the insecticide use pattern for cabbage cultivation reported in Accra. Standards of permethrin, deltamethrin, cypermethrin, chlorpyrifos, pirimiphos-methyl were purchased from EQ Laboratories Inc., Atlanta, USA. Purity of standards was 100%. Stock solutions of standards were prepared in hexane.
   Run-off water was collected in duplicates into 2.5 litre Winchester bottles from mosquito breeding sites inside cabbage farms and immediately transported to the laboratory. Samples were filtered and concentrated to 500 ml, using Eyela rotary vacuum evaporator (Rotavap) at 40 ℃. Soil samples were collected from 0-15 cm of surface soil in duplicates at various points on the cabbage farm during water sampling, and transported immediately to the laboratory in bottles wrapped in aluminum foil and then air-dried for 48 h. The soil was then ground in a mortar to render it fine using a pestle and then sieved using a 1-mm sieve. Both soil and water were stored separately at 4 ℃ till ready for use.

Extraction and clean up of residues
   Soil samples. The extraction procedure of Singh *et al*. (1999) was used with minor modifications. Air-dried soil (10 g) was extracted in 25.0 ml hexane-ethyl acetate solution (9:1) with stirring for 12 h. The solvent phase was carefully taken out and extraction was repeated with fresh solvent. The two fractions were pooled and centrifuged at 3000 r.p.m. for 5 min, and the supernatant transferred into clean 100-ml flasks containing anhydrous sodium sulphate to remove any moisture present.
   Water samples. Extraction procedure of Handa *et al*. (1999) was used with minor modifications. Fifty millilitres of run-off concentrate was taken into a separating funnel in duplicates and shaken with 50 ml, hexane:ethyl acetate mixture (9:1) for 2-3 min. The organic phase was carefully transferred into clean flasks. The process was repeated three times with fresh solvents and all three extracts were pooled and transferred into clean flasks containing anhydrous sodium sulphate. Both soil and water extracts were concentrated to 5 ml using Buchi Rotavapour (Buchi, Switzerland) at 40 ℃, transferred into clean pre-weighed vials and evaporated under a stream of nitrogen gas to dryness and then reconstituted in hexane. Differential fractionation and clean up of extracts was done using C_{18} solid phase extraction (SPE) columns. Methanol, ethyl acetate and hexane were selected as eluting solvents, based on the differences in polarity. The SPE columns were preconditioned with methanol followed by the addition of 100 µl of each sample.
   Two millilitres of each solvent in the order methanol, ethyl acetate and hexane, was added to the columns and the eluents were separately collected in 2-ml fractions into 10 ml vials and then evaporated to dryness under a stream of nitrogen gas. The weight of each extract recovered after clean-up was determined and reconstituted in appropriate volume of solvent to obtain a 10-mg/ml solution. In order to determine the solvents that the selected insecticides best eluted in, the insecticide standards were similarly fractionated by SPE using the three eluting solvents, methanol, ethylacetate and hexane.

Determination of residue levels
   The method of Meyer *et al*. (1982) was used with minor modifications. Brine shrimp eggs, purchased from Brine shrimp Direct, California, USA were cultured in a hatching tank containing filtered sea salt water and placed under light. After 48 h the hatched and mature shrimp nauplii were then used for lethal toxicity assay. Five microlitres of each extract was aliquoted separately into 10 ml vials (with marking at 5 ml level) and evaporated to dryness under

a stream of nitrogen gas. The residue was dissolved in 40 µl of acetone and 2 ml of the saline water was added to each vial to make the initial solution of the extract or insecticide standard. Ten shrimp nauplii were transferred to each vial and the volume made up to the 5 ml mark with sea water. The control contained only acetone and sea water. The set up was placed under light and number of dead shrimp nauphii after 24 h was recorded. Each test was replicated four times.
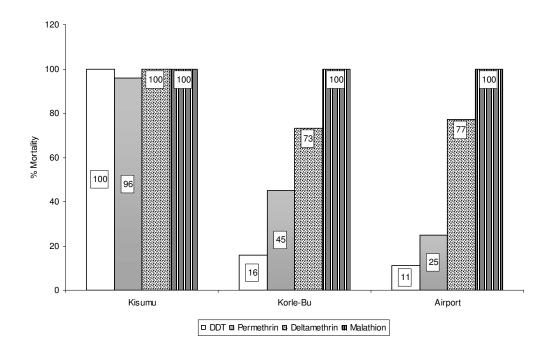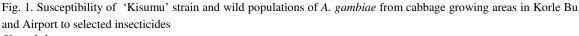
Data analysis

All data was analyzed using Minitab version 12 for Windows™ software. Abbott's formula (Abbott, 1925) was used to correct for natural mortality in mosquitoes and brine shrimp nauplii. The KT_{50} and KT_{95} values for mosquitoes were estimated from the time-mortality regression using probit analysis (Finney, 1971). Standard dosage-brine shrimp mortality curves for the insecticide standards were obtained and then used to estimate their concentrations in soil and water extracts.

