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Selection for and biochemical characterization of DDT resistance in laboratory strains of *Anopheles arabiensis*

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Resistance to conventional insecticides still constitutes a major obstacle to control of malaria vectors. Xenobiotic pollutants encountered by aquatic stages of natural populations of malarial vector species in agricultural and domestic environment are often selected due to resistance to various insecticides. The Laboratory Matatuine (MAT) and Kayamba (KGB) strains of *Anopheles arabiensis* were subjected to controlled dosages of DDT for over twenty generations. WHO insecticide susceptibility protocols was used to monitor changes in mortality between generations. The selected lines of both strains developed resistance to DDT and cross resistance to permethrin. Polymerase Chain Reaction (PCR) detection of knock-down resistance (kdr) gene and sequencing revealed absence of L1014F mutations. Biochemical analysis of detoxification enzymes showed significant Glutathione S transferase (GST) activity in the selected lines [MAT: 0.236 (P>0.001) and KGB: 0.221 (P>0.014)], thus suggesting the presence of GST-based resistance mechanism.

Key words: Dichloro-diphenyl-trichloroethane (DDT), *Anopheles arabiensis.*

INTRODUCTION

Anopheles arabiensis is the second most efficient malaria vector species of the *An*. *gambiae* complex and it occurs in sympatry with *An*. *gambiae* sensu stricto in most areas. The two species form the most efficient malaria vectorial system in Africa (Powell et al., 1999; Coetzee et al., 2013). It often breeds in pesticide contaminated rice irrigation ecosystems found at malaria endemic areas in East and West Africa and adapt faster to man-made ecological habitats in urban cities (Coluzzi et al., 1979; Ijumba and Lindsay, 2001; Kamau and Vulule, 2006). It contributes considerably to malaria transmission in South Africa, Sudan, Nigeria and is the major malaria vector in Tanzania (Onyabe and Conn, 2001; Nardini et al., 2013; Matawo et al., 2014; Tarig et al., 2018). Current global strategy for control of malaria is based on chemoprophylaxis, treatment of diagnosed infected

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persons using effective anti-plasmodial drugs and insecticidal based methods to control the malaria vectors. The World Health Organisation (WHO) Pesticides Evaluation Scheme (WHOPES) approves eleven insecticides including permethrin and DDT to be used in public health, particularly malaria control programmes (WHO, 1998). Development of resistance to insecticides by malaria vector species still remains the major obstacle to malaria control globally (Karunaratne et al., 2018; Ranson et al., 2011). Resistance is the ability of an insect to withstand toxic effects of an insecticide by means of natural selection and mutations and is a heritable genetic trait passed down through generations (Davidson, 1957; Corvel and Nguessan, 2013). Increasing selection pressure on malaria vector populations caused by xenobiotic pollutants presence in agricultural and domestic environments necessitates selection for resistance to various insecticides (Nkya et al., 2012; Matawo et al., 2015). Resistance to DDT in field populations of *An. arabiensis* began to appear in South Africa (Hargreaves et al., 2003), Nigeria (Kristian et al., 2003), and subsequently, in other countries. Resistance to deltamethrin, permethrin and DDT have been reported in Ethiopia (Balkew et al*.,* 2010; Yewhalaw et al., 2011) and Sudan (Abdullah et al., 2008).

The resistance to DDT in mosquitoes is generally associated with one of two mechanisms; increasing DDT dehydrochlorination catalysed by GSTs or decreased target site sensitivity (Hemingway and Ranson, 2000; Ranson et al., 2011; Karunaratne et al., 2018). Some of the previous studies on insecticide resistance in *An. arabiensis* have used populations selected in the laboratory for many generations (Hemingway, 1983; Matambo et al*.,* 2007). The use of laboratory strains (MAT and KGB) in comparison with field strains to study resistance mechanisms advantageously exclude factors such as effect of temperature, larval diet and exposure to agricultural pesticides that can confound diagnosis (Nardini et al*.,* 2013). The present study was aimed at selecting MAT and KGB laboratory reared strains of *An. arabiensis* for DDT resistance. The specific objectives were to (i) test for succeptibility of parental lines of MAT and KGB strains to DDT (ii) select subsequent generations for resistance to DDT (iii) test the selected lines for cross resistance to permethrin, and (iv) compare levels of activity of the detoxifying enzymes GSTs, esterases and monooxygenases in mosquitoes sampled from parental and selected lines of both strains.

