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USAID *Okoa Maisha Dhibiti Malaria* (OMDM)/Save Lives, End Malaria

ENTOMOLOGICAL ASSESSMENT ANNUAL REPORT: YEAR 3

September 29, 2021

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Prepared for:

USAID/Tanzania
U.S. Agency for International Development
Office of Acquisition and Assistance
686 Old Bagamoyo Road, Msasani
P.O. Box 9130
Dar es Salaam, Tanzania
Telephone: 255-22-229-4490

Attn: **Chonge Kitojo (ckitojo@usaid.gov)**
Agreement Officer's Representative (AOR)
Naomi Serbantez (nserbantez@usaid.gov)
Alternate AOR
Dennis Foster (dfoster@usaid.gov)
Deputy Director, Office of Acquisition & Assistance (OAA)

Prepared by

RTI International
P.O. Box 12194
Research Triangle Park, NC 22709-2194

ZANZIBAR MALARIA ELIMINATION PROGRAMME

ENTOMOLOGICAL MONITORING ANNUAL REPORT

(October 2020 - September 2021)

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List of abbreviation

CS	Circum sporozoite antigens
CDC	Centers for Disease Control
DNA	Deoxyribonucleic Acid
EIR	Entomological Inoculation Rate
ELISA	Enzyme-linked immunosorbent assay
HBI	Human blood index
HLC	Human landing Catch
HBR	Human biting rate
IRS	Indoor Residual Spraying
IBR	Indoor biting rate
ITNs	Insecticide treated nets
kdr	Knockdown Resistance
LLINs	Long-lasting Insecticide-treated bed nets
OBR	Outdoor biting rate
PTC	Pit trap catches
PBO	Piperonyl Butoxide
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction Restriction Fragment Length Polymorphism
PSC	Pyrethrum Spray Catch
PMI	Presidential Malaria Initiative
TMIS	Tanzania Malaria Indicator survey
USAID	United States Agency for International Development
WHO	World Health Organization
ZAMEP	Zanzibar Malaria Elimination Programme

1.0 Introduction

For the past ten years, malaria in Zanzibar has remained stable, with a parasite prevalence of less than 1%. The last survey reported a further reduction in malaria prevalence to 0.2% in children under five years, with considerable variation between the two islands (0.4% in Unguja and 0.0% in Pemba-TMIS 2017). Malaria transmission in Zanzibar is bimodal with two peaks following rainfall patterns, heavy rain (Masika) in March-May and short rain (Vuli) in October to December. The distribution of malaria cases varies between islands, being higher in Unguja than in Pemba. In 2020/2021, Unguja island reported 86% (8,033) compared to 14% (1300) of the total malaria cases (n= 9,2290) .

Insecticide-treated nets (ITNs) and indoor residual spraying (IRS) are the front line malaria vector interventions in Zanzibar. In the 2021 IRS operation, ZAMEP used Clothianidin/sumishield with the spray coverage of 94.5% (n = 46,316) structures protecting the 94.5% of the population(n = 230,708) in 67 shehia (56 in Unguja and 11 in Pemba). These interventions have greatly reduced malaria morbidity and mortality because the most important mosquito vectors, exclusively An gambiae s.l, are synanthropic and are closely associated with humans wherever they are. Insecticidal contact with vectors is at the maximum level if they go indoors.

The recent and historical reports in Zanzibar show that widespread use of ITNs or IRS changes the species composition of residual vector populations by progressively diminishing densities of each species in proportion. For example, in 2005, before the intensive application of insecticide interventions. The entomological surveillance documented *An gambiae s.s* as predominant malaria vectors with anthropophagic, endophagic and endophilic behaviour. The current report shows that *An arabiensis* is a dominant malaria vector exhibiting exophagic behaviour forcing the ZAMEP to have complementary interventions to fight against outdoor biting vectors.

Pyrethroid resistance of *Anopheles gambiae s.l* was described in 2010 in Pemba for the first time. However, two years later, Unguja reported the same situation as in Pemba. The strength of resistance varies across the sentinel sites, being higher in Pemba than in Unguja. The phenotypic data indicates the pyrethroid resistance is likely due to increases in the rate of insecticide metabolism. The cytochrome P450 is the most likely candidate for conferring this resistance. The cause of resistance remained unclear. However, high LLINs coverage within the population was suspected to be the causal.

