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First report of insecticide resistance in *Anopheles gambiae* sensu lato from Sahel eastern Chad, central Africa

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ABSTRACT

Objective: Malaria is the main driver of sickness and death in Chad. To facilitate pre-elimination efforts in the Sahel region, researchers characterized the composition of anopheline species and their role in malaria transmission, examined the *Anopheles gambiae sensu* lato (*An. gambiae s.l.)* population in eastern Chad, and investigated the insecticide resistance status of field populations.

Methodology and results: Anopheline larvae were collected from sampling sites located in eastern Chad and reared in an insectary. Emerging *An. gambiae s.l.* adults were sorted to assess insecticides resistance profile using WHO tube assays. Overall 395 female mosquitoes collected by Pyrethrum spray catches, *An. gambiae s.l.* was the predominant, followed by *An. rufipes*, *An. squamosus* and *An. pharoensis*. ELISA detection revealed a low *Plasmodium falciparum* infection rate. Bioassays were carried out with adult female *An. gambiae s.l.* revealed resistance to pyrethroids and DDT (dichlorodiphenyltrichloroethane) and susceptibility to malathion and carbamates. TaqMan detected 1014F *kdr* mutation in *An. gambiae s.l.* at lower frequency.

Conclusion and application of findings: Vector control is the cornerstone of malaria control/elimination agenda, but information on molecular basis of insecticide resistance in the major malaria vectors *An. gambiae s.l.* from Sahel eastern Chad is grossly lacking. Such data underpin evidence-based vector control and resistance management. Here, we established the role of a major malaria *An. gambiae s.l.* from Sahel eastern Chad in malaria transmission. The results showing pyrethroid and DDT resistance in *An. gambiae s.l.* from eastern Chad highlight challenge associated with deployment of malaria control tools using pyrethroid bed nets and indoor residual spraying with DDT in the Sahel area of this country. Organophosphate and carbamate insecticides could be alternative for control strategies in this locality given their full susceptibility. Switching current DDT-IRS to organophosphate and carbamate-based IRS using such as pirimiphos-methyl for organophosphate or bendiocarb for carbamates appears as a promising alternative. For LLINs, priority should be given to dual-AI nets incorporating either pyrethroid plus PBO or pyrethroid

plus chlorfenapyr. Rotation across insecticide classes for organophosphate and carbamate should be performed annually for IRS in order to main effectiveness of control strategies and break potential emerging resistance. Mosquito susceptibility and molecular characterization of target mutation should be monitored each semester. Moreover, larval source management, housing improvements, and strict safety measures to prevent human toxicity should complement current interventions.

Keywords: *Anopheles gambiae* s.l., Malaria, Insecticide Resistance, Biltine, Chad.

