# THE PMI VECTORLINK PROJECT UGANDA
# ANNUAL ENTOMOLOGICAL REPORT JANUARY- DECEMBER 2019
# ADDENDUM

## (INCORPORATING MOLECULAR ASSAY DATA)

**Recommended Citation:** The PMI VectorLink Project Uganda. 2019. Addendum to *Uganda Entomological Monitoring Annual Report, January 1 -December 31, 2019*. Rockville, MD, USA: The PMI VectorLink Project, Abt Associates Inc.

**Contract: AID-OAA-I-17-00008**

**Task Order: AID-OAA-TO-17-00027**

**Submitted to: United States Agency for International Development/PMI**

**Submitted: January 10, 2021**

**Approved: February 24, 2021**

Abt Associates Inc. | 6130 Executive Blvd.
Rockville, MD 20852 | Suite 800 North | T. 301.347.5000 | F. 301.913.9061 |
www.abtassociates.com

# CONTENTS



## LIST OF TABLES



## LIST OF FIGURES

# ACRONYMS

| | |
|---|---|
| **Ace-1** | Acetylcholinesterase |
| **bp** | base pair |
| **DNA** | Deoxyribonucleic acid |
| **IDT** | Integrated DNA Technology |
| **IRS** | indoor residual spraying |
| **kdr** | knockdown resistance |
| **PCR** | polymerase chain reaction |
| **PBS** | phosphate buffered saline |
| **PMI** | President's Malaria Initiative |
| **PSCs** | pyrethrum spray catches |
| **PBO** | piperonyl butoxide |
| **SNP** | single nucleotide polymorphism |
| **WHO** | World Health Organization |

# I. INTRODUCTION

This addendum report presents the additional results from the laboratory analysis of entomological samples associated with the original results presented previously in the President's Malaria Initiative (PMI) VectorLink Uganda Entomological Monitoring Annual Report for 2019 and submitted on February 28, 2020. It includes: data on molecular species; identification of mosquitoes collected and used for insecticide susceptibility tests; mosquito blood meal source identification from the *Anopheles gambiae* s.l. collected through pyrethrum spray catches (PSCs); and allelic frequencies of genetic markers of insecticide resistance of susceptibility tested mosquitoes. The report covers the period from January to December 2019 and the activities conducted during this period by the PMI VectorLink Uganda Project.

# 2. METHODOLOGY

## 2.1 SAMPLE DNA EXTRACTION

Deoxyribonucleic acid (DNA) from a leg or a wing of a mosquito was extracted following the protocol developed by Musapa *et al.*[1]. Briefly, 20µl of de-ionized water was added in a sample tube containing the mosquito part. A pipette tip was used to grind mosquito part in tube into a uniform suspension. This was followed by adding 100µl of an autoclaved 1X phosphate buffered saline (PBS), 1% Saponin solution to the sample homogenate and gently vortexed to mix. The samples were then incubated for 20 minutes at room temperature; then centrifuged at 20,000 x g for 2mins. The supernatant was discarded, and pellets re-suspend in 100µl of 1X PBS; centrifuged at 20, 000 x g for 2mins. The supernatant was again discarded and 75µl of sterile de-ionized water and 25µl of 20% weight per volume of Chelex 100 resin suspension in de-ionized water added to the pellet. Pellet was gently vortexed before being boiled on the heating block for 10 minutes. Extracted DNA was transferred into a final tube and stored at -20ºC before analysis.

## 2.2 POLYMERASE CHAIN REACTION (PCR) SPECIES IDENTIFICATION

Subsamples of adult *An. gambiae* s.l. emerging from collected larvae and tested for susceptibility were selected per site for molecular analysis. The selected mosquitoes were sampled from the eight tested sites: Apac, Arua, Kanungu, Lira, Rakai, Bugiri, Moroto and Tororo. A total of 587 randomly selected *An. gambiae* s.l. among the dead and the surviving mosquitoes from the World Health Organization (WHO) susceptibility tests insecticides (pirimiphos-methyl, bendiocarb, alpha-cypermethrin, deltamethrin and permethrin) were analyzed for species identification. A polymerase chain reaction (PCR) mix of species primers, enzymes and extracted DNA were prepared and run following the protocol of Scott *et al.* [2]. Amplified DNA samples were run on a 1.5% agarose gel stained with Ethidium Bromide. Expected amplicon fragments of 153 base pair (bp) or 415bp for *An. quadriannulatus*, 464bp/466bp for *An. melas/merus*, 390bp for *An. gambiae* s.s. and 315bp for *An. arabiensis* were used to identify each species [2].

