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First Evidence of High Knockdown Resistance Frequency in *Anopheles arabiensis* (Diptera: Culicidae) from Ethiopia

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Abstract. The status of knockdown resistance (*kdr*) mutation was investigated in the major malaria vector *Anopheles arabiensis* Patton (Diptera: Culicidae) from Ethiopia. Among 240 mosquito samples from 15 villages of southwestern Ethiopia that were screened by allele-specific polymerase chain reaction for *kdr* mutations, the West African *kdr* mutation (L1014F) was detected in almost all specimens (98.5%), whereas the East African *kdr* mutation (L1014S) was absent. Moreover, the mortality of *An. gambiae* s.l. to diagnostic dosages of 4% DDT, 0.75% permethrin, and 0.05% deltamethrin from bioassay results was 1.0%, 18.1%, and 82.2%, respectively. We report here the highest *kdr* allele frequency ever observed in *An. arabiensis* and its implications in malaria vector control in Ethiopia are discussed.

INTRODUCTION

In Ethiopia, the control of malaria relies on early diagnosis, effective treatment of malaria patients, vector control by indoor residual spraying (IRS), and large-scale distribution of insecticide-treated nets (ITNs) or long lasting insecticide nets (LLINs).[1] DDT was and is still the primary agent used for IRS, whereas pyrethroids are used to treat mosquito nets. *Anopheles arabiensis* is the most important malaria vector in southwestern Ethiopia.[2] Insecticide resistance to DDT and permethrin in *An. arabiensis* has been reported from different parts of Ethiopia.[3,4] An important mechanism that is associated with both DDT and pyrethroid resistance is knockdown resistance. In *Anopheles gambiae sensu stricto* two mutations at the domain II of the voltage-gated sodium channel gene have been associated with resistance to DDT and pyrethroids.[5,6] The first mutation, West African *kdr* (L1014F), involves a nucleotide change resulting in the substitution of leucine residue (TTA) to a phenylalanine (TTT). This mutation is widespread in West Africa at variable frequencies.[7,8] The second mutation, East African *kdr* (L1014S), consists of a leucine (TTA) serine (TCA) substitution at the same codon and was originally described in Western Kenya.[6,9] The presence of both East and West African *kdr* mutations in *An. gambiae* s.s. populations has been reported from different countries in Africa.[10,11] The geographic restriction of both mutations is less definite than previously thought.[12]

The two mutations, West and East African *kdr* in the major malaria vector *An. arabiensis*, have not been described yet in Ethiopia. Therefore, in this study the occurrence of the *kdr* mutation and its allele frequency were determined in *An. arabiensis* population from Ethiopia. Such information is essential in the light of the ongoing efforts to scale-up the use of LLINs in Ethiopia.

MATERIALS AND METHODS

Study area. The study was conducted in the framework of a longitudinal study on malaria incidence and transmission in the surroundings of the Gilgel-Gibe hydroelectric dam, southwestern Ethiopia. This study showed that children living near the man-made reservoir (within 3 km from dam, designed as "high-risk" villages) were at higher risk of malaria compared with those living farther away (5–8 km from dam, designed as "low-risk" village).[13] The study area lies between latitudes 7°42′50″N and 07°53′50″N and between longitudes 37°11′22″E and 37°20′36″E, at an altitude of 1,671 to 1,864 m above sea level. The area has a sub-humid, warm to hot climate, with a mean annual rainfall between 1,300 and 1,800 mm and a mean annual temperature of 19°C. The primary economic activity of communities in both groups of villages is subsistence farming. Vector control intervention in the study area is similar to other parts of the country relying on IRS using DDT and ITNs/LLINs distributed through both the public and private sector.

Mosquito collection. Adult female anopheline mosquitoes were collected from houses located in 15 study villages from September to October 2008 by hand capture collection of indoor resting mosquitoes (IRCs) using oral aspirators.[14] Identification of collected mosquitoes was carried out morphologically using the standard key of Gillies and Coetzee.[15] Mosquitoes were individually preserved in Eppendorf tubes (Eppendorf Intl., Germany) over silica-gel for further molecular assays.

