DETECTION OF INSECTICIDE RESISTANCE TO MALARIA VECTORS IN SELECTED SENTINEL SITES IN SOUTH SUDAN[1]

NATIONAL MALARIA CONTROL PROGRAM

MINISTRY OF HEALTH, REPUBLIC OF SOUTH SUDAN

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I. ABBREVIATIONS/ACRONYMS

DDT	DICHLORODIPHENYLTRICHLOROETHANE
ERB	ETHICAL REVIEW BOARD
GF	THE GLOBAL FUND
IRS	INDOOR RESIDUAL SPRAYING
ITN	INSECTICIDE TREATED NET
KDR	KNOCKDOWN RESISTANCE
LLIN	LONG-LASTING INSECTICIDAL NET
MOH	MINISTRY OF HEALTH
NIMR	NATIONAL INSTITUTE FOR MEDICAL RESEARCH
NMCP	NATIONAL MALARIA CONTROL PROGRAMME
PCR	POLYMERASE CHAIN REACTION
PSI	POPULATIONA SERVICE INTERNATIONAL
USA	UNITED STATES OF AMERICA
VSS	VECTOR SUSCEPTIBILITY STUDY
WHO	WORLD HEALTH ORGANIZATION

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[2] Based on WHO criteria for insecticide susceptibility levels [i.e., Mortality-rate based criteria was used to determine the levels of mosquito susceptibilities: Susceptible (≥ 98%); resistant to be confirmed (97 – 90%) and resistant (≤ 90%).

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EXECUTIVE SUMMARY

Introduction: Malaria remains a disease of public health significance and a major cause of morbidity and mortality particularly in pregnant women and children under 5 years of age in South Sudan. Following the signing of the comprehensive peace agreement in 2005, the National Malaria Control Programme (NMCP) in South Sudan, implements long lasting insecticidal nets (LLINs) as the frontline vector control intervention. However, the extensive use of insecticide-based interventions (LLINs) has led to the development and spread of insecticide resistance, making these frontlines tools less effective and limiting the available options for disease prevention and control particularly for the insecticide treated bed nets. In addition, there are very limited data available on the relative spatiotemporal bionomics and insecticide resistance status of these major malaria vectors to guide targeted and effective scaled up deployment of transmission-reducing tools. This invariably poses a huge challenge for the establishment of a rational and multi-pronged malaria vector control strategy and clearly underscores the need for studies to monitor different primary entomological parameters across the country. The current vector susceptibility study (VSS) was carried out between March and August 2020 in 6 sentinel sites for the detection of malaria vectors resistance to insecticides of public health relevance in Republic of South Sudan.

Methods: During the current report, VSS was conducted in 5 sentinel sites of which mosquito larvae were collected and reared to adults for susceptibility tests. Molecular analysis was only done to mosquito samples from three sentinel sites only (Juba, Aweil and Wau). Adult female mosquitoes of 3-5 days old were exposed to insecticides following standard World Health Organisation (WHO) protocol. The discriminating dosages of 0.75% Permethrin, 0.05% Deltamethin, 0.75% Pirimiphos-Methyl, 4% DDT and 0.1% Bendiocarb were used. The distribution of *An. gambiae* sub-species and pyrethroid target-site mutations (*kdr*) were investigated using molecular assays i.e. Standard and Real time Polymerase Chain Reaction (PCR). Biochemical assays were used to detect the enzyme-based resistance mechanisms in mosquitoes across all the sentinel sites.

Results: During the current report, A total of 1,875 collected adult female *Anopheles* mosquitoes from the five sites were morphologically identified as *Anopheles gambiae* sansu lato. Susceptibility tests showed the widespread of pyrethroid resistance across the country. Bendiocarb showed the highest level of susceptibility in Aweil and Yambio sentinel sites (mortality rate of 98 -100%). However highest level of resistance to all insecticides was observed in Juba and Renk (mortality rate of 12-88%). Specifically, in Wau and Aweil, *An gambiae* s.l. were resistant to Permethrin, Deltamethrin and Pirimiphos-Methyl insecticides; in Yambio, *An gambiae* s.l. were resistant to Permethrin insecticide. Of the collected mosquitoes, 1125 mosquitoes were subjected for PCR for species identification. Majority belonged to *Anopheles gambiae* s.s. were 38% and *Anopheles arabiensis* were 62%. Majority of *Anopheles gambiae* were recorded in Juba while mosq of *Anopheles arabiensis* were recorded in Wau and Aweil. L1014S east-kdr-mutation was recorded in *An gambiae* s.s. in Juba (54%) and Wau (10%) while L1014F west-kdr-mutation was recorded in Wau (40%), Juba (40%) and Aweil (30%). For *An arabiensis*, L1014S east-kdr-mutation was only recorded in Juba (40%) while L1014F west-kdr-mutation was recorded in Wau (10%), Juba (30%) and Aweil (10%).