## 2.3 BLOOD MEAL ANALYSIS

A total of 152 mosquitoes from the five sites were analyzed by PCR and five blood sources were assessed (cow, dog, goat, pig and human). The blood meal source of mosquitoes collected through PSCs was also determined using PCR following the methods of Kent and Norris, 2005 [2]. The human blood index was calculated using the number of mosquitoes found with human blood out of the total number of mosquitoes tested. All the blood samples on filter paper were analyzed.

## 2.4 CHARACTERIZATION OF INSECTICIDE RESISTANCE MARKERS: KNOCK DOWN RESISTANCE AND ACETYLCHOLINESTERASE RESISTANCE SINGLE NUCLEOTIDE POLYMORPHISM (SNP) ANALYSIS

Samples were randomly selected from the eight districts namely: Bugiri, Arua, Apac, Moroto, Lira, Kanungu, Tororo and Rakai (Table 3 & 4). Priority was given to mosquito samples that remained alive after exposure to insecticides at the highest concentration to estimate the frequency of resistance allele among the surviving mosquitoes. For knockdown resistance (*kdr*) west and east mutations, a total of 101 samples from dead and alive mosquitoes after being exposed to permethrin, deltamethrin, alpha-cypermethrin and pirimiphos-methyl were selected from each district for the analysis (Table 3). For

Acetylcholinesterase (*Ace*-1), 25 samples were selected from mosquitoes (both alive and dead) that were exposed to bendiocarb to express the *Ace-1* resistance allele frequency. In the case where bendiocarb was not tested at a site, pyrethroids tested mosquitoes were used for the *Ace-1* mutation detection. The project used Integrated DNA Technology (IDT) PCR-based SNP genotyping called the rhAmp SNP Genotyping System [4] to analyze *kdr* and *Acetylcholinesterase* SNPs mediating resistance in the mosquitoes. The rhAmp Genotyping Design Tool was used to design rhAmp SNP primers for SNPs in knockown resistance 'East' / *kd*r-e / *kdr L1014S*, knock down resistance 'West' /*kdr*-w/*kdr L1014F*, and *Acetylcholinesterase* gene / *AChE* / *Ace-1*R using FASTA format of target sequence. All rhAmp SNP assays consist of two allele-specific forward primers and a locus-specific reverse primer. Genotyping by rhAmp assay was performed following the manufacture's instruction using extracted DNA from mosquito mixed with rhAmp Genotyping Mix, composed by rhAmp Genotyping Master Mix (catalogue number: 1076016, Integrated DNA Technology, USA), rhAmp Reporter Mix (catalogue number: 1076022, Integrated DNA Technology).