Insecticide resistance poses challenges in insecticide-based malaria vector control interventions in many areas in sub-Saharan Africa, where pyrethroid resistance is documented. Therefore, it is crucial to understand the local malaria vector species, their behavior, disease incrimination, susceptibility to insecticides and residual effectiveness of insecticide in treated surfaces for planning effective malaria control interventions.

1.1 Objectives of entomological surveillance

- To assess the quality of IRS operation and insecticide decay rate
- To determine insecticide susceptibility status of malaria vectors to insecticides
- To determine the intensity and mechanisms of resistance
- To assess malaria vector density, species, behavior (feeding and resting), seasonality, distribution, infection rate and entomological inoculation rate.

1.2 Sentinel sites

Entomological surveillance at sentinel sites was conducted from October 2020 to September 2021 whilst IRS quality assessment and insecticide residual efficacy were carried out from February 2021 to September 2021. Ten entomological (10) sentinel sites are used for entomological monitoring. These sites are located in ten different districts i.e. one site per district. There were six (6) sites in Unguja and four (4) in Pemba. The selection of entomological sentinel sites considered the following criteria:

- Disease incidence/ prevalence
- Topography of the area
- Agricultural practices (Rain fed rice, Irrigation etc)
- Urban or rural setting

Table 1 : Sentinel sites in Zanzibar

Location	District	sentinel site	Geo Coordinate	IRS status in 2021	Entomological parameters collected
Unguja	Mjini	Stone town	Longitude 39.1936844 Latitude -6. 1583878	Not sprayed	Vector abundance, species distribution, seasonality, feeding time and location and infection rate
	Kaskazini B	Bumbwini	Longitude 39.2229625 Latitude -6.1775842	Sprayed	Vector abundance, species distribution, seasonality, feeding time and location and infection rate. Insecticide decaying rate
	Kaskazini A	Donge	Longitude 39.239423 Latitude -5. 9305385	Not Sprayed	Vector abundance, species distribution, seasonality, feeding time and location and infection rate,
	Magharibi	Mwera	Longitude 39.4623959 Latitude -6. 3722427	Not sprayed	Vector abundance, species distribution, seasonality, feeding time and location and infection rate
	Kusini	Muyuni	Longitude 39.2231848 Latitude -6. 1776179	Not Sprayed	Vector abundance, species distribution, seasonality, feeding time and location and infection rate
	Kati	Cheju	Longitude 39. 3730012 Latitude -6. 2223636	Not Sprayed	Vector abundance, species distribution, seasonality, feeding time and location and infection rate.
	Micheweni	Tumbe	Longitude 39. 7895961 Latitude -4.9473016	Sprayed	Vector abundance, species distribution, seasonality, feeding time and location and infection rate
	Wete	Bopwe	Longitude 39.725216	Not sprayed	Vector abundance, species distribution, seasonality,

			Latitude -5.0487204		feeding time and location and infection rate
	Chake Chake	Uwandani	Longitude 39.8243398 Latitude -5.1959127	Not sprayed	Vector abundance, species distribution, seasonality, feeding time and location and infection rate
	Mkoani	Wambaa	Longitude 39.7270355 Latitude -5.4011374	sprayed	Vector abundance, species distribution, seasonality, feeding time and location and infection rate, insecticide decaying rate

1.3 IRS Quality assessment & Insecticide decaying rate rate sites

ZAMEP collaborated with Vectorlink to conduct targeted spraying operation using Clothianidin/Sumishield in sixty-seven shehia (67) in February 2021, in Unguja and Pemba. Fifty-six shehias were from Unguja, and eleven were from Pemba.The operation covered 94.5% (46,316 /49,238) structures protecting the population of 230,708 (94.5%). Of the shehia sprayed, the team selected six shehias (3 in Pemba and 3 in Unguja) for IRS Quality assessment & Insecticide decaying rate rate (see the table below:

Table 2 :Sites for clothianidin residual efficacy

Location	District	Shehia	Geo Coordinate
Pemba	Wete	Gando	Longitude39.6962757. Latitude -4.9888278
	Mkoani	Wambaa	Longitude 39.7270355 Latitude -5.4011374
	Micheweni	Makangale	Longitude39.6903829. Latitude -4.9102173
Unguja	Magharibi B	Dimani	Longitude39.29717. Latitude -6.24008
	Kaskazini B	Bumbwini	Longitude39.2229625. Latitude -6.1775842
	Kati	Umbuji	Longitude39.36731. Latitude -6.12301

1.4 Vector bionomics

The objective of entomological monitoring was to assess the impact of IRS/ITNs at sentinel sites regarding potential mosquito parameters for malaria transmission. These include vector abundance, species, seasonality, density, infection rate, feeding and resting behaviour and susceptibility of vectors to insecticides. Methods used for vector sampling were Pyrethrum spray catches, Human landing catches, CDC light traps and Pit traps.