DNA extraction, molecular identification, and detection of *kdr* alleles. DNA extraction from mosquitoes was carried out individually applying the procedure described elsewhere.[16] DNA was re-suspended in 25 mL sterile TE-buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). The members of the *An. gambiae* complex were identified molecularly using polymerase chain reaction (PCR) techniques including the primers for *An. gambiae* s.s., *An. arabiensis, An. quadriannulatus A* and *B*.[17] The protocol used for the detection of the West and East African *kdr* alleles was adapted from established protocols.[5,6,11] Sequencing of the fragment of the domain II of the voltage-gated sodium channel gene of at least one specimen per genotype was performed from amplified products obtained with primers Agd1 and Agd2 to confirm the genotyping done by the allele-specific PCR. The PCR products were sequenced by Genoscreen (Lille, France). Deviation from Hardy-Weinberg equilibrium and population differentiation was tested using Genepop 3.4 exact tests.[18]

Bioassays. Bioassays were done on wild-caught adult *An. gambiae* s.l. collected by indoor resting catches from the same study area in August 2009 to assess the importance of

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the findings on *kdr* allele frequency in terms of phenotypic resistance. Standard World Health Organization (WHO) susceptibility test procedures were done,[19] using permethrin 0.75%, DDT 4%, and deltamethrin 0.05% at discriminating concentrations. These insecticides were chosen as *kdr* causes cross-resistance to DDT and pyrethroids. On average, 20 batches of mosquitoes in five replicates were exposed in test kit tubes for 1 hour against DDT and deltamethrin (four replicates for permethrin) and knockdown was recorded at the end of the exposure period. An equal number of mosquitoes were exposed to untreated papers impregnated with oil to serve as a control. Mortality was recorded 24 hours post-exposure. As insects in the same tube share common "tube-related" characteristics, we included "tube" as a clustering effect in the calculation of the confidence intervals for the mortalities. The clustering effect was taken into account by using the Taylor series linearization variance estimation for complex survey data by using the svy: commands in the Stata 11 software (Stata Corp., College Station, TX).

RESULTS

Of the 284 *An. gambiae* s.l. collected (September to October 2008), 265 were molecularly identified as *An. arabiensis. Anopheles gambiae* s.s and *An. quadriannulatus* were not detected and DNA of 19 specimens could not be amplified. In total, 240 *An. arabiensis* could be scored for both the West (L1014F) and East (L1014S) African *kdr* alleles. No East African *kdr* mutation was detected, whereas the allele frequency of the West African *kdr* mutation was greater than 98% (Table 1). The West African *kdr* mutation was found in each of the 15 study villages. The *kdr* homozygous genotype occurred with a very high frequency in both groups of villages (96.75% in "high-risk" and 99.15% in "low-risk" villages). No significant differences in West African *kdr* allele frequency was observed among the study villages ($P = 0.086$) or between the "high-risk" and "low-risk" groups ($P = 0.452$). A deviation from the Hardy Weinberg equilibrium was found in both the "low-risk" ($P = 0.004$) and "high-risk" ($P = 0.041$) groups.

The sequencing confirmed the genotyping by allele-specific PCR and sequences were deposited in GenBank with the following accession nos.: homozygote (L1014F) GU248311, heterozygote GU248312, and homozygote wild type GU248310.

Anopheles gambiae s.l. was highly resistant against DDT 4% and permethrin 0.75% with mortality of 1% and 18%, respectively. The mortality rate in the deltamethrin-treated group was 82% with 71.96% knockdown at 60 minutes (Table 2).

DISCUSSION

In this study, the major malaria vector *An. arabiensis* from southwestern Ethiopia was screened for both East and West African *kdr*. A very high frequency of the West African *kdr* mutation was observed, which contrasts with the observations in other African countries for this species. Knockdown resistance was absent in *An. arabiensis* from Mali[7] and Cameroon,[20] whereas the West African *kdr* mutation (L1014F) was reported in *An. arabiensis* from Burkina Faso,[21] Tanzania,[12] and Sudan,[22,23] at very low to moderate frequencies. In the current study, no East African *kdr* mutation was observed. The East African *kdr* (L1014S) mutation was found for the first time in *An. arabiensis* from Uganda.[11]

The current high frequency of West African *kdr* mutation observed in *An. arabiensis* populations of southwestern Ethiopia may be attributed to the long intensive use of DDT in indoor residual spraying by the malaria vector control program and/or by the illegal extensive use of DDT for the control of pests of kat (*Katha edulis*) and green pepper (*Capsicum annuum*) crops and also for the control of storage pests of maize (*Zea mays*) and sorghum (*Sorghum bicolor*) in the study area (Yewhalaw, pers. obs.). Moreover, the use of pyrethroids as aerosol for control of household pests and vectors could also be implicated to the observed selection of a high level of *kdr* resistance.[24]