Conclusion: The current report evidently shows a widespread of insecticide resistance particularly to pyrethroids. The resistance mechanisms reported here was target site resistance (kdr). This phenomenon of resistance mechanisms in the malaria mosquito population may complicate the resistance management strategies that need to be explored further. With such observation, the systematic resistance monitoring and surveillance is very important and that operational impact of resistance to the effectiveness of the LLINs in the control of malaria vectors should be determined.

1 INTRODUCTION AND BACKGROUND

Malaria remains a disease of public health significance and a major cause of morbidity and mortality particularly in pregnant women and children under 5 years of age in all parts of South Sudan with the entire population at risk of the infection. The country experiences year-round transmission with peaks towards the end of the rainy season from July to November. Malaria accounts for 30% to 50% of all visits to health facilities and 40% of all hospital admissions (Annual HMIS report, 2019 draft). In 2019 malaria accounted for over 56% of all inpatient deaths (HMIS 2019). current data from population-based malaria indicator survey conducted in 2017 found that the Greater Bahr el Ghazal region had the highest prevalence with an average 39% followed by Greater Equatorial Region at 35% and the Greater Upper Nile with lowest prevalence of 17%. The prevalence had been on increase since first MIS was conducted in 2009[3]. Long lasting insecticidal nets (LLINs) have been the frontline malaria vector control intervention in the country.

However, the extensive use of insecticide-based interventions (LLINs) has led to the development and spread of insecticide resistance, making these frontlines tools less effective and limiting the available options for disease prevention and control particularly for the insecticide treated bed nets [1–4]. In addition, there are very limited data available on the relative spatiotemporal bionomics and insecticide resistance status of these major malaria vectors to guide targeted and effective scaled up deployment of transmission-reducing tools. This invariably poses a huge challenge for the establishment of a rational and multi-pronged malaria vector control strategy and clearly underscores the need for studies to monitor different primary entomological parameters across the country.

An integrated vector management (IVM) strategy is being established by the Ministry of Health (MOH) as the main malaria transmission-reducing approach to combat malaria transmission. However, rational and evidence-based decision-making is critical for vector control to optimise utilization of the limited available resources and to ensure that this strategy is effective. Thus, rigorous monitoring and surveillance of fundamental entomological indicators; Species identification, Vector density, Vector behaviour, Vector susceptibility and Insecticide decay rates across the country is crucial to ensure successful deployment of present and future tools.

[3] South Sudan Malaria Indicator Survey – 2017 https://www.malariasurveys.org/surveys.cfm

Here we report and discuss preliminary results of the first field surveys on detection of insecticide resistance carried out in 5 out of 13 sentinel states between March and August in 2020. Specifically, we report the preliminary findings on the susceptibility status of the mosquitoes against five standard insecticides as per WHO guidelines and the molecular identification and mechanism of resistance among malaria vectors in three sentinel sites.

2 STUDY OBJECTIVES

2.1 Main objective

The main objective of the study was to monitor the spatiotemporal bionomics and insecticide resistance status of *Anopheles gambiae s.*l., mosquitoes in different malaria epidemiological settings of South Sudan.

2.2 Specific objectives

The specific objectives of the survey were:

1) To determine the susceptibility levels of local malaria vectors to the insecticides used both for public health and agricultural purposes in the sentinel states.
2) To document species composition of malaria vectors and their distribution in the sentinel states.
3) To determine the resistance mechanisms and their distribution among malaria vector populations in the surveillance sentinel states.

3 METHODOLOGY

3.1 Study design

This was a cross-sectional countrywide survey, which was conducted between March and August 2020 in selected five sentinel states for detecting malaria vectors resistance to insecticides of public health relevance in South Sudan.