Results

Mortality

Apart from permethrin to which the 'Kisumu' strain demonstrated 96% mortality, full susceptibility to deltamethrin, DDT and malathion was observed (Fig. 1). The wild *Anopheles* populations for Airport and Korle-Bu, like the 'Kisumu' population, demonstrated complete susceptibility (100% mortality) to the organophosphate, malathion. Contrarily, the wild populations gave lower mortality rates against the pyrethroids, deltamethrin (77% for Airport and 73% for Korle-Bu) and permethrin (25% for Airport and 45% for Korle-Bu), with the lowest mortality being recorded for DDT (11% for Airport and 16% for Korle-Bu).

Fig. 1. Susceptibility of 'Kisumu' strain and wild populations of *A. gambiae* from cabbage growing areas in Korle Bu and Airport to selected insecticides

Knockdown

The KT_{50} and KT_{95} values obtained for the susceptible 'Kisumu' strain were compatible with the susceptibility of these mosquitoes to the tested insecticides. The KT_{50} of the two wild populations were compared with those obtained for the susceptible mosquitoes to obtain the

resistance ratio. The resistant ratio (RR_{50}) at KT_{50} level for permethrin was 10.6 and 9.4 for Airport and Korle-Bu, respectively, while relatively lower levels were obtained for deltamethrin (Table 1). The RR_{50} for DDT was not calculated because the number of mosquitoes knocked down in the wild populations at the end of the exposure time was less than 15%.

TABLE 1
Knockdown time (KT) and resistance ratio for bioassays of the susceptible and two wild populations of female A. gambiae

Insecticide	Population	$KT_{50}(min)$	$KT_{95}(min)$	N	RR_{50}
Permethrin (0.75%)	Kisumu	9.6	28.8	100	–
	Airport	101.5	151.6	100	10.6
	Korle-Bu	89.8	159.8	100	9.4
Deltamethrin (0.05%)	Kisumu	17.3	33.0	100	–
	Airport	54.7	89.1	100	3.2
	Korle-Bu	44.8	82.2	100	2.6
DDT (4%)	Kisumu	23.4	43.1	80	–
	Airport	No kd	No kd	100	ND
	Korle-Bu	No kd	No kd	100	ND

Resistance ratio was calculated at KT_{50} level with reference to the susceptible 'Kisumu' strain as RR_{50}= resistance ratio (KT_{50} Wild/KT_{50} Kisumu) at $P < 0.05$.

N = number of treated mosquitoes; No kd = complete loss of knockdown effect (< 15% of mosquitoes knockdown after 1 h of exposure); ND = Indeterminate.

Mosquito species and molecular forms

Of the 60 *A. gambiae* s.s. identified from Korle-Bu, 10 (17%) were found to be the S form while 50 (83%) were the M form. In contrast, out of the 60 *A. gambiae* s.s. identified from Airport, only three (5%) were the M form while 57 (95%) were the S form.

Residue levels

Relative to the other pyrethroids, cypermethrin was observed to have the lowest LC_{50} value. The concentrations of toxic components in extracts were estimated from log concentration-brine shrimp mortality regression equations of cypermethrin, chlorpyrifos and pirmiphos-methyl. Concentration of residues in hexane fractions of extracts were estimated from the calibration curve of cypermethrin while calibration curve of chlorpyrifos and pirmiphos-methyl were used for determina-tion of residues in the methanol and ethyl acetate fractions, respectively. The concen-tration of active residue in fraction (residual bioactivity) was estimated as a percentage of estimated concentration of toxic component relative to the lethal concen-tration of 10 µg/ml, i.e. concentration found to give at least 20% brine shrimp mortality (Tables 2 and 3). Run-off extracts from Korle-Bu area showed mortality ranging from 20-100% while for Airport area, brine shrimp mortality ranged from 28-50% at 10 µg/ml concentration. For soil extracts, mortality was between 5–75% and 5–30% at 10 µg/ml concentration for Korle-Bu and Airport areas, respectively.