INTRODUCTION

Malaria remains the most predominant vector borne diseases and a major public health challenge with endemicity reported in 80 countries worldwide, including Chad. The disease continues to cause significant morbidity and mortality globally each year, including refugee populations despite reported control progress in various settings (WHO, 2025). In Chad , malaria is endemic and accounts for 30% deaths out of all hospital deaths (NMCP, 2017) with some variation in the transmission intensity in specific areas such as the Sahel region characterized by seasonal patterns linked to the rainy season (Djaskano *et al.,* 2023). Prevention based on vector control strategies has largely contributed to reduction in malaria burden over the last two decades (WHO, 2022). Malaria vector control relies primarily on long-lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS) (WHO, 2018). The global technical strategy for malaria 2016-2030 calls for a reduction of malaria case incidence and mortality rate by 90% (WHO, 2024). Chad Republic relies on mass distribution of pyrethroid-based LLINs every three years as primary method for vector control, while organophosphate (Actellic® 300 CS, pirimiphos-methyl) and carbamate (Ficam®, bendiocarb) based-IRS campaign has been implemented in some eastern districts since 2015 (Kodindo *et al.,* 2021). However, widespread insecticide resistance in the main malaria vectors is reversing the effectiveness of these control measures (Riveron *et al.,* 2018). The main mosquitoes that transmit malaria have been identified as *An. arabiensis*, *An. gambiae*, *An. coluzzii* (Ibrahim *et al.,* 2019a; Kerah-Hinzoumbé *et al.,* 2009; Kodindo *et al.,* 2021), and identified secondary vectors identified include *An*. *funestus*, *An*. *pharoensis* and *An*. *ziemanni* (Coetzee *et al.,* 2013; Kerah-Hinzoumbé *et al.,* 2009). Several studies have reported the emergence and the spread of insecticide resistance in the *An*. *arabiensis*, *An*. *gambiae* and *An*. *coluzzii* in Chad Republic and established the molecular basis of the resistance (Dadzie *et al.,* 2016; Ibrahim *et al.,* 2023). The presence of *L1014F*, *L1014S* and *N1575Y* knockdown resistance (*kdr*) mutations has also been detected in the several *An*. *gambiae*, *An*. *coluzzii* and *An*. *arabiensis* from southern to central Africa Sahel region (Antonio-Nkondjio *et al.,* 2019; Fadel *et al.,* 2024). Genome-wide transcriptomic and population genetic analyses using RNA-Seq supported with qRT-PCR identified *CYP6Z2* and *GSTe2* genes contributing to metabolic insecticide resistance in Sahel region of Chad Republic (Fadel *et al.,* 2024; Ibrahim *et al.,* 2023). Also, there is the concern that escalation in pyrethroid resistance in the major malaria vectors *An. gambiae s.l.* population from southern endemic region and *An*. *coluzzii* from soudano-sahelian region could jeopardize malaria control efforts in Chad. To achieve malaria pre-elimination from such eastern region of Chad Republic, data-informed policymaking by national malaria control program requires temporal and spatial surveillance of insecticide resistance. We report first-hand data on populations of the key malaria vector *An. gambiae s.l.* from eastern Sahel region of Chad. The role of these

vectors in malaria transmission, their resistance status to various public health insecticide, and the occurrence of the *1014F* knockdown resistance mutation in the field was assessed.

MATERIALS AND METHODS

Study sites and populations: The Ministry of Public Health of Chad, through the National Malaria Control Programme (NMCP) provided authorization for field work in Wadi Fira Province (Clearance number: 1426/MSP/SE/DGSP/PNLP/2022), that has been hosting large number of refugees and internally displaced people for several years. The study was carried out in September 2022 in Biltine (14°31'39"N, 20°55'36"E), the capital city of Wadi Fira Province which border on Sudan (Figure1), a small peri-urban area with limited access to health care facilities. Based on the last recent population and housing census in Chad in 2009 (the newest population and housing census in Chad is ongoing since January 2026), the population of Biltine is estimated to be over 25,000. Biltine is located in Sahelian zone in eastern Chad with a short rainy season (July to September) and characterized with seasonal malaria transmission. The average annual rainfall is about 500 mm, and the average temperature is 37°C with an average relative humidity of about 25% (De Zborowski I and Beauvillain A, 1996).