# 3. RESULTS

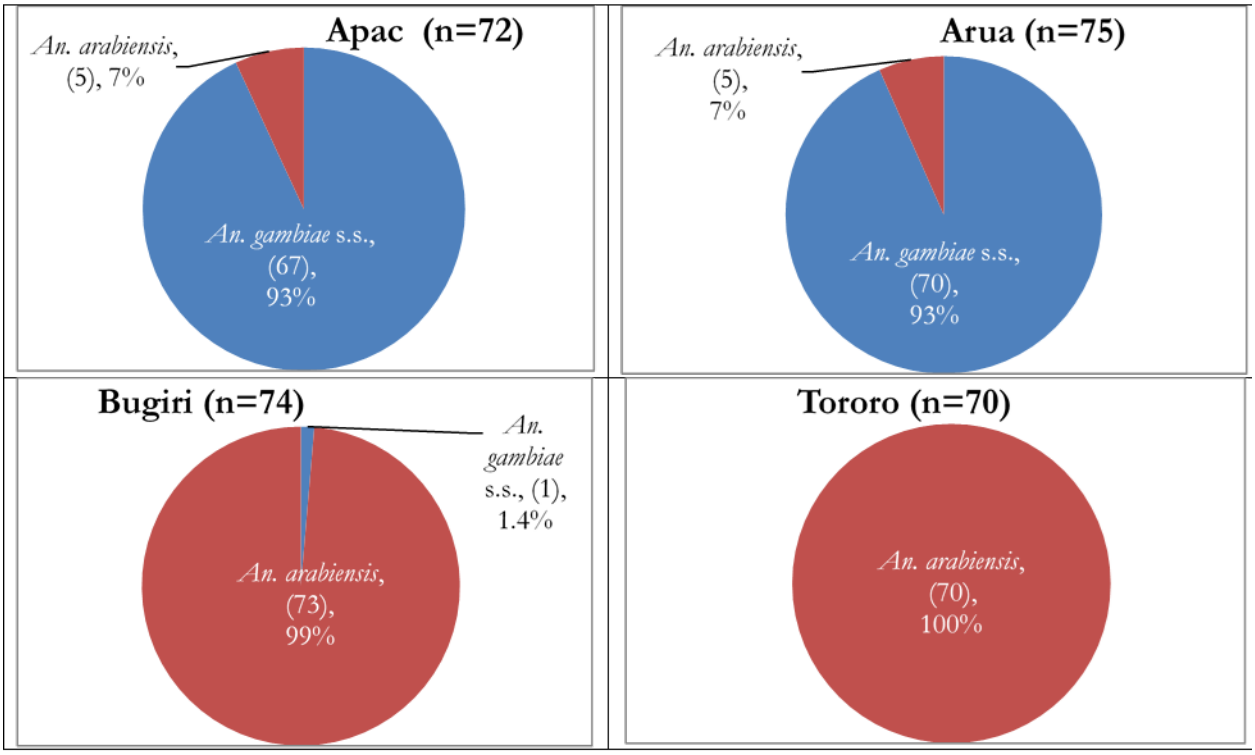
## 3.1. SPECIES IDENTIFICATION OF WHO SUSCEPTIBILITY TESTED MOSQUITOES

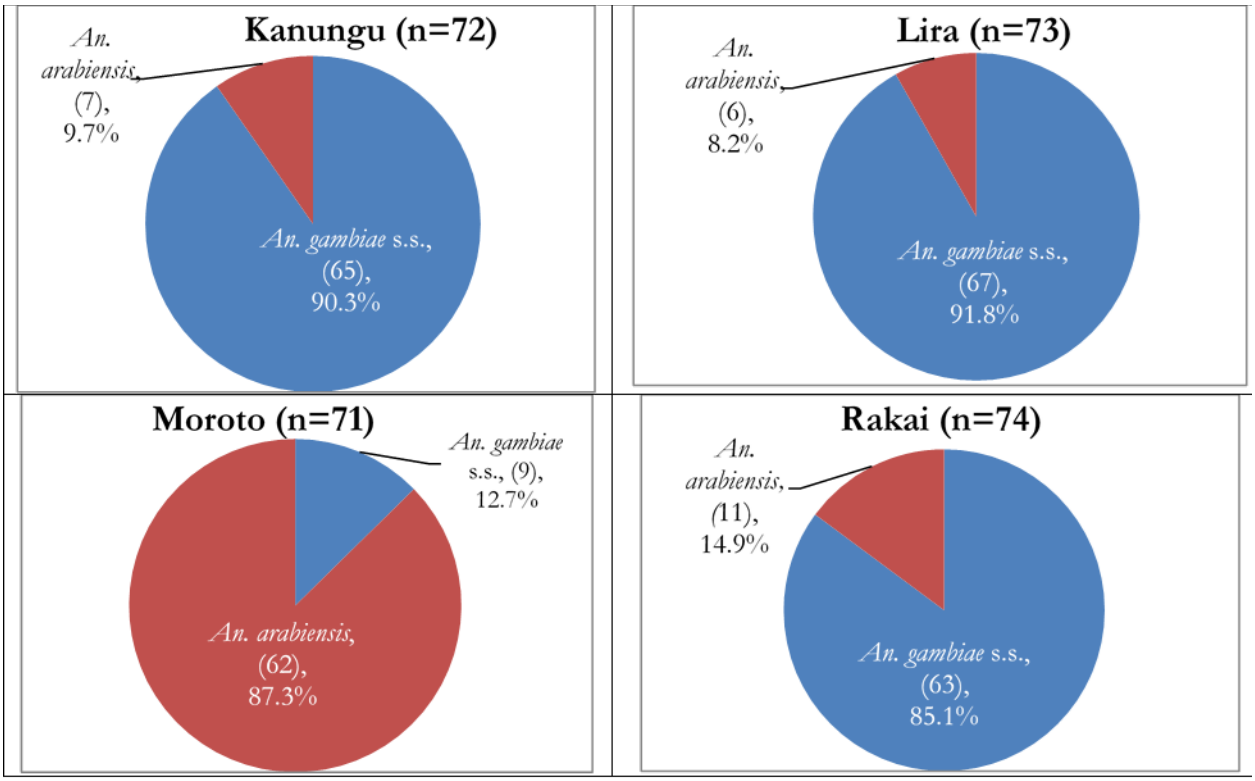
Of the 587 *An. gambiae* s.l. analyzed from mosquitoes used for WHO susceptibility tests, *An. gambiae* s.s. was the predominant species in the districts of Apac, Arua, Kanungu, Lira and Rakai, while *An. arabiensis* was the predominant species in the districts of Bugiri, Moroto and Tororo (Table 1, Figure 1).