3.2 Study sites and selection criteria

3.2.1 *Sentinel states and selection criteria*

The vector susceptibility study was conducted in five selected sentinel sites as shown on Table 1. The selection criteria of sentinel states for the insecticide resistance surveillance were based on the WHO recommendations (WHO, 2012):

a) History of insecticides use by communities in the areas (both for agricultural and public health use).

b) Malaria endemicity in the area (priority was given to the districts with high malaria prevalence).

c) Accessibility to the sites.

Table 1: The Sentinel states used for the Insecticide Resistance detection for the year 2020

#	State	County	Malaria[4] prevalence (%)
1	Northern Bahr el Ghazar	Aweil	53
2	Western Bahr el Ghazar	Wau	49
3	Central Equatoria	Juba	44
4	Western Equatoria	Yambio	14
5	Upper Nile	Renk	10
6	Unity State	Bentiu	16

[4] South Sudan Annual HMIS report, 2019 draft

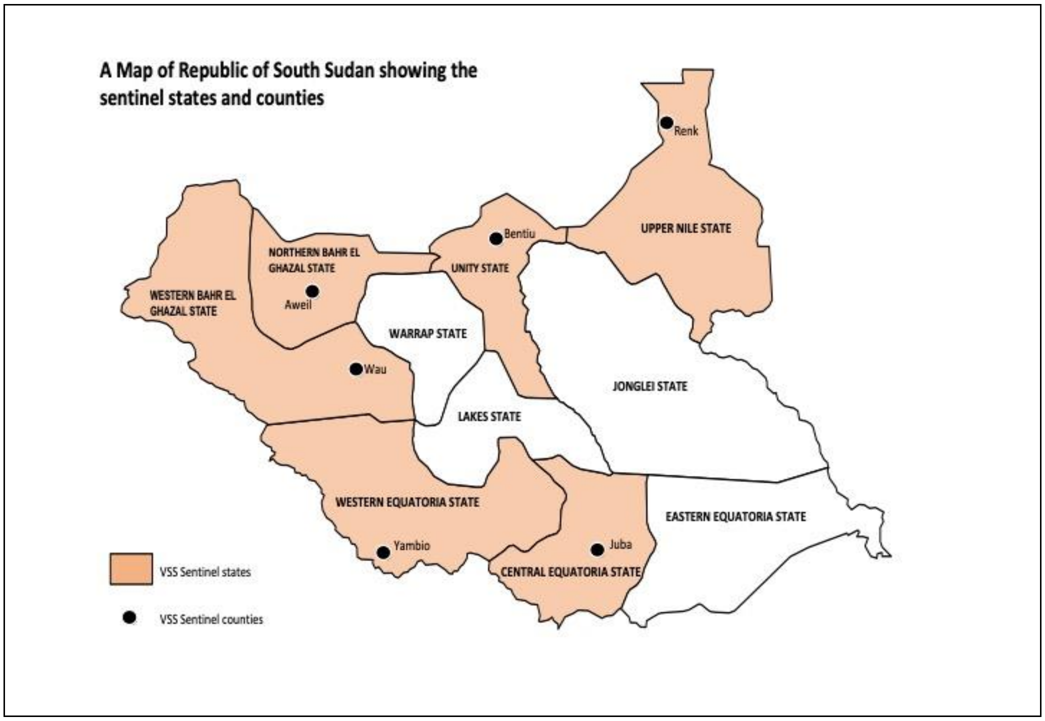

Figure 1: Distribution of sentinel sites for insecticide susceptibility surveillance in 2020

3.3 Hands-on training of field assistants for vector surveillance

Insecticide resistance surveillance activities were preceded by training of field assistants on basic field entomology and familiarization of the detection techniques for insecticide resistance under field conditions. A consultant medical entomologist in support with colleagues from National Malaria Control Program (NMCP) conducted a one-week hands-on training workshop of field implementations on the standardized protocol for vector surveillance. The major aim of this training was to impart basic entomological skills, with a particular emphasis on mosquito resistance detection. Training on basic entomological skills included mapping and characterization of breeding sites (in areas where larvicides can potentially be used), larval collection and rearing techniques under field conditions, adult mosquito collection and morphological identification, estimation of vector density, conducting susceptibility tests, malaria vector control and resistance management techniques.