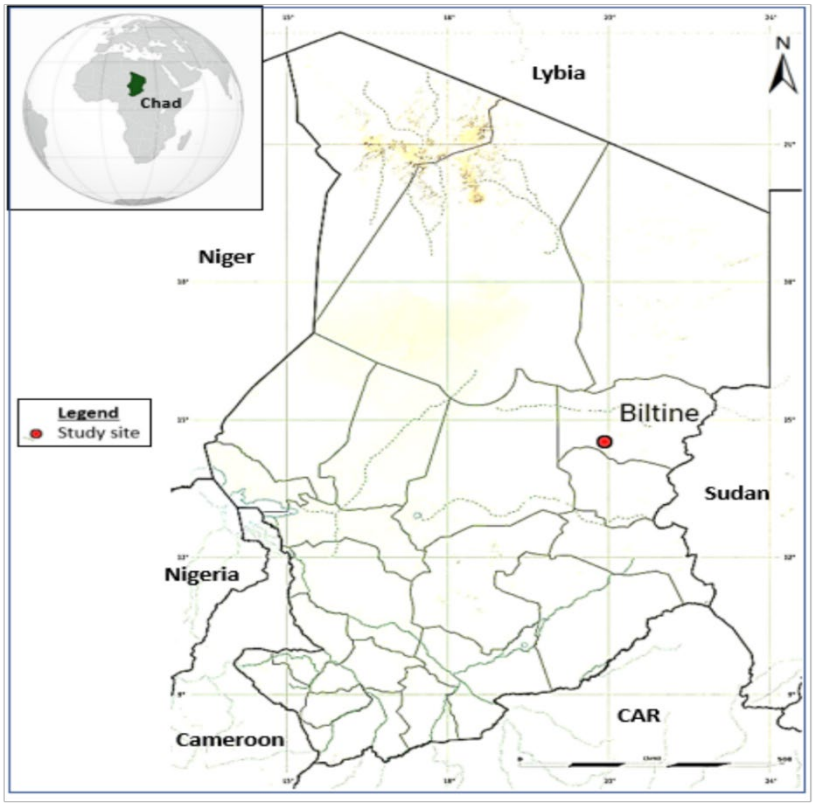

Figure 1 A map of the sampling locality, Biltine, eastern Chad.

Mosquito sampling

Larval collection: Field-wild *An. gambiae s.l.* mosquitoes were collected during the rainy season in temporary rain pools, semi-permanent breeding sites including puddles, foot and hoof prints on pond margin, tire tracks

and rice fields. The larval were reared separately, supplemented with Tetramin™ baby fish food to adults' stage. Following morphological identification with the Gillies key (Gillies and Coetzee, 1987; Gillies and De Meillon, 1968), emerging specimens were sorted and maintained on 10% sucrose solution. Female *An. gambiae s.l.* were exclusively used for experiments described in following sections.

WHO conventional insecticide susceptibility assays: Susceptible tests were conducted according to the WHO protocol (WHO, 2016) with discriminating concentration of deltamethrin (0.05%), permethrin (0.75%), DDT (4%), bendiocarb (0.01%), malathion (5%) and propoxur (0.1%). These insecticide papers (reference: WHO/VBC/81/806) were purchased from University of Sains Malaysia. Four replicates of 25 females emerged from larval collection (2-4 day old, unfed) per tube were exposed to each insecticide for 1 hour. Post-exposure survivors were moved to holding tubes and provided with 10% sucrose solution. Mortality rate was scored 24 hours post-exposure. Two replicated of 25 females unexposed females each were used as control. Populations were deemed susceptible to an insecticidal compound when the mortality rate was > 98%, suspected to be resistant (moderately resistant) when mortality was between 90-98%; and resistant where death rate was recorded to be <90% (WHO, 2016).

Imago sampling and field processing : Field-wild *An. gambiae s.l.* adult mosquitoes were captured using pyrethrum spray catches (PSCs), tools that target endophilic mosquito vectors. This PSCs approach allows estimation of key parameters of malaria transmission at the local population level. PSCs were conducted over four consecutive days in 48 randomly households. Twelve households were sampled each day between 6:00 am and 9:00 am following a verbal consent from the household owners. A white cloth was laid to cover the entire floor and bed in each room prior to spraying. Pyrethroid spray insecticide containing a synergist PBO, was used to spray the room and collect all indoor mosquitoes. Households with open eaves were sprayed on the outside first, then indoor, to prevent mosquito escape. Ten minutes after spraying, sheets were taken outside and mosquitoes knocked down were collected with forceps into petri dishes for morphological identification. Collected mosquitoes were identified morphologically to species under a binocular microscope and standard taxonomic keys (Gillies and Coetzee, 1987; Gillies and De Meillon, 1968). The number of female of *Anopheles* mosquitoes was recorded for each collection day. Abdominal status was recorded and the proportion of blood-fed mosquitoes was noted during identification. Morphologically anopheline mosquitoes identified are then individually deposited in microtubes containing silica gel, a desiccator for transport and storage. Identified mosquitoes were transferred to labelled 1.5 mL Eppendorf tubes, placed in storage bags, and kept at minus 20°C for subsequent analyses to the Centre for Research in Infectious Diseases (CRID), Yaoundé, Cameroon.