The observed high *kdr* frequency in Ethiopia might result in a phenotypic resistance as defined by WHO. The bioassays using the discriminating concentration of permethrin 0.75%, DDT 4%, and deltamethrin 0.05% indicate that the population was highly resistant, especially to DDT and permethrin (99% and 81.9%, respectively). The observed resistance was by far higher than reported from eastern Ethiopia (around 30% survival for DDT[4,25] and 25% for permethrin[4]) and from South and southwestern Ethiopia[3] (70% or lower survival for DDT). In South Africa, lower survival of *An. arabiensis* (37%) for DDT was also detected 24 h post-exposure.[26] The mortality level for deltamethrin (82.2%) also suggests resistance despite the fact that deltamethrin is not yet used for indoor residual spraying in the malaria control program in Ethiopia. However, the lower resistance of *An. arabiensis* to deltamethrin may be attributed to the large-scale distribution of LLINs (PermaNet, Vestergaard Frandson, Denmark) by the Ministry of Health throughout the country (20 million LLINs were distributed between 2005 and 2007) and/or could result from cross-resistance to DDT.

The West African *kdr* mutation is closely associated with DDT and pyrethroid resistance in the major malaria vector *An. gambiae* s.s. and is considered to be the cause of the resistance genotype.[27] However, *kdr* may not always have a significance to control interventions and the protective efficacy of nets may remain high. Various studies have shown that insecticide-treated nets can provide protection despite the presence of *kdr* resistance. This could be attributed to the prolonged contact of resistant mosquitoes to impregnated substrate before taking off as a result of diminished sensitivity to the irritant effect of the insecticide. Hence, the resistant mosquitoes would die of the high dose of the pyrethroid deposit.[28–30] In contrast, a

TABLE 1

Frequency of West African *kdr* mutation (L1014F) among wild populations of *Anopheles arabiensis* from southwestern Ethiopia

Type village	Number villages	Number tested	Homozygote mutation	Heterozygote	Homozygote wild type	*Kdr* allele frequency
Low-risk	7	117	116	0	1	0.992
High-risk	8	123	119	3	1	0.980
Overall	15	240	235	3	2	0.985

TABLE 2

Mortality rate and knockdown in field populations of *Anopheles gambiae* s.l. for the different insecticides, southwestern Ethiopia

Insecticide	Number tested	% Knockdown at 60 minutes [95% CI]	Percentage mortality [95% CI]
DDT 4%	100	0% [0–4.3]*	1.0 [0.9–2.9]
Permethrin 0.75%	83	0% [0–3.6]*	18.1 [0.5–35.7]
Deltamethrin 0.05%	107	71.96% [65.1–78.8]	82.2 [77.2–87.3]

*= exact (Clopper-Pearson) binomial confidence intervals (all other confidence intervals [CIs] are calculated, including the "tubes clustering" as specified in the text).

study conducted in Benin showed that high frequency of West African *kdr* correlates to reduced efficacy of pyrethroid-based vector control efforts using insecticide-treated nets and indoor residual spraying.[31] Moreover, other resistance genes along *kdr* may considerably increase the level of insecticide resistance.[20,32] It should also be noted that the observed deviation from Hardy-Weinberg equilibrium in the current study would suggest that selection against *kdr* heterozygotes is still ongoing. This might be caused by the efficacy of pyrethroid insecticides against heterozygotes, as *kdr*-type resistance to mortality and knockdown were reported to be semi-dominant.[28]

The high prevalence of *kdr* resistance highlighted in the current study may call for initiating programs designed to monitor the distribution and spread of this resistance and to study the operational implications of the observed *kdr* frequency. This information is needed to guide the further use of insecticides in malaria control programs and vector resistance management interventions such as the need for alternatives to the currently used DDT and perimethrin/deltamethrin for IRS and treatment of mosquito nets, respectively.

In conclusion, our study represents the first evidence of the occurrence of high frequency of the *kdr* allele in *An. arabiensis*. The impact of the resistance on the efficacy of DDT and pyrethroids on vector control interventions in Ethiopia should be further investigated. It is also imperative to evaluate the status of *kdr* resistance throughout the country to implement vector control strategies designed to manage insecticide resistance.

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