3.4 Data Collection from the field and WHO Susceptibility tests

3.4.1 *Larval collections and rearing*

Mosquito larvae searches were done and Anopheles larvae identified from their horizontal position on the surface of water were carefully collected with a 350 ml dipper and transferred into plastic containers which were then loosely capped to allow aeration. These were transported in cool boxes to the laboratory where they were reared at 27 - 30^{o}C and 76±5% relative humidity with a 12h: 12h light and dark cycle. The larvae were fed with grinded Tetramin® fish food. The development of the larvae was monitored regularly and all those that pupated were transferred into shallow plastic cups/small beakers using Pasteur pipettes, and then placed in appropriately labeled cages for adult emergence. Using the Global Positioning System (GPS; Trimble Geoexplorer II, Trimble Navigation Limited, Sunnyvale, CA, USA) the geographical coordinates of each sampling site was determined.

3.4.2 *WHO insecticide susceptibility tests*

The susceptibility tests were carried out using the World Health Organization (WHO) test kits for adult mosquitoes (WHO, 1998). The kit is basically comprised of insecticide impregnated test papers and non-impregnated papers for control and plastic tubes that are marked with a red dot for exposure and a green dot for the holding tubes. Test papers impregnated with the WHO-recommended discriminating dosages of 0.75% Permethrin, 0.05% Deltamethrin, 4% DDT, and 0.1% Bendiocarb were used. The quality of the test paper was checked against a laboratory susceptible *An. gambiae s.s.* Kisumu strain. Knockdown effect and mortality were measured in standard WHO susceptibility tests. The standard methods were used for insecticide susceptibility tests (WHO, 1998). For each test, batches of 15- 20 adult female mosquitoes reared from field-collected larvae were aspirated from paper cups and transferred into the holding tubes where they were held for 1 hour. They were then transferred into exposure tubes (through the open space between the exposure and the holding tubes). Exposure tubes were lined with the insecticide impregnated papers to which mosquitoes were exposed for 1 hour. During the exposure period, the number of mosquitoes knocked down was recorded after 10, 15, 20, 30, 40, 50 and 60 minutes for pyrethroid and organochlorine insecticides only. A mosquito was considered knocked down if it lay on its side on the floor of the exposure tube and unable to fly at the end of exposure period mosquitoes were then transferred into holding tubes (lined with untreated papers) by gently blowing them through the open space between the exposure and the holding tubes. A cotton pad soaked in 10% sugar was placed on top of the holding tube. This is to avoid death by starvation.

The mortality was scored 24 hours post-exposure and each test at each site was replicated at least four times. The resistance or susceptibility status were evaluated based on the WHO criteria i.e. 98-100% mortality indicate susceptibility; 90-97% mortality required confirmation and less than 90% mortality indicate possible resistance (WHO, 2013). When the control mortality was scored between 5% and 20%, the mean observed mortality was corrected using Abbott's formula (Abbott, 1925). Tested mosquitoes were preserved with *silica gel* in 1.5 ml eppendorf tubes and transported to National Insectary laboratory in Juba for preserving in fridge before they can be shipped for further laboratory analysis (molecular species identification and detection of biochemical/molecular mechanisms of insecticide resistance).

3.4.3 *Molecular Analyses of Mosquito Samples in the Laboratory*

The molecular analyses were conducted to the samples of the mosquitoes after standard WHO susceptibility testing at a WHO reference insectary site outside the country. Main molecular activities conducted included;

(a) Molecular identification of members of the Anopheles gambiae species complex

(b) Detection of knock down resistance (kdr) alleles in Anopheles gambiae complex

4 PRELIMINARY RESULTS

4.1 Susceptibility levels of *An. gambiae sensu lato* to insecticides

The preliminary results of the resistance status of the collected mosquitoes from five sentinel sites are shown on Table 2. A total of 1600 *An. gambiae* s.l mosquitoes reared to adult and tested against standard WHO insecticides on their susceptibility status. Preliminary findings showed that, *An. gambiae* s.l was susceptible to 0.1% Bendiocarb in Aweil and Yambio sentinel sites and 0.25% Pirimiphos Methyl in Yambio only with mortality rate range of 98-100% (Table 3). At the same time, *An. gambiae* s.l was highly resistant to all insecticides in Renk and Juba ranging from 12-88%). Resistance to 0.75% Permethrin was also recorded in Aweil, Wau and Yambio sentinel sites. Resistance was also seen to 0.05% Deltamethrin and 0.25% Pirimiphos-Methyl in Aweil and Wau sentinel sites ranging from 55%-88%.