Molecular characterization and sporozoite detection: A random sample of 252 females dead and surviving susceptibility evaluation were selected and used for PCR-based molecular identification of *Anopheles* sibling species. Among all dead and surviving mosquitoes from WHO tube insecticide susceptibility assays, a subset of 49 randomly selected samples was used to detect the presence of L1014F knockdown resistance gene.

Genomic DNA extraction and species identification : Members of known *An. gambiae* complex in Chad (*An. gambiae* sensu stricto (s.s.), *An. coluzzii* and *An. arabiensis*) were identified to molecular species level using the SINE PCR assay described by Santolamazza et al. (Santolamazza *et al.,*

2008). PCR amplicons were separated by electrophoresis on a 1.5% agarose gel stained Midori Green® (Gene flow, UK) and visualized under ultraviolet transillumination.

Circumsporozoite infection detection : Additionally, all the 395 samples field preserved PSC-collected mosquitoes that were morphologically identified as *An. gambiae* s.l, *An. rufipes*, *An. squamosus* and *An. pharoensis* were used to determine sporozoite infection rates by testing the head and thorax of each mosquito using indirect enzyme-linked immunosorbent assay (ELISA). The extraction of whole genomic DNA (gDNA) from each mosquito sample was performed following the LIVAK method (Livak, 1984) and stored at -20°C. DNA concentration and purity were assessed using a NanoDropTM spectrophotometer (Thermo-Scientific, Wilmington, USA). Sporozoite infection rates of adult mosquitoes collected using PSCs were determined using circumsporozoite ELISA (csELISA) following the method described by Burkot *et al.* (Burkot *et al.,* 1981) and adapted by Wirtz *et al.* (Wirtz *et al.,* 1987).

Detection of *L1014F* knockdown resistance gene: To assess the role of the *L1014F* knockdown resistance mutation in pyrethroid/DDT resistance, its frequency was established in total of 49 female mosquitoes surviving bioassay tests using TaqMan assays, as previously described (Bass *et al.,* 2007). Ten microliters containing 1X Sensimix (Bioline, TN, USA), 80X primer/probe mix and 1 µl of template gDNA were used. The primers *kdr*_Forward (5'-CAT TTT TCT TGG CCA CTG TAG TGA T-3') and *kdr*_Reverse(5'-CGA TCT TGG TCC ATG TTA ATT TGC A-3') were used without modification. TaqMan probes were labelled with two specific fluorophores, FAM and HEX: FAM to detect resistant allele (5'-ACG ACA AAA TTT C-3' for *1014F kdr*) and HEX: (5'-CTT ACG ACT AAA TTT C-3') to detect susceptible allele. The assay was carried out on an Agilent MX3005 real-time PCR machine with cycling conditions of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Data analysis: The data were compiled into Microsoft Excel® 2007 datasheets. Species composition was expressed as the percentage of each identified species relative to the total number of *Anopheles* mosquitoes collected. The mean of indoor resting density (IRD) of each *Anopheles* species collected using PSCs was calculated by dividing the total number of mosquitoes collected by the total number of houses visited. The sporozoite infection rate, defined as the proportion of mosquitoes positive for circumsporozoite antigen by ELISA, was calculated as the number of positive mosquitoes divided by the total number tested. Bioassays results were analysed as continuous variables with normal distributions and percentage mortalities ± standard error of mean (SEM) calculated based on the WHO protocol (WHO, 2016). For the polymorphism analysis of the fragment of the voltage-gated sodium channel allele frequency was calculated using the formula $f(R)=(2xRR+RS)/2N$ for individuals carrying the *kdr* mutation, and $f(S)=1-f(R)$ for the susceptible individuals; where RR = total number of homozygote resistant; RS = total number of heterozygote resistant; N, total number of individuals investigated. Genotype frequency was determined as relative frequencies of the homozygote resistant and heterozygote resistant individuals. All data were visualised by using GraphPad Prism 8 (GraphPad Inc., La Jolla, CA, USA).