There was variation in residual bioactivity in the three extracting solvents for both run-off and soil extracts within and between the two study areas (Tables 2 and 3). Methanolic fractions of run-off extracts at 10 µg/ml showed the lowest residual bioactivity for both sites while hexane and ethylacetate fractions recorded the highest residual bioactivity for Korle-Bu and Airport,

respectively. However, the highest residual bioactivies for soil extracts were recorded for the methanol and ethyl acetate fractions for Korle-Bu and ethyl acetate and hexane fractions for Airport area. A tenth dilution of both soil and run-off water extracts in either site essentially recorded low or no activity. The total residue concentration for both run-off and soil was higher (Table 4) in Korle-Bu relative to the Airport area.

TABLE 2

Residual bioactivity in run-off and soil extracts from Korle Bu, Ghana

Extracting solvent	% Residual bioactivity* Concentrations of extracts			
	Run-off		Soil	
	10 µg/ml	*1 µg/ml*	*10 µg/ml*	*1 µg/ml*
Methanol[a]	0.7	Low	0.2	Low
Ethylacetate[b]	5.7	Low	2.4	N
Hexane[c]	8.2	Low	Low	N

[a] Methanol fractions estimated from the standard curve for chlorpyrifos.
[b] Ethylacetate fractions estimated from standard curve for pirimiphos-methyl.
[c] Hexane fractions estimated from standard curve for cypermethrin.
*Residual bioactivity = Estimated concentration (µg/ml) of toxic component/lethal concentration for each fraction.
**Lethal concentration = 10 µg/ml (concentration found to give at least 20% mortality).
Low = Residual bioactivity so low it could not be estimated from calibration curve.
N = No residual bioactivity.

TABLE 3

Residual bioactivity in run-off and soil extracts from Accra Airport, Ghana

Extracting solvent	% Residual bioactivity* Concentrations of extracts			
	Run-off		Soil	
	10 µg/ml	*1µg/ml*	*10 µg/ml*	*1 µg/ml*
Methanol[a]	0.5	N	N	Low
Ethylacetate[b]	6.04	N	4.2	N
Hexane[c]	2.3	N	4.62	N

[a] Methanol fractions estimated from the standard curve for chlorpyrifos.
[b] Ethylacetate fractions estimated from standard curve for pirimiphos-methyl.
[c] Hexane fractions estimated from standard curve for cypermethrin.
*Residual bioactivity = Estimated concentration (µg/ml) of toxic component/lethal concentration** for each fraction.
**Lethal concentration =10 µg/ml (concentration found to give at least 20% mortality).
Low = Residual bioactivity so low it could not be estimated from calibration curve.
N = No residual bioactivity.

TABLE 4

Estimates of insecticide residue levels in run-off and soil from cabbage growing areas within Korle-Bu and Airport

Site	Run-off		Soil	
	Total weight of*	*Residue[a]*	*Total weight of*	*Total estimated[b]*

	residue extracted (mg)	concentration (µg/ml)	residue extracted (mg)	residue level (mg/g)
Korle-Bu	6.4	25.7	8.8	0.89
Airport	6.1	24.3	4.0	0.43

Volume of run-off = 2500 ml; Weight of air-dried soil = 10 g; [a]Residue concentration = Total weight of residue extracted/volume of run-off; [b]Total estimated residue level = Total weight of residue extracted/weight of air dried soil.

*Total weight of residue is the average of four replicates.

Discussion

Bioassay data obtained for permethrin, deltamethrin, malathion and DDT confirmed resistance in mosquitoes population in Accra as previously reported by Adasi *et al.* (2000) and Adeniran (2002). Relative to the susceptible strain, the wild populations were observed to be highly resistant to the selected pyrethroids and DDT but showed full susceptibility to malathion. The increase in knockdown time was more marked with permethrin than deltamethrin. For example, in the Airport population, the KT_{50} for permethrin increased by 10.6-fold but only 3.6-fold for deltamethrin while the KT_{50} for Korle-Bu population increased by 9.4-fold and 2.6-fold for permethrin and deltamethrin, respectively. These results agree with earlier reports that permethrin is a better indicator of knockdown-based resistance than deltamethrin (Chandre *et al.*, 1999b). The reduced mortality with deltamethrin in populations displaying a very high permethrin resistance suggests that *kdr* mutation confers cross resistance to various pyrethroids. Cross-resistance to DDT was observed in these populations, although these cabbage farmers have not used this insecticide for pest control.