Table 2: Mortality rates[5] (%) of the female adult Anopheles *gambiae sl* after exposure to WHO discriminatory dosages of 0.75% Permethrin, 0.05% Deltamethrin, 0.75% Pirimiphos-Methyl, 4% DDT and 0.1% Bendiocarb

States	Permethrin 0.75%	Deltamethrin 0.05%	Bendiocard 0.1%	DDT 4%	Pirimiphos Methyl 0.25%
Renk	48	88	25	52	60
Juba	30	30	12	58	27
Aweil	70	55	98	87	100
Wau	2	78	97	92	88
Yambio	21	95	74	15	100

[5] Based on WHO criteria for insecticide susceptibility levels [i.e., Mortality-rate based criteria was used to determine the levels of mosquito susceptibilities: Susceptible (≥ 98%); resistant to be confirmed (97 – 90%) and resistant (≤ 90%).

4.2 Mosquitoes Identification and distribution

A total of 1,875 mosquitoes (from five sentinel sites i.e. Juba, Yambio, Wau, Renk and Aweil) were morphologically identified as *An. gambiae* s.l. Of these, 1,125 (from Juba, Aweil and Wau sites only) were subjected for PCR analysis to identify the *An. gambiae* s.l. sibling species. Out of these 1,125 mosquitoes, 38% and 62% were identified as *An. gambiae s.s* and *An. arabiensis* respectively (Figure 2). The presence of An. gambiae s.s and An. arabiensis were indicated by the diagnostic size of amplified DNA fragments, which are 390bp and 315bp respectively. The distribution of these two sibling species at each of the sentinel districts is shown in figure 3 below. By sites, more *An. gambiae s.s* were found in Juba while *An. Arabiensis* were more found in Aweil and Wau sites.

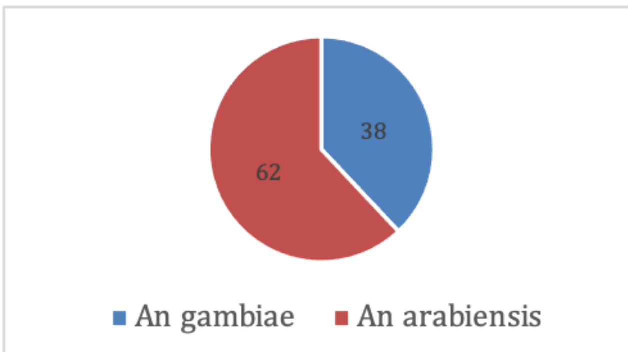

Figure 2: Percentage distribution of the *An. gambiae* sibling species

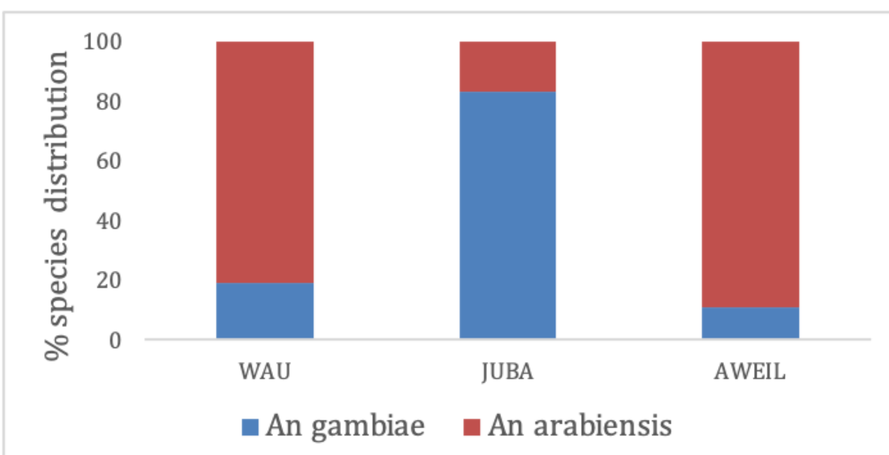

Figure 3: Percentage distribution of the *An. gambiae* sibling species in surveyed sentinel town.

4.3 Insecticide resistance mechanisms (Kdr genes detection in the *An. gambiae* s.l)

Mosquitoes from all the three sentinel sites (Juba, Aweil and Wau) were analyzed using Taqman assay (Bass et al., 2008) for kdr east and kdr west and results are shown on Table 3 and Table 4. Knock down resistance east was detected in mosquitoes with allelic frequencies ranging from 0% in Aweil to 50% in Juba. This shows that kdr gene mutation intensity is increasing and is also geographically spreading widely. This trend depicts that the resistance selection pressure is still not adequately contained hence more emphasis needed for resistance management.