RESULTS

Insecticide resistance profile of *An. gambiae s.l.* populations: Results of insecticide susceptibility assays for six insecticides tested, are presented in Figure 2. Overall *An. gambiae s.l.* populations at Biltine are resistant to pyrethroids, with mortalities of 84% ±1.63 for permethrin and 66.01 ±0.86 for deltamethrin. Higher resistance was observed with DDT with mortality of 41% ±1.19. However, 100% mortality was obtained for all mosquito populations exposed both to bendiocarb and propoxur. A similar susceptibility profile was observed with organophosphate, with malathion producing 100% mortality.

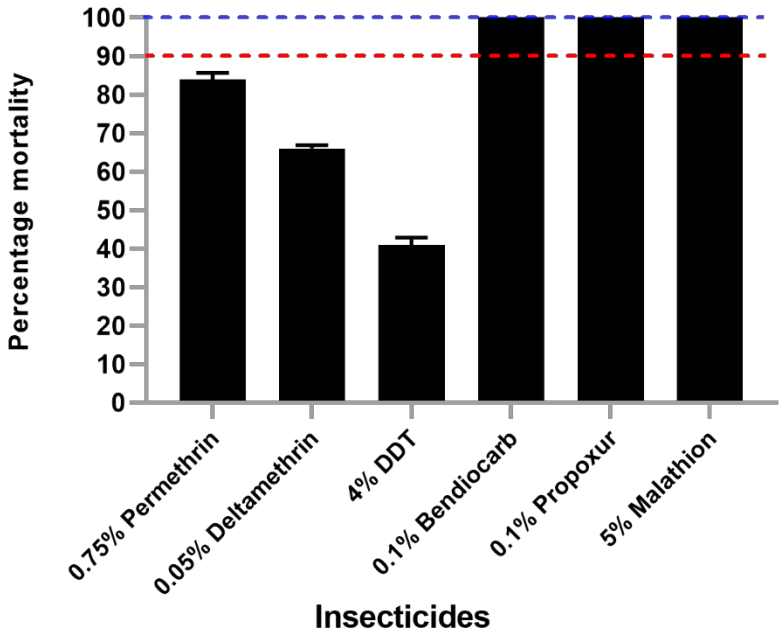

Figure 2 Susceptibility profile of female Biltine *An. gambiae s.l.* field populations following exposure to diagnostic doses of public health insecticides from different classes. Error bars represent standard error of the mean (SEM).

***Anopheles* mosquito species composition:** Overall, 395 *Anopheles* female mosquitoes representing four distinct species were collected by PSC. *An. gambiae s.l.*. (252; 63.79%) was the predominant specie, followed by *An. rufipes* (134; 33.92%). Seven *An. squamosus* (1.77%) and two *An. pharoensis* (0.52%) were found at lower proportions within indoor collected mosquitoes, respectively (Figure 3A). A total of 237 (94.04%) out of 252 females dead and surviving susceptibility evaluation were successfully tested by PCR for molecular identification of species of *An. gambiae* complex. Among extracted gDNA, 15 (5.06%) did not amplify representing PCR negative results obtained. Two species from *An. gambiae* complex were identified in Biltine including *An. arabiensis* (96.20%) found at high frequency and *An. coluzzii* (3.80%) at lower frequency, respectively (Figure 3B). No hybrid specimen was detected among *An. arabiensis* and *An. coluzzii*.