2.0 Methodology

2.1 Assessment of Quality of IRS programme and insecticide decay rate

The team conducted Quality assurance of IRS programs of *Sumishield 50WG* on the first seven days of the operation to assess the spray quality and sprayer performance. It was then followed by monitoring the insecticide decay rate every month for seven months. Six shehias were involved in both Unguja and Pemba. The shehias were selected randomly per district. The exercise involved fifteen houses per shehias built up of different wall surfaces such as mud, cement, oil and water paint and stone block. The team tested three house structures per shehia made of each wall surface type.

Test procedures for assessment of IRS quality and insecticide decay rate

- Bioassays were conducted in accordance with WHO (2006) guidelines. A susceptible colony of laboratory-reared *An. gambiae* s.s. (R-70 strain) was used for the cone bioassays on the different wall surface types.
- Two to five days old, non-blood fed females of *An. gambiae* s.s. R-70 from the ZAMEP insectary were exposed to sprayed wall surfaces in batches of ten mosquitoes for 30 minutes under WHO cones.
- Cones were attached to walls at two different heights in each of the two rooms sampled in a house: at a lower level (1.0-meter-high) and an upper level (1.5 meters high).
- At the end of the test, mosquitoes were transferred to paper cups and supplied with cotton pads soaked with sugar solution.
- First knockdown was scored after 30 minutes followed by 60 minutes post exposure and mortality scored and recorded after 24 hours holding period for seven days. Moribund and dead mosquitoes were counted as dead.
- Negative control bioassays were conducted on unsprayed surfaces covered with manila sheet layers. When control mortality was scored between 5% and 20%, experimental mortality was corrected using Abbott's formula (Abbott, 1925). If the mortality score was more than 20%, the experiment was discarded.

2.2 Methods used in mosquito collection and processing for vector bionomics

The table below summarizes the methods used in mosquito collection and the frequency of sampling

Method	Purpose	Sentinel site	≠ of Households	Days/month	Time	Indicator assessed
HLC	Indoor & outdoor biting behavior	10	2 house/ site	2 days/site	1800-0600	Species composition, sporozoite rate

PSC/Proko pack	Indoor resting behavior	10	5 houses/site	2 days/site	0600-0800	Species composition, sporozoite, rate HBI
CDC light trap	Indoor abundance	10	2 houses/site	2 days/site	1800-0600	Species composition, sporozoite rate
PTC	Outdoor resting behavior	10	2 pits/site	2 days/site	0600-0800	Species composition, sporozoite rate ,HBI

Human landing catches (HLC)

Human-landing catches (HLC) (WHO, 1975) were conducted between 18.00 hrs – 06.00 hrs outdoors and indoors twice per month at each site. Catches from the collections were kept in paper cup/hour/collectors. Two collectors were collecting mosquitoes outdoor and the other two were doing the same indoor in two houses per site. They were then kept in a cool box until sorted, counted and recorded in the following morning.

Pyrethrum Spray Catches (PSC)

Pyrethrum spray catch was carried out in five houses during each mosquito collection morning twice per month. White sheets were laid on the entire floor and over the furniture within one room where people slept in previous night in each selected house. White sheets facilitate visibility of the knocked down mosquitoes. The doors and windows of the houses were shut then the rooms sprayed with pyrethrum (0.3%) synergised with Piperonyl-butoxide (PBO) as described by Gimnig et al. (2003). Briefly, a collector outside the house sprayed around the eaves with insecticide to prevent the mosquitoes inside the houses from escaping and another collector sprayed the roofs and the walls inside the house. The houses were then closed for 10-15 minutes. The white sheets were removed from all the rooms of the houses and the knocked down mosquitoes collected using forceps. Knocked down mosquitoes for each room were recorded and then transferred onto moist filter paper inside labelled petri dishes indicating the date and house number. The same procedure was repeated for all the five houses and collected mosquitoes put in a cool box and transported to the laboratory for further processing.