MATERIALS AND METHODS

Establishment of *An. arabiensis colony*

Eggs of *An. arabiensis* MAT strains were collected from a field site at Matatuine located 10 km from Maputo in Mozambique. The colony was first established at the Institute Nacional de Saude Mozambique in May 2000 and transferred to The Liverpool School of Tropical Medicine in 2002. No information was available on the resistance status of this colony to any class of insecticide. Adult females of *An. arabiensis* KGB stains were caught at Kayamba, Zambesi Valley in Zimbabwe in 1975 and a colony established at The South African Institute for Medical Research. Eggs were brought on request to Liverpool in September 2004 and a colony was re-established there.

Mosquito rearing

The colonies of MAT and KGB *An. arabiensis* mosquito strains were maintained in the insectaries at the Liverpool School of Tropical Medicine. The mosquitoes were reared at temperature range of 27 to 28°C , 80 to 85% relative humidity with 12-h day/night light regime and 45-min dusk/dawn cycles. The duration of development from eggs to emerging adults ranged from 7-12 days amongst both strains. All mosquito larvae were fed on Tetramin fish food flakes, using ground-up flakes for the first instar. Extreme care was taken to avoid contamination between the different lines and strains of *An. arabiensis.* All larval trays were cleaned with hot water after each rearing cycle, when all the pupae had emerged into adults. Pipettes, egg pots and larval trays were colour-coded for each line of *An. arabiensis* strains. Adult mosquitoes were constantly provided with cotton wool soaked in saturated 10% sugar solution formed by using tap water. Females of both strains were given guinea pig blood twice a week (Hunt et al., 2005). Samples of the adult mosquitoes were identified to species using the polymerase chain reaction method described by Scott et al*.* (1993).

Susceptibility to WHO bioassays

Adult mosquitoes from both colonies of the parental lines of *An. arabiensis* MAT and KGB strains were tested for susceptibility to DDT. Bioassays were performed according to WHO protocols using standard WHO susceptibility test kits and 4% DDT impregnated papers (WHO, 1998). Survivors from each test were placed in a separate cage and used to establish subsequent generations.

Selection for resistance to DDT

Mosquitoes from *An. arabiensis* MAT and KGB strains, which survived previous exposures to DDT, were reared and their progeny subjected to selection using 4% DDT. Adult mosquitoes from *An. arabiensis* MAT strain were maintained under selection pressure with 4% DDT continuously for eight months and selection was interrupted for five months due to a crush in the colony, but thereafter the selection pressure was continued further for eight months. Mosquitoes from *An. arabiensis* KGB strain were similarly selected for resistance to DDT for a period of twenty three months without interruption.

Testing for cross resistance to permethrin

Batches of mosquitoes from *An. arabiensis* MAT and KGB selected lines were tested for resistance to DDT and cross-resistance to permethrin. Samples of adult mosquitoes from F10 and F20 selected generations of *An. arabiensis* MAT and *An. arabiensis* KGB respectively were exposed to 4% DDT and 0.75% permethrin for one hour. Knocked-down mosquitoes were recorded at intervals of ten minutes. The KDT50 and KDT90 knock-down times were calculated by probit analysis. The data was entered into Minitab 14 and LDP line software programmes for the analysis.

Knockdown resistance (kdr) assay

A PCR assay described by Martinez-Torres et al*.* (1998) was used to

Table 1. Susceptibility of adult mosquitoes in parental MAT colony to DDT.

Time (min) exposure	Number tested	Number dead	Number alive	% Mortality 24 post-exposure
15	58	16	42	27.5
30	80	53	27	66.3
45	135	116	19	85.9
60	65	57	8	87.6

Initial scores for mortality from WHO diagnostic test kit for 4% DDT tested against adult mosquitoes (n=338) sampled from the F1 generation of parental line.