Table 3: Distribution and allelic frequencies of kdr-east (L1014S) and kdr-west (L1014F) mutation genotypes in *Anopheles gambiae* s.s

Sentinel sites	No.	kdr East Genotype count				kdr West Genotype count			
		RR	RS	SS	Allelic frequency	RR	RS	SS	Allelic frequency
WAU	52	6	0	17	0.1	14	15	17	0.4
JUBA	269	131	26	11	0.54	95	5	11	0.4
AWEIL	33	0	0	18	0	5	10	18	0.3

Table 4: Distribution and allelic frequencies of kdr-east (L1014S) and kdr-west (L1014F) mutation genotypes in *Anopheles arabiensis*

Sentinel sites	No.	kdr East Genotype count				kdr West Genotype count			
		RR	RS	SS	Allelic frequency	RR	RS	SS	Allelic frequency
WAU	232	0	0	189	0	2	41	189	0.1
JUBA	55	19	2	17	0.4	13	4	17	0.3
AWEIL	294	0	0	228	0	16	50	228	0.1

5 DISCUSSION

In this baseline study of insecticide resistance detection, we confirm the presence of insecticide resistance in the surveyed sentinel sites. The predominant malaria vector in South Sudan, *An. gambiae* s.l., has shown to be resistance to deltamethrin, permethrin, Pirimiphos-methyl, DDT and Bendiocarb. Among the *An. gambiae* s.l., we report high proportions of *An. arabiensis* in almost all of the sites. Resistance mechanisms that we report are eastern knockdown resistance commonly referred to as “kdr east” and western knockdown resistance commonly referred to as “kdr west”.

There is evident that, *An. Arabiensis* (which are the exophilic mosquitoes) is becoming predominant in some areas while the *An. gambiae* s.s. (traditional endophilc malaria vector) is decreasing in terms of population in some areas. Such a scenario can be attributed to the vector control strategies that have been geared towards the indoor resting *An. gambiae* s.s [5].

With these baseline findings, we can mention that, the malaria transmission observed in some parts of the country (like in Aweil and Wau counties), can be accounted by the presence of high proportion of *An. arabiensis* and also by the resistance of these mosquitoes to the standard insecticides of public health [6–10].

We confirm the high levels of resistance to Permethrin, Deltamethrin, Pirimiphos-methyl, DDT and Bendiocarb in Renk and Juba areas. These sites, in other words, were not resistance to only one insecticide; rather they had resistance across all the five tested insecticides. Hence one would expect that some sites to have more than one resistance mechanisms to explain such resistance profile. Our overall molecular analysis results evidently showed that one mechanism can be sufficient to express phenotypic resistance. In addition, resistance to Permethrin and Deltamethrin (Pyrethroids), Bendiocarb (Carbamates). DDT (Organochlorines) and Pirimiphos-methyl (Organophosphates) were observed to all sentinel sites with exception of Aweil and Yambio (for Pirimiphos methyl) and Aweil (for Bendiocarb). In addition, “kdr east” was detected in Juba and Wau sites. All of these sites have preliminary information of phenotypic resistance to all the tested insecticides. Similar evidence have been reported in other areas [11–16]. Our findings also support that one resistance mechanism in a mosquito population, is sufficient enough to cause high levels of phenotypic resistance across different classes of insecticides. This was observed in Juba and Wau sites (with *An. arabiensis*), that showed elevated levels of “kdr-east” mutation and also high pyrethroid resistance (deltamethrin and permethrin). The lack of observing other resistance mechanisms could be explained by the sample preservation limitations in these sites that need to be ascertained in the subsequent surveys.

The information on the presence of kdr- mechanisms (target site resistance mechanisms) in malaria vector populations may complicate the resistance management strategies in South

Sudan and need further explorations particularly on their distributions among vector populations in different epidemiological settings in the country. In addition, more molecular analysis need to be done to determine the presence of other resistance mechanisms i.e. metabolic resistance mechanism in all sites. Information on presence of resistance mechanisms is very important as they can be used to establish the operational impact of resistance mechanisms on the effectiveness of LLINs and IRS for malaria vector control in the country.

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