Pit Trap Catches (PTC)

A rectangular pit was dug in the ground (1.5 m in depth, 1.2 m in length and 1 m in width) within 10 m of each selected residential house. In each of the four vertical sides, about 50–60 cm and 90–100 cm from the bottom of the pit, 5-8 little cavities were dug into a depth of about 30 cm. The main pits were then shaded by an artificial framework thatched with locally available coconut palms on top to provide shade. Resting mosquitoes were sampled from 06:00 to 09:00 hrs inside the cavities by using hand-held mouth aspirators or Prokopack. Collected mosquito samples were kept in paper cups before being processed accordingly in the laboratory. The collection was done twice per month at each site.

CDC light trap

Indoor host-seeking mosquitoes were collected using Centre for Disease Control and Prevention (CDC) light traps (John W. Hock Ltd, Gainesville, FL., USA). In every mosquito trapping night, mosquitoes were collected from 2 randomly selected houses from a single collection site. In each house, light traps were hanged a meter off the ground and approximately 150 cm from an occupied mosquito net. A single trap was set per room and a total of two traps were set each sampling night/HOUSE. These traps were positioned at the head side of the sleeper. Light traps were switched on at 1800 hrs and switched off at 0600 hrs the next morning. Mosquitoes were then collected in the morning. Live mosquitoes were aspirated into clearly labelled paper cups and transported to the laboratory for further processing.

2.3 Morphological identification of mosquitoes

Morphological identification was done using the taxonomic keys of Gillies and Coetzee (1987) at laboratory once the samples are arrived from field. Based on morphological characteristics, Anophelines were sorted to generally known species i.e. *An. gambiae* s.l., *An. funestus*, *An. coustani*, *An. pharoensis*, etc.

2.4 Detection of sporozoites in mosquitoes by CSP-ELISA technique

A sandwich ELISA schematic principle for malaria vector infection rate
Enzyme-linked immunosorbent assays (ELISAs) were developed to detect *Plasmodium falciparum*, *P. vivax*-210, and *P. vivax*-247 circumsporozoite proteins (CSP) in malaria-infected mosquitoes. The sensitivity and specificity of the ELISAs are based on the monoclonal antibodies (Mabs) used. The ELISAs detect CS proteins, which can be present in the developing oocysts, dissolved in haemolymph, and on sporozoites present in the haemocoel or in the salivary glands.
The ELISA assays were carried out using dried mosquitoes. The specimens were processed and preserved in Eppendorf tubes with silica gel to keep them dry. The specimens were grinded using grinding solution containing Blocking Buffer (BB) and Igepal CA-630, combination was mixed properly so that the Igepal is dissolved in BB.

Mosquito grinding

Each mosquito sample was ground separately (one mosquito per well), only the head-thorax part was placed in a labelled 1.5ml micro centrifuge grinding tube. Grinding buffer was then added at this point for the specimen to be well grinded. The pestles were rinsed with Grinding Solution; to make sure that the rinses were held in the tube containing mosquito triturate.

The “sandwich” begun by absorption of the capture Monoclonal antibodies (Mab) to the wells of a micro plate. After the capture Mab had bound to the plate, the well contents were aspirated, and the remaining binding sites were blocked with blocking buffer containing IGEPAL CA- 630 and an aliquot was tested. Positive and Negative Control were also added to specific well at this time. If circum-sporozoite (CS) antigen is present it will form AgAb complex with the capture Mab. After 2hrs

incubation at room temperature, the mosquito triturate was aspirated, and the wells were washed. Peroxidase – linked Mab was then added to the wells, completing the formation of the "sandwich". After 1hr, the well contents were aspirated, the plate was washed again, and the clear peroxidase substrate solution was added. As the peroxidase enzyme reacts with the substrate, a dark green product is formed; the intensity of the colour is proportional to the amount of CS antigen present in the test sample.

2.5 Detection of blood meal sources by ELISA

The blood meal origins of freshly fed Anophelines mosquitoes collected inside houses were determined using a direct enzyme-linked immunosorbent assay (ELISA) following the method of Beier et al. (1988) using human and bovine antibodies. Each mosquito abdomen was crushed in 50-µl phosphate buffered saline (PBS) solution (pH 7.4), which was further diluted by adding 950 µl PBS. Fifty microliters of sample added to each well in a 96-well microtitre plate and incubated overnight at room temperature. Each well was washed twice with PBS containing Tween-20 solution, and 50-µl host specific conjugate (either human or bovine) was added to each well and incubated for one hour. After one hour, each well was washed three times with a PBS–Tween-20 solution. Finally, 100 µl of peroxidase substrate was added to each well and after 30 minutes the absorbency at 405 nm was recorded with an ELISA plate reader. Each blood meal sample was considered positive if the absorbance value exceeded the mean plus three times the standard deviation of the four negative controls (from a laboratory colony of *An. gambiae* Kisumu strain adults not fed with blood). Positive controls contained human and bovine blood.