Table 2. Susceptibility of adult mosquitoes in parental KGB colony to DDT.

Time (min) exposure	Number tested	Number dead	Number alive	% Mortality 24 post- exposure
15	67	13	54	18.6
30	85	38	47	44.7
45	98	65	33	66.3
60	148	121	27	81.6

Initial scores for mortality from WHO diagnostic test kit for 4% DDT tested against adult mosquitoes (n=398) sampled from the F1 generation of parental line.

test for the presence of the typical kdr mutations in individual mosquitoes sampled from parental and selected lines of both strains.

Biochemical assays

Unexposed mosquito samples taken from the parent stock and F15 selected generation of *An. arabiensis* MAT and KGB strains were kept at -80°C for biochemical analysis. Biochemical assays were performed according to the standardized procedures described in the manual by Hemingway (1998). Batches of 22 one-day old, frozen mosquitoes were individually homogenized in 200 µl of distilled water in 1.5 ml Eppendorf tubes. The crude homogenate was spun at maximum speed of. 10,000 rev min^{-} for two minutes in a microfuge. After centrifugation, the supernatant from each Eppendorf tube was then transferred to a well of a micro titre. Esterases, monooxygenases and GST assays were carried out in line with WHO (1998).

Protein assay

Protein assays were conducted according to the method of Bradford (1976). Microfuged homogenate (10 µl) from each mosquito was added to 300 µl Bio-Rad Protein assay reagent (diluted 5 times from stock), incubated for 5 min and end point absorbance measured at 570 nM. Protein concentration was determined by converting the absorbance into concentration based on a bovine serum albumin standard curve.

RESULTS

WHO susceptibility assays

The susceptibility levels to DDT of adult mosquitoes of the original parental populations of the *An*. *arabiensis* MAT and KGB strains were determined. A total of 338 adult mosquitoes aged two to three day old from the parental *An*. *arabiensis* MAT strains were exposed to 4% DDT for different time periods (Table 1). The 87% mortality after exposure to DDT for one hour indicates the presence of low level of resistant genotypes in the MAT parental colony.

In the KGB strain, 389 adult mosquitoes in batches of 20 to 25 were exposed at the four different time points and mortality was recorded 24 h post exposure as shown (Table 2).

The 81.6% mortality after exposure to DDT for 1 h suggests higher level of resistant genotypes in KGB than in the MAT colony.

Selection of resistant genotypes

The mortality decreased from 73.5% in the F3 to 51.4% in the F6. Due to rearing problems, selective pressure was not applied in generations from F7 to F13. The mortality rose back to 61.8% in F14 but decreased gradually to 48.3% in generation 16. Selection at 45 min exposure period raised the mortality to 69.4% but subsequently decreased to 53% in F20 (Figure 1).

The KGB colony did show a similar pattern of response to DDT. However, the selection pressure was gradually increased from 30 min in F1 generation to 60 min over 20 generations. Over these selected generations, the mortality decreased from 56.4% in F6 to 28.4% in F14 (Figure 2).