**TABLE 1. SPECIES IDENTIFICATION BY PCR OF AN. GAMBIAE S.L. USED FOR WHO SUSCEPTIBILITY TESTS**

| District | Total analyzed | An. gambiae s.s. | An. arabiensis | Not amplified |
|---|---|---|---|---|
| Apac | 74 | 67 (90.5%) | 5 (6.8%) | 2 (2.7%) |
| Arua | 76 | 70 (92.1%) | 5 (6.6%) | 1 (1.3%) |
| Bugiri | 74 | 1 (1.4%) | 73 (98.6%) | 0 (0.0%) |
| Kanungu | 72 | 65 (90.3%) | 7 (9.7%) | 0 (0.0%) |
| Lira | 73 | 67 (91.8%) | 6 (8.2%) | 0 (0.0%) |
| Moroto | 72 | 9 (12.5%) | 62 (86.1%) | 1 (1.4%) |
| Rakai | 74 | 63 (85.1%) | 11 (14.9%) | 0 (0.0%) |
| Tororo | 72 | 0 (0.0%) | 70 (97.2%) | 2 (2.8%) |
| Total | 587 | 342 (58.3%) | 239 (40.7%) | 6 (1.0%) |

**FIGURE 1: SPECIES COMPOSITION OF *AN. GAMBIAE* COMPLEX USED FOR WHO SUSCEPTIBILITY STUDIES IN EIGHT SITES IN UGANDA, 2019**






## 3.2. HOST PREFERENCE

Of the 152 mosquitoes analyzed, ninety four percent (94.1%) of the mosquitoes analyzed had evidence of a human blood meal (n= 143). Cow blood represented the second most prevalent blood source (5.9%), while none of the mosquitoes fed on either dog, goat or pig. The highest human blood index was observed in Otuke and Soroti (100%), followed by Bugiri (97.4%) and Tororo (80.5%) (Table 2). The results also showed other blood meal sources. Only Tororo and Bugiri had mosquito samples that had fed also on cows along with humans (Table 2).

**TABLE 2. BLOOD MEAL SOURCES AND HUMAN BLOOD INDEX OF *AN. GAMBIAE* S.L. COLLECTED BY PSCS IN FOUR OF THE SURVEYED SITES**

| District | No Analyzed | Source of Blood Meal | | | | | Human Blood Index (%) |
|---|---|---|---|---|---|---|---|
| | | Cow | Dog | Goat | Pig | Human | |
| Bugiri | 39 | 1 | 0 | 0 | 0 | 38 | 97.4 |
| Otuke | 52 | 0 | 0 | 0 | 0 | 52 | 100 |
| Soroti | 20 | 0 | 0 | 0 | 0 | 20 | 100 |
| Tororo | 41 | 8 | 0 | 0 | 0 | 33 | 80.5 |
| Total | 152 | 9 | 0 | 0 | 0 | 143 | 94.1 |

## 3.3. INSECTICIDE RESISTANCE MARKERS

Of the total 101 samples analyzed, both L1014F (*kdr*-west) and L1014S (*kdr*-east) alleles were present, with the *kdr*-east being more prevalent in Ugandan mosquito population compared to *kdr*-west (Table 3). The average frequency of *kdr*-east all sites included was 0.19 and *kdr*-west 0.12. Additionally, few samples carried both *kdr* alleles (Apac: 2; Arua: 5, Lira: 2). All 200 samples collected from different districts and analyzed for identification of *ace*-1R SNP mediating resistance to carbamates and organophosphate insecticides were wild types (Table 4).