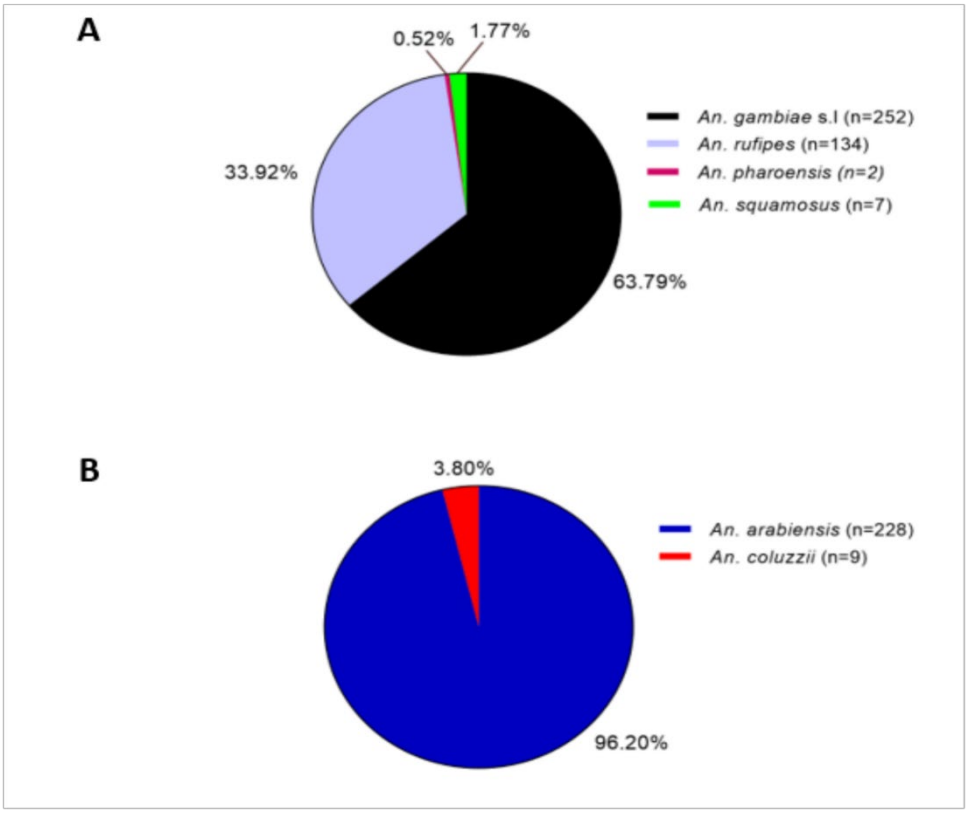

Figure 3 Anopheline mosquito species composition. (A) Vector composition among *Anopheles* mosquitoes collected by pyrethrum spray catches and (B) Identification of sibling species of *An. gambiae* complex by SINE-PCR.

Indoor resting density and sporozoite infection rate : A total of 395 *Anopheles* mosquitoes including 252 *An. gambiae s.l.* and 143 other *Anopheles* were screened by ELISA, of which six *An. gambiae s.l.* were positive for *Plasmodium* circumsporozoite antigen, for a total average infection rate of 1.5% (Table 1). The average density per room of *Anopheles* mosquitoes resting indoors (IRD) was 9.8 females/room (f/r) (395 total females/ 40 rooms visited), and the mean IRD for *An. gambiae s.l.* in Biltine was 6.3 females/room.