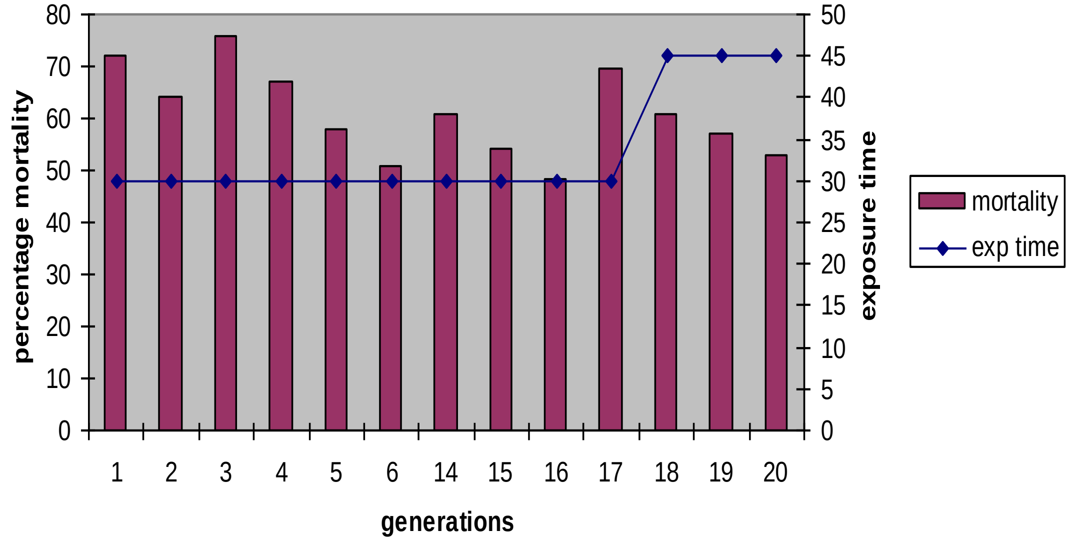

Figure 1. Selection of *Anopheles arabiensis* MAT strain with DDT.

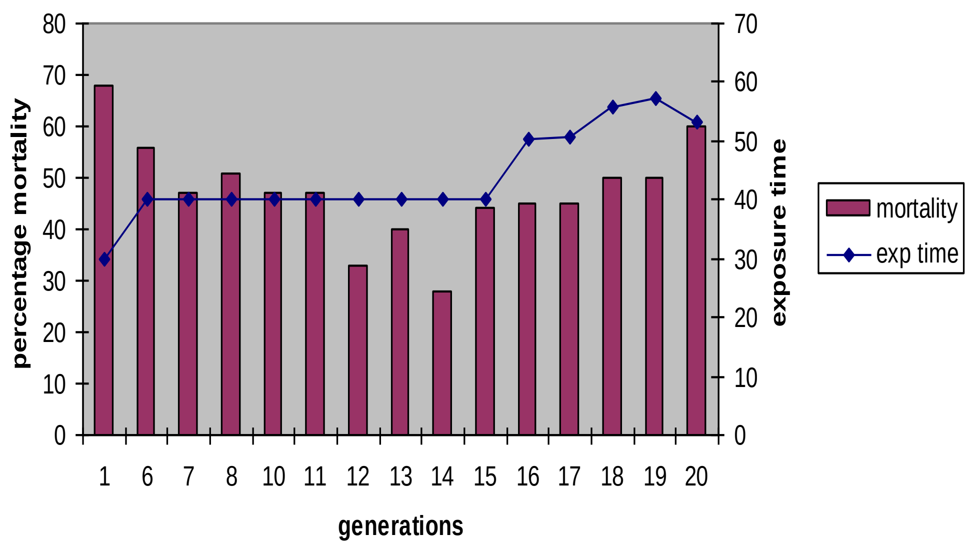

Figure 2. Selection of adult *Anopheles arabiensis* KGB strain with DDT.

After selection, the susceptibility tests with the diagnostic dose of DDT (4%) were repeated at different time points for the parental and selected populations of both *An. arabiensis* MAT and KGB strains. The LT50 values for DDT were 23.4 min and 33.2 min (resistant ratio 1.4) in the parental and selected colonies of *An. arabiensis* MAT strain. The slopes of the regression lines are 2.96 in the parental and 2.4 in the selected lines respectively (Table 3). In the KGB strain, the LT50 values were 33.5 min and 50.8 min and the corresponding slopes of the regression lines were 3.26 and 1.7 in the parental and selected populations respectively (Table 3). The change in slope of regression lines between KGB selected and parental indicates increased resistance in the selected population.

Cross-resistance to permethrin

The populations of *An. arabiensis* DDT selected in both MAT and KGB parental strain were also tested against the diagnostic dosage of 0.75% permethrin to check for cross resistance or increased tolerance (Figure 3). Significantly more mosquitoes were knocked down by permethrin at 30 min, 40 min, ($P < 0.001$) and at 50 min ($P = 0.072$) in the parental line than in the DDT selected

Table 3. Relative susceptibility of DDT (4%) based on time mortality relationships tested against parental and selected lines of *An*. *arabiensis* MAT and KGB strains.