2.6 Methods used for Insecticide susceptibility test

The entomology team assessed the susceptibility of local vectors against common insecticides thus guiding the IRS campaign on insecticide to be applied. The test was carried out across the sentinel sites in 10 districts. (six in Unguja and four in Pemba). The tests used WHO discrimination doses of Permethrin (0.75%), Deltamethrin (0.05%) and Bendiocarb (0.01%) Alpha-cypermethrin 0.05% Clothianidin 2% and Pirimiphos methyl 0.25%.

Strength/intensity of pyrethroid resistance against malaria vectors was determined at concentrations of 5x to 10x of Alpha-cypermethrin deltamethrin, Permethrin and lambda-cyhalothrin in WHO impregnated paper in Pemba.

Mosquito collection

Wild Anopheles larvae and pupae were collected in various breeding sites from established entomological surveillance sites ranging from home yards, cultivated land and rice fields. Therefore, mosquitoes obtained could be representative of the vectors available in the areas. Mosquito larvae were 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 ZAMEP insectary in both Unguja and Pemba where they were reared at 27-30°C. Larvae collected from several breeding sites in the same village were pooled together for rearing and testing. The larvae were fed with 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 labelled cages for adult emergence. Female adult mosquitoes aged 2-5 days were used for WHO susceptibility tests and PBO synergy testing.

Insecticide Resistance Test procedures

The susceptibility tests were carried out using the World Health Organization test kits for adult mosquitoes (WHO, 2016). Two to five-day old female F1 generation mosquitoes were tested using standard WHO insecticide susceptibility procedures with four replicates of 25 wild adult female mosquitoes per test tube. Mosquitoes were exposed to papers impregnated with the WHO-recommended discriminating concentrations of deltamethrin (0.05%), Bendiocarb (0.1%), Permethrin (0.75%) Alpha alpha-cypermethrin (0.05%), Pirimiphos-methyl (0.25%) and Clothianidin (2%) .

At the end of the exposure period, mosquitoes were transferred into holding tubes (lined with untreated papers) by gently blowing them through the open space between the exposure tube and the holding tubes. Cotton 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 except for clothianidin whereby the score and record continued for seven days. The susceptibility status was evaluated based on the WHO criteria i.e. 98-100% mortality indicate susceptibility; 90-97% mortality required confirmation and less than 90% mortality indicate resistance (WHO, 2016). When the control mortality between 5% and 20% was recorded, the mean observed mortality was corrected using Abbott's formula (Abbott, 1925). All tested mosquitoes were preserved with silica gel in 1.5 ml Eppendorf tubes and transported to ZAMEP molecular laboratory for further laboratory analyses (molecular species identification and detection of mechanisms of insecticide resistance).

We determined the strength/intensity of pyrethroid resistance against malaria vectors at two concentrations of 5x Deltamethrin (0.25%) and Permethrin (3.75%) in WHO impregnated paper

PBO – Synergist Bioassays

The aim of the test was to ascertain the involvement of mixed function oxidases in the observed phenotypic resistance. In this test, 2–5 days old F1 adult mosquitoes were pre-exposed to 4% Piperonyl butoxide (PBO) paper for 1 h and immediately exposed to 0.75% Permethrin, or 0.05% Deltamethrin for 1 h. Two controls were used during this experiment: control constituted mosquitoes exposed to clean papers neither with insecticides nor with PBO, while control 2 constituted mosquitoes exposed to papers treated with PBO only. Mortalities were later assessed after exposure; the PBO synergised group was compared to the un-synergized group after 24 h post-exposure. This comparison was used to evaluate the potential role of Monooxygenase enzyme in the observed resistance.

2.7 Molecular identification of *An. gambiae* s.l. and *An. funestus*

Anopheles gambiae sibling species identification was carried out according to the standard polymerase chain reaction (PCR) method (Scott *et al.,* 1993). Five oligonucleotide primers, GA, ME, AR, QD and UN designed from the DNA sequences of the intergenic spacer region of complex ribosomal DNA (rDNA) were used to amplify species-specific DNA sequences. The UN-primer is universal and anneals to the same position on the rDNA sequences of all five species, the GA anneals specifically to *Anopheles gambiae s.s.*, the ME anneals to either *Anopheles merus* or *Anopheles melas*, AR to *Anopheles arabiensis* and the QD to *Anopheles quadriannulatus.* The PCR reaction mix of 25 µl contained 1 X PCR buffer (constituents), 200 µM of each of the deoxyribonucleotide triphosphates (dNTPs), 20 µM of oligonucleotide primers, 0.125 units of Taq Polymerase enzyme (Sigma, USA) and 0.5 µl of the extracted genomic DNA. Sterile double distilled water was added to make up the volume to 25 µl. The reaction mix was spun down briefly at 14,000 rpm and overlaid with mineral oil to avoid evaporation and refluxing during thermo-cycling.