Resistance to DDT must have occurred because of similarity in the mode of action of DDT and pyrethroid, which is basically on the voltage-dependent sodium channel of nerve (Brooke *et al.*, 1999; Bloomquist, 1996). Extensive use of pyrethroids for control of insect pests of cabbage may explain the high level of resistance to pyrethroids observed in these *A. gambiae* populations (Odhiambo, 2005). Molecular identification of *A. gambiae* s.l. revealed only *A. gambiae* s.s. as the sibling species. These results are consistent with that of other studies carried out in Accra (Adasi *et al.*, 2000; Adeniran, 2002). Both the 'M' and 'S' molecular forms of *A. gambiae* s.s. were present and occurred in sympatry across the two collection sites. Unlike the Korle-Bu area, the 'S' form occurred at higher frequencies than the 'M' form in the Airport area, and this has serious implications for malaria control efforts since the 'S' form has been strongly associated with the *kdr* mechanism of resistance (Chandre *et al.*, 1999b).

Brine shrimp lethality was observed in both soil and run-off extracts, i.e. 10 µg/ml from the two sites, indicative of the presence of insecticide residues in these extracts. The absence or low residual activity, recorded in a 10th dilution of soil and run-off extracts from both sites, suggest that 10 µg/ml is the concentration at which biological activity is observed. Based on the results obtained by differential fractionation studies of standard insecticides, using SPE, the bulk of insecticide residues in run-offs and soil extracts from cabbage farms in the two sites are likely to be pyrethroids and organophosphates, which agrees with the insecticide use pattern reported in Accra (Ninsin, 1996; Odhiambo, 2005). The variation of residual bioactivity between the two sites may be explained by the different farming practices of individual cabbage growers whose choice of pesticide and application rate might be different, depending on the level of insect pest infestation encountered on their farms (Ninsin, 1996; Odhiambo, 2005).

Generally, residual activity was observed to be higher in run-off than in soil extracts within Korle-Bu and Airport areas. The bulk of pesticide residues in soil have been reported to be confined to the upper 5 cm of the topsoil and agrochemicals in soil can move from the surface when they are dissolved in run-off water, or when they percolate down through the soil (FAO,

2000). This may explain the differences in residual activity of soil and run-off extracts observed in this study. It has been reported that the persistence of a pesticide in the soil depends on the soil's pH, texture and microoraganisms (El Beit *et al*., 1981). It is, therefore, likely that the variation in residue levels in soil extracts from the two sites may be due to differences in the physiochemical properties of the different soils.

No correlation was, however, observed between the residual bioactivity and total residue level and the resistance levels in the *A. gambiae* s.s. populations in the two study sites. For instance, in the Airport Residential Area, the mosquitoes showed a high level of resistance to permethrin, deltamethrin and DDT relative to the Korle-Bu population. In contrast, the residual activity and total residue level were observed to be higher for the Korle-Bu than for the Airport areas. Mechanisms of resistance are known to develop as a result of long periods of exposure to particular insecticides (Chandre *et al*., 1999a). Thus, the increase in knockdown resistance may have evolved due to prolonged exposure to sub-lethal doses of the organophosphates and pyrethroids within these farming environ over time. The high level of resistance observed in *A. gambiae* from the Airport area, accom-panied by lower residue levels, indicates that resistance might have been selected for a longer period of time, since cabbage is grown in this area all year round and the area has been under cultivation for close to 30 years (Odhiambo, 2005).

Soil and run-off water may not have much accumulation of insecticide residues at the Airport area partly because of good drainage and partly because of observance of better agronomic practices by farmers at the Airport area compared to those at Korle-Bu (Odhiambo, 2005). Some farmers in the Airport area use water drawn from deep wells and also water running through the big drains to the Odaw river. Farmers at Korle-Bu, however, obtain water mostly from shallow wells. Furthermore, the frequency of application of insecticides by individual farmers in the two sites may be different, resulting in different levels of selection pressures on the mosquitoes. Thus, pools of water in the farms which may serve as breeding places may contain varying levels of insecticide residues, leading to the differences noted in the development or enhancement of resistance in mosquito larvae, since insecticides used by these cabbage farmers for crop protection are similar to those used for vector control programmes.

The study indicates the role of agricultural use of insecticides in the build up of resistance in malaria vectors, with its attendant implications. There is need, therefore, to consider the role of agricultural use of insecticides and farming practices in resistance management strategies for malaria vectors in order to improve the effectiveness of malaria control pro-grammes. Such studies will provide useful information for planners and implementers of malaria control programmes in Ghana.

Acknowledgement

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