Strain	Line	Sample	No. tested	LT50 (min)	CI	LT90	Slope	RR	X^2
MAT	Parental	F20	355	23.4	(20.3 - 26.1)	63.40	2.9 ± 0.3	1	0.703
	Selected	F20	290	33.2	(29.2 - 37.7)	88..96	2.40 ± 0.3	1.4	1.301
KGB	Parental	F20	360	35.1	(28.2 - 37 .3)	95.06	3.26 ± 0.5	1.5	0.821
	Selected	F20	581	50.8	(44.1 - 66.6)	139.56	1.70 ± 0.4	2.2	1.25

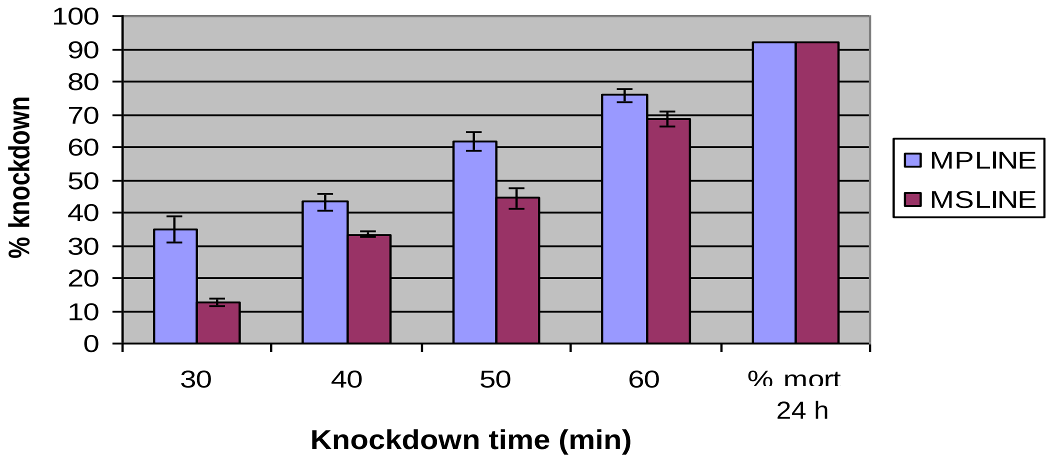

Figure 3. Percentage knockdown and percentage mortality of 1 to 3 day-old adult F18 on 0.75% permethrin. MPLINE (MAT parental line) (n = 261) and F18 MSLINE (MAT selected line) (n = 144) during 30 – 60 minute exposure to 0 .75% permethrin and 24 h after exposure respectively.

Table 4. Comparisons of geometric means (with 95% confidence limits) for GST activity in *An*. *arabiensis* MAT and KGB strains.

Variable	Line	N	Mean (95% confidence interval)		Test statistic	p-value
	Mparental	59	0.139	(0.120 - 0.161)		
	Mselected	61	0.236	(0.209 - 0.267)	Mp vs Ms	< 0.001
GSTact	KGBPG6	60	0.183	(0.170 - 0.198)		-
	KGBSG9	70	0.221	(0.202 - 0.241)	Kgbg6 vs g9	0.014
	KGBSG15	92	0.189	(0.173 - 0.205)	Kgbg6 vs g15	0.022

Test statistics: GSTact:- F(118) = 5.579 MAT p < 0.001, F(2,219) = 5.002 KGB p = 0.014. KGBPG6 denotes KGB parental generation 6, KGBSG9, KGB selected generation 9, KGBSG15, KGB selected generation 15.

population but both showed > 97% mortality at 24 h after exposure (Figure 3).

Biochemical assays

Glutathione S – transferase activity

The geometric mean GST activity was significantly higher (p < 0.001, 0.014) in populations under selection pressure than in the parental (unselected) populations in *An*. *arabiensis* MAT and the ninth generation KGB strains respectively (Table 4).