**TABLE 3: FREQUENCY OF INSECTICIDE RESISTANCE MARKERS ACROSS SITES**

| Site | Total tested | Status | Total per status | Kdr-east Resistance allele | | | Kdr-east freq | Kdr-west Resistance allele | | | Kdr-west freq |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | RR | RS | SS | | RR | RS | SS | |
| Apac | 12 | Dead | 1 | 0 | 0 | 1 | 0.17 | 0 | 0 | 1 | 0.13 |
| | | Alive | 10 | 0 | 4 | 6 | | 0 | 3 | 7 | |
| | | Missing status | 1 | 0 | 0 | 1 | | 0 | 0 | 1 | |
| Arua | 17 | Dead | 0 | - | - | - | 0.24 | - | - | - | 0.44 |
| | | Alive | 16 | 1 | 4 | 10 | | 5 | 5 | 6 | |
| | | Missing status | 1 | 1 | 0 | 0 | | 0 | 0 | 1 | |
| Bugiri | 16 | Dead | 2 | 0 | 0 | 2 | 0.0 | 0 | 0 | 2 | 0.03 |
| | | Alive | 10 | 0 | 0 | 10 | | 0 | 1 | 9 | |
| | | Missing status | 4 | 0 | 0 | 4 | | 0 | 0 | 4 | |
| Kanungu | 12 | Dead | 0 | - | - | - | 0.71 | - | - | - | 0.0 |
| | | Alive | 12 | 5 | 7 | 0 | | 0 | 0 | 12 | |
| Lira | 11 | Dead | 7 | 0 | 2 | 5 | 0.14 | 0 | 1 | 6 | 0.18 |
| | | Alive | 4 | 0 | 1 | 3 | | 1 | 1 | 2 | |
| Moroto | 9 | Dead | 0 | - | - | - | 0.11 | - | - | - | 0.0 |
| | | Alive | 7 | 0 | 2 | 5 | | 0 | 0 | 7 | |
| | | Missing status | 2 | 0 | 0 | 2 | | 0 | 0 | 2 | |
| Rakai | 12 | Dead | 12 | 1 | 0 | 11 | 0.08 | 0 | 0 | 12 | 0.0 |
| | | Alive | 0 | - | - | - | | - | - | - | |
| Tororo | 12 | Dead | 0 | - | - | - | 0.08 | - | - | - | 0.04 |
| | | Alive | 12 | 0 | 2 | 10 | | 0 | 1 | 11 | |
| **Total** | | | **101** | **8** | **22** | **70** | **0.19** | **6** | **12** | **83** | **0.12** |

**Key:** f= frequency; RR= homozygous resistant; RS= heterozygous resistant; SS= homozygous susceptible

## TABLE 4: NUMBER OF MOSQUITOES WITH DIFFERENT GENOTYPES OF ACETYLCHOLINESTERASE RESISTANCE (ACE-1) USING PCR METHODS

| District | Status | Total | Resistance genotypes | |
|---|---|---|---|---|
| | | | Wild type (SS) | Resistant (RR) |
| Apac | dead | 23 | 23 | 0 |
| | alive | 2 | 2 | 0 |
| Arua | dead | 14 | 14 | 0 |
| | alive | 11 | 11 | 0 |
| Bugiri | dead | 23 | 23 | 0 |
| | alive | 2 | 2 | 0 |
| Kanungu | dead | 24 | 24 | 0 |
| | alive | 1 | 1 | 0 |
| Lira | dead | 25 | 25 | 0 |
| | alive | 0 | 0 | 0 |
| Moroto | dead | 25 | 25 | 0 |
| | alive | 0 | 0 | 0 |
| Rakai | dead | 10 | 10 | 0 |
| | alive | 15 | 15 | 0 |
| Tororo | dead | 23 | 23 | 0 |
| | alive | 2 | 2 | 0 |

**Key: RR** = homozygous resistant; **SS** = homozygous susceptible

# 4. DISCUSSION AND CONCLUSIONS

Molecular analysis of entomological samples collected from January to December 2019 under the PMI VectorLink Uganda project including bionomics and susceptibility monitoring in the sentinel sites showed that both *An. gambiae* s.s. and *An. arabiensis* were found in the country with varying predominance throughout the sites. As described in the main report, *An. gambiae* s.s. was predominant in two sites (Apac – 65.5% and Lira – 60.6%) while *An. arabiensis* was predominant in four sites (Bugiri – 85.5%, Otuke – 58.1%, Soroti -79.7% and Tororo – 99.3%) among the samples collected through human landing catches and PSCs. The mosquitoes tested for insecticide susceptibility followed by molecular species identification showed that *An. gambiae* s.s is still the major vector in five sites: Apac – 93.1%, Arua – 93.3%, Kanungu – 90.3%, Lira – 91.8% and Rakai – 85.1%, while *An. arabiensis* was predominant in three sites: Buguri – 98.6%, Moroto – 87.3% and Tororo – 100%.