Table 1 Sporozoite infection rate of *Anopheles* mosquitoes collected in Biltine, eastern Chad

Locality	**Species**	**N**	**N (+)**	**Infection rate**	
Biltine	*An. gambiae s.l.*	252	6	2.38	
	An. rufipes	134	0	0	
	An. pharoensis	2	0	0	
	An. squamosus	7	0	0	
	Total	**395**	**6**	**1.52**	

N: total number of individuals investigated, N (+**):** total number of positive samples

Presence of the knockdown resistance mutation: To establish the frequency of 1014F *kdr* mutation in Biltine population, a TaqMan genotyping assaywas performed using DNA samples for extracted from 49 surviving *An. gambiae s.l.* mosquitoes included *An. arabiensis* and *An. coluzzii*. Analysis revelead the presence of 1014F kdr mutation at lower frequency of 10% (Table 2). Four heterozygote individuals (8.1%, 4/49), and three homozygote resistant individuals (6.1%, 3/49) were found for the resistant allele (RR). The susceptible allele 1014L was detected with 42 homozygote susceptible individuals (85.7%, 42/49). Segregated by species belonging to tested *An. gambiae* complex, the 1014F *kdr* mutation occurred in *An. arabiensis* only. All tested *An. coluzzii* were found to carry susceptible allele.

Table 2: Genotype and allele frequency of the 1014F *kdr* mutation in the Biltine *An. gambiae s.l.* population

Population	**Genotype**					**Allele**	
	1014F kdr						
	RR (%)	RS (%)	SS (%)	Total	2N	f(R)	f(S)
Biltine	3 (6.12)	4 (8.16)	42 (85.71)	49	98	0.10	0.90

RR, homozygous resistant, RS, heterozygous resistant, SS, homozygous susceptible, N, total number of individuals investigated, f(R), frequency of resistant allele calculated using formula f(R)=(2xRR+RS)/2N, f(S), frequency of susceptible allele calculated using formula f(S)=1-f(R).

DISCUSSION

Evidence-based malaria control measures and resistance management in Chad's Sahel region require routine surveillance of local vectors to assess their role in transmission and resistance status. In Wadi Fira Province characterized by its harsh semi-arid environment combined with its mass population displacement and limited access to water, hygiene and sanitation particularly, there is a lack of research on malaria in this Province, making it an important site for conducting studies to inform public health interventions. This study conducted in Biltine during rainy season captured level and variation in *Plasmodium* infection and pattern of insecticide resistance in malaria vectors from eastern Chad province, border on Sudan. However, data provided from this survey are drawn from Biltine, a single collection at one site due to fact that heavy rains and the inadequacy of infrastructures for access to roads, leading to a risk of flooding and sites are becoming increasingly inaccessible. Four species of *Anopheles* were identified in Biltine including *An. gambiae s.l.*, *An. rufipes*, *An. squamosus* and *An. pharoensis*. This finding is consistent with previous reports in Chad, e.g. in Goulmoun located in southern Chad (Kerah-Hinzoumbé *et al.,* 2009), in Douguia located in western Sahel Chad (Diarra *et al.,* 2017), and in Sahel of Cameroon, e.g. in Maroua (Saotoing *et al.,* 2014) and in Simatou (Fondjo *et al.,* 2023). The finding of *An. arabiensis* as the major malaria vector in Biltine contrasts with previous observations reported *An. coluzzii*, as the predominant vector in Sahel of central Chad (Ibrahim *et al.,* 2019a), in Sahel of northern Nigeria (Ibrahim *et al.,* 2019b), in Sahel of far north Cameroon (Fadel *et al.,* 2024), and in Sahel of Niger Republic (Ibrahim *et al.,* 2019c). Indeed, studies conducted a decade ago have reported *An. arabiensis* as the major malaria vector of the *An. gambiae* complex that tend to predominate in drier areas in Sub-Saharan African countries (Ranson *et al.,* 2009) and in arid savannah in Chad, e.g. in N'Djamena, in Mandelia, and Bongor (Foster *et al.,* 2016). Malaria parasite *Plasmodium falciparum* was the only *Plasmodium* specie detected in *An. gambiae s.l.*, which suggests