Esterase activity

The geometric mean values of α-esterase and β-esterase

Table 5. Comparisons of geometric means (with confidence limits) for alpha and beta esterase activities between adults sampled from the parental and selected lines of *An. arabiensis* MAT and KGB strains.

Variable	Line	N	Mean (95% confidence interval)		p-value
Alfact	Mparental	59	0.000399	(0.000363 – 0.000438)	
	Mselected	61	0.000653	(0.000551 – 0.000774)	< 0.001
	KGBPG6	60	0.000700	(0.000655 – 0.000749)	-
	KGBSG9	69	0.000862	(0.000791 – 0.000940)	0.004
	KGBSG15	107	0.000583	(0.000539 – 0.000630)	<0.001
Betact	Mparental	59	0.000271	(0.000247 – 0.000297)	
	Mselected	61	0.000447	(0.000373 – 000535)	<0.001
	KGBPG6	60	0.000536	(0.000504 – 0.000571)	
	KGBSG9	70	0.000670	(0.000615 – 0.000729)	< 0.001
	KGBSG15	92	0.000423	(0.000392 – 0.000456)	<0.001

Table 6. (Geometric) mean monooxygenase activity levels (with 95% confidence intervals) in *An. arabiensis* MAT and KGB strains.

Strain	Line	N	Mean (95% confidence interval)		p-value
MAT	Parental	38	0.000192	(0.000163 – 000227)	-
	Selected	32	0.000410	(0.000300 – 0.000559)	0.001
KGB	KGBGP6	72	0.000171	(0.000158 – 0.000184)	-
	KGBSG9	114	0.000186	(0.000173 – 0.000201)	0.148
	KGBSG15	89	0.000161	(0.000150 – 0.000172)	<0.677

activities were significantly higher ($p < 0.001$) in the selected than in the parental lines of the MAT strains. In the KGB strain, the esterase activities were higher in the KGBS9 compared to KGBP6 ($p < 0.001$). However, the activities in KGBSG15 were lower than that in KGPG6 ($P < 0.004$) (Table 5).

Monooxygenases activity

The geometric means of monooxygenases in the selected and parental lines in the *An. arabiensis* MAT and KGB strains were not significantly different ($p = 0.148$) (Table 6).

DISCUSSION

The results of this study indicate that a low level of physiological resistance to DDT in *An. arabiensis* is developed under selection pressure in the laboratory. The LT50 and LT90 values of DDT increased significantly over 15 generations of selection pressure in both MAT and KGB *An. arabiensis* strains. The LT50 and LT90 recorded for the twentieth generation of KGB selected line are similar to the values reported for the DDT resistant field populations of *An. gambiae* sl (Tarig et al., 2018). In the selection of *An. arabienisis* MAT strain, high variation characterised the mortality values during the first four generations. This might have been due to the error made initially in the selection process by putting the survivors from the first two selection experiments back in the same cage with the parental colony. Mating between the two populations might have resulted in dilution of the selected resistant genotypes; therefore making the population more susceptible to DDT as was earlier hypothesized (Prasittisuk and Curtis, 1982). Alternatively, it has been suggested that high variation in mortalities is perhaps typical of populations in early stages of selective pressure (Theeraphap et al., 2002).

The general patterns of the selection for DDT resistance are similar in both strains. This observation is typical of most laboratory regimes which tend to select within existing phenotypic distributions often at 80 – 90%