Of the four indoor residual spraying (IRS) districts where bionomics studies were conducted, the insecticide susceptibility tested mosquito's species identification confirmed *An. gambiae* s.s. as the predominant species in Lira and *An. arabiensis* in Bugiri and Tororo. Furthermore, it is known that *An. arabiensis* prefers drier areas than *An. gambiae* s.s. which may explain the higher proportion of *An. arabiensis* in the localities of Bugiri, Moroto and Tororo. Bugiri and Tororo have a bimodal rainfall season while Moroto district experiences only one rain season and one dry season. Moroto and Rakai fall into what is called the cattle corridor and usually drier than the rest of other study districts. Also, the ongoing intervention of IRS tends to control the more endophagic and endophilic mosquitoes such as *An. gambiae* s.s. more than the zoophilic and exophagic vectors. *An. arabiensis.* was more predominant in IRS districts (Bugiri, Tororo) than other non-IRS districts such as Apac, Arua, Kanungu and Rakai where primary *An. gambiae* s.s. was collected.

The blood meal analyses of the mosquitoes collected indoors using PSCs showed that *An. gambiae* s.l. fed on both humans and cows, but predominantly on humans, exhibiting a high human blood index. The fact that the mosquitoes were collected inside the houses may contribute to the source of human blood, but it's also known that the vectors can bite outside and then rest indoors. The high human blood index indicates a high vector-human contact and reiterates the need for increased awareness among the communities regarding the consistent and correct use of long-lasting insecticidal nets every night, as well as encouraging and mobilizing communities in the IRS districts to support and accept IRS.

The characterization of the different mechanisms involved in vector resistance to insecticides confirms the presence of both *kdr* L1014F (*kdr*-west) and *kdr* L1014S (*kdr*-east) alleles. The kdr-east was present in seven of the eight sites surveyed and the *kdr*-west in six of the eight sites. Though the resistance allele frequency is still very low in the majority of the sites, the *kdr*-east frequency in Kanungu and the *kdr*-west frequency in Arua were particularly higher than all the other sites. These trends should be monitored closely to avoid fixation of the resistance allele within the vector population, which threaten the efficacy of vector control interventions. Interestingly, the resistance allele frequencies recorded within the predominant *An. arabiensis* populations from Bugiri, Moroto and Tororo were the lowest, compared to those of the *An. gambiae* s.s predominant population sites like Arua and Kanungu, except in Rakai. Furthermore, resistance to pyrethroid insecticide was recorded in all sites during the susceptibility testing, which necessitates additional investigation to fully understand the other resistance mechanisms, such as metabolic resistance, that may be involved in the resistance of the vectors, per the piperonyl butoxide (PBO) exposure results recorded during the susceptibility test and reported in the main 2019 Uganda entomological report.

No *ace-1* mutation was observed in all eight sites in Uganda, showing that carbamate and organophosphate insecticides could still be used for vector control in the country. These results will support the National Malaria Control Division's decision-making on insecticide resistance management plan and vector control activities in Uganda.

# 5. LIMITATIONS

- The species identification of the sub-species of the *An. gambiae* complex could not be fully completed to enable differentiation between *An. gambiae* and *An. coluzzii* due to lack of appropriate species primers due to a procurement issue. This is unfortunate as both species sometimes exhibit different malaria transmission efficiency and insecticide resistance allele frequency that could contribute to the vector control intervention decision making. Furthermore, both species may show different insecticide resistance mechanisms.
- The number of samples tested for *kdr* is deemed to be too low to enable concrete interpretation of the resistance allele present among the population of each site.

# 6. REFERENCES


1. Musapa, M., et al., A simple Chelex protocol for DNA extraction from Anopheles spp. J Vis Exp, 2013(71).
2. Scott, J.A., W.G. Brogdon, and F.H. Collins, Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction. Am J Trop Med Hyg, 1993. 49(4): p. 520-9.
3. Kent, R.J. and D.E. Norris, Identification of mammalian blood meals in mosquitoes by a multiplexed polymerase chain reaction targeting cytochrome B. Am J Trop Med Hyg, 2005. 73(2): p. 336-42.
4. Ayalew, H., et al., Comparison of TaqMan, KASP and rhAmp SNP genotyping platforms in hexaploid wheat. PLoS One, 2019. 14(5): p. e0217222.