even low but potential for malaria transmission intensity at this site. This circumsporozoite infection rate is similar to that obtained in 2006 in *An*. *gambaie* s.l. from Goulmoun, south-western Chad (Kerah-Hinzoumbé *et al.,* 2009), but lower than those obtained in 2012 in *An. gambiae s.l.*. from Douguia located in western Sahel zone in Chad (Diarra *et al.,* 2017) and in 2016 in *An. gambiae s.l.*. from Moïssala in southern region of Chad (Kodindo *et al.,* 2021). However, when placed in the Sahelian context of Biltine, eastern Chad marked by humanitarian, migratory, and security crisis, this low Plasmodium infection rate obtained from subset samples should not strictly be interpreted as a low transmission risk. The high population density, with people forced to live in dwellings less than 2 kilometers apart, maintains proximity favourable to the human-vector-human cycle. Thus, even a limited human reservoir may be sufficient to sustain, or even amplify, malaria transmission within these vulnerable communities (Cotter *et al.,* 2013). Additional longitudinal surveillance is required to clarify the role of this vector to malaria transmission in this area. The study highlighted the existence of permethrin, deltamethrin and DDT resistance in *An. gambiae s.l.* populations from eastern Chad, but completely susceptibility to bendiocarb, propoxur and malathion. This pattern is in line with our findings of pyrethroids and DDT resistance as well as susceptibility to carbamates and organophosphates observed at southern savannah and western Sahel regions in Chad (Dadzie *et al.,* 2016; Foster *et al.,* 2016; Ranson *et al.,* 2009) and west and central Africa regions (Corbel and N'Guessan, 2013; Riveron *et al.,* 2018). However, mortalities for permethrin, deltamethrin and DDT recorded with *An. gambiae s.l.* from eastern Chad are higher than those reported in recent studies of *An. coluzzii* from central Chad (Ibrahim *et al.,* 2019a) and neighbouring Sahel region sharing the similar ecological characteristics, e.g. in Cameroon (Fadel *et al.,* 2024; Fadel *et al.,* 2019), in Nigeria and Niger (Ibrahim *et al.,* 2019b; Ibrahim *et al.,* 2019c). This various level of insecticide susceptibility as observed in Biltine, eastern Chad may reflect differential insecticide selection pressure exerted on field mosquito populations. One of the main findings of this present study is the second report of *L1014F kdr* mutation in wild *An*. *arabiensis* populations from Chad. The *1014F* mutation has been previously identified in *An*. *arabiensis* in Kome, southern Chad at lower frequency (Dadzie *et al.,* 2016). The frequencies of individuals with resistant allele are similar than those reported in southern Kome (Dadzie *et al.,* 2016). The presence of *1014F kdr* mutation in *An*. *arabiensis* Biltine populations may suggest the spreading of this resistant allele from southern to northern east region of Chad. Further investigations establishing correlation between the genotype *kdr* locus and the expression of insecticide resistance are needed to explore the implication target-site modification in crossed DDT and pyrethroids resistance observed in the field.

CONCLUSION AND APPLICATION OF RESULTS

This study revealed variable levels of resistance to pyrethroids and detected *L1014F kdr* mutation in major malaria vector *An*. *arabiensis* from eastern Chad, which will seriously pose threat to malaria control using pyrethroid impregnated bed nets. The finding of susceptibility to carbamate and organophosphate makes them important alternative for indoor residual spraying, which could help in pre-elimination of malaria in the Sahel of Chad. The main recommendation is to switch current DDT- IRS to organophosphate and carbamate based IRS. Insecticides such as pirimiphos-methyl for organophosphate or bendiocarb for carbamate could be considered. For LLINs, priority should be given to dual-AI

nets incorporating either pyrethroid plus PBO or pyrethroid plus chlorfenapyr. Rotation across insecticide classes for organophosphate and carbamate should be performed annually for IRS in order to main effectiveness of control strategies and break any potential raising resistance. In addition, mosquito susceptibility and molecular characterization of target mutation should be monitored each semester. Larval source management, housing improvements, and strict safety measures to prevent human toxicity should also complement the existing interventions.

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