mortality in order to provide survivors for the next generation (Martins et al., 2012; Roush and McKenzie, 1987). The dosage for selection was closely controlled between 30 to 45 min and 30 – 60 min for the MAT and KGB respectively to permit discrimination among similar genotypes within the physiological distribution of phenotypes (Roush and McKenzie, 1987). However, in previous similar studies, 3 laboratory colonies G1, SENN and MBN of *An*. *arabiensis* have been selected for resistance to DDT at higher doses and adults were reported to have survived exposure to DDT for 8 h (Hemingway, 1981; Matambo et al., 2007). This suggests that the KGB and the MAT strains were at comparatively low level of DDT resistance. Theoretically, a susceptible colony comprising of totally susceptible individuals will produce the highest slope for a regression line of dose – response data. With selective pressure from the exposures to insecticides, a population will become heterozygous for resistant genotypes and as the frequency of resistant genotypes increases, the slope of the regression line will shift to the right (Brown and Brogdon, 1976). There was a shift to the right in regression lines from dose response data for the populations under DDT selection in both the MAT and KGB strains and the slopes of regression lines based on the data from these experiments continuously declined over time in the two strains. This suggests that the resistance to DDT in the selected populations was not due to vigour tolerance but reflects true physiological resistance (Oliver and Brooke, 2014; Brown and Brogdon, 1976).

Evidence for cross resistance to permethrin was observed in the DDT – selected colony of *An*. *arabiensis* MAT strain Various previous studies have shown some evidence for resistance to permethrin in colonies of *An. arabiensis* strains selected for DDT resistance (Nardini et al., 2013; Matambo et al., 2007). In addition, cross resistance between pyrethroids and DDT has been reported in natural populations of *An. arabiensis* (Abdulla et al., 2008), *An. gambiae* (Matawo et al., 2015), and *An. funestus* (Tchouakui et al., 2019). The similar mode of action of DDT and pyrethroids can result in cross-resistance if the mechanism is due to kdr mutations in the sodium ion channel target sites (Martinezz-Torre et al., 1998; Tene et al., 2013). The West African L1014F mutation has previously been reported in *An. arabiensis* from Sudan (Abdullah et al., 2008) and SENN-DDT resistant laboratory strain (Matambo et al., 2007), although in both the correlation between the L1014F genotype and DDT resistant genotype, it was not clear.

In this study, analysis of sequence data for the gene revealed absence of *kdr* mutations in both MAT and KGB colonies. Similarly, the kdr mutation has not been observed in the M form of *An. gambiae* ss and *An. arabiensis* despite high levels of resistance to pyrethroid and DDT although it was found in the S form (Diabete et al., 2002). The kdr mutations have been documented in DDT resistant field populations of *An. coluzzi*, *An. gambiae* ss and *An. arabiensis* (Cisse et al., 2015). The combined effects of detoxifying enzymes and potential mutations have been associated with resistance to multiple insecticides in *An. funestus* and *An. arabiensis* (Menze et al., 2016; Matawo et al., 2014) Nevertheless, the absence of the *kdr* mutations in the colonies of *An. arabiensis* studied here is not conclusive considering the low number of samples used in the assay.

The results of biochemical analysis have shown that more individuals with high GST activity are present in the selected than in the parental lines of both the MAT and KGB strains. The order of magnitude of change in GST activity observed in the selected populations of MAT and KGB strains is consistent with our recent report on involvement of the epsilon gste2 gene in DDT resistance in the colonies (Yayo et al., 2018, 2019). Previous studies have severally associated resistance to DDT with increased levels of GST activities in several species of mosquitoes including *An. subpictus* (Hemingway et al., 1991) and *An*. *gambiae* (Karunaratne et al., 2018). Elevated esterase activity was also detected in the MAT populations under DDT selection compared to the unselected population.

The results in absolute unit for the alpha and beta esterase were similar to those of Hargreaves et al. (2003). Casimiro et al. (2006) found lower average esterase activities with the two substrates in DDT susceptible populations of *An. arabiensis* from Mozambique. The monooxygenase activity was low in the selected populations in KBG strain, suggesting that the p450 enzyme system may not be involved in DDT resistance in this strain. However, the monooxygenase activity was significantly higher in some individuals from selected line compared to the parental line in MAT strain, but the small sample size was low to derive a conclusion.

Conclusion

Two laboratory strains of *An. arabiensis* exposed to controlled doses of DDT have developed resistance to DDT and cross resistance to permethrin. Analyses of the detoxification enzymes have shown significantly high GST activity in the selected line of both strains. Preliminary investigations revealed absence of kdr suggesting the possible role GST-based DDT resistance mechanism in the colony.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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