The amplification reactions were carried out using PTC 100 thermal cycler (MJ Research Inc., USA) and the cycling parameters were as follows: 3 minutes at 94ºC (initial denaturation), followed by 35 cycles with denaturation at 94ºC for 30 seconds, annealing at 50ºC for 30 seconds and extension at 72ºC for 60 seconds and ended with a final cycle at 94ºC for 30 seconds, annealing at 50ºC for 30s and extension at 72ºC for 10 minutes. For each reaction, a positive control containing 0.5 µl of PCR products of *An. gambiae s.s* as template DNA and a negative control that contained no DNA template were included.

The amplified products were analysed by agarose gel electrophoresis. Ten micro-litres of each PCR product were added to 1µl of 10x Orange-G loading dye and electrophoresed in 2% agarose gel stained with 0.5 µg/ml of ethidium bromide. The electrophoresis was run in 1X Tris acetate-EDTA (TAE) buffer at 100V for one hour and were visualized and photographed over a UVP dual intensity trans-illuminator at short wavelength using a digital camera fitted with an orange filter and a hood. The amplified PCR product was identified to the sibling species on the basis of the diagnostic band size determined by comparison with the mobility of a standard 100bp DNA ladder (Sigma, USA).

2.8 Detection of target site resistance mechanisms

The PCR-based standard protocol used for the detection of the L1014S or L1014F kdr alleles was adapted from the protocols developed (Martinez-Torres, Chandre et al. 1998; Ranson, Jensen et al. 2000). All wild-caught *An. gambiae* s.l. from each of the above-mentioned sentinel sites were tested for KDR mutation.

Amplification was performed in a 25 µl reaction containing 2 µl of template DNA, GoTaq 5X PCR Buffer (containing 15 mM MgCl2), MgCl2 (25mM), dNTP (2-2.5 mM mix), dNTP (2-2.5 mM, and 1 5 U/µl of Go-Taq DNA polymerase, 2.5 pmol/µl for both IPCF and IPCR, 2.5 pmol/µl East primer and 8.8 pmol/µl for West primer.

The *kdr* genotyping of susceptible and resistant individuals was possible after amplifying the DNA template from mosquitoes following the PCR conditions of 95°C for 5 minutes (initial denaturation), followed by 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds. For the East Africa *kdr* amplification, there was a final extension cycle of 72°C for 5 minutes followed by 4°C coolingFor west African *kdr* amplification, the PCR conditions involved an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 59°C for 30 seconds and 72°C for 30 seconds. There was a final extension cycle of 72°C for 5 minutes followed by cooling at 4°C. The products were electrophoresed through 2% agarose gel with ethidium bromide stain and visualized under UV light. Knock down resistant (*kdr*) genotypes of both the susceptible and resistant individuals were then recorded. All successful reactions should contain a band of 285 bp. In addition, a band of 210 bp indicates the susceptible (wild type) allele and one of 188 bp the resistant allele.

3.0 Results

3.1 Residual efficacy of Sumishield 50WG

Quality assurance of the IRS programme was done in the first week of the operation to assess the quality of indoor residual spraying and spray team performance, followed by an insecticide decaying rate assessment monthly.
The average mosquito mortality for the wall cone bioassays from six (6) shehia in Pemba and Unguja is still effective (≥80% mortality) against susceptible *Anopheles gambiae s.s* R70. However, in Pemba, many walls surfaces reached equal to or above 80% mortality on days three to four, indicating that the residual efficacy of clothianidin is gradually decreasing compared to the first four months of spraying, whereby all exposed mosquitoes died within 48 hours following insecticide exposure (see graph below). The situation in Unguja was different as the 97% - 100% mosquito mortality at all wall surfaces reached with 24 hrs, indicating the high toxicity of insecticide to susceptible malaria vectors.

Figure 1 : Residual Efficacy of Sumishield 50WG in Treated Walls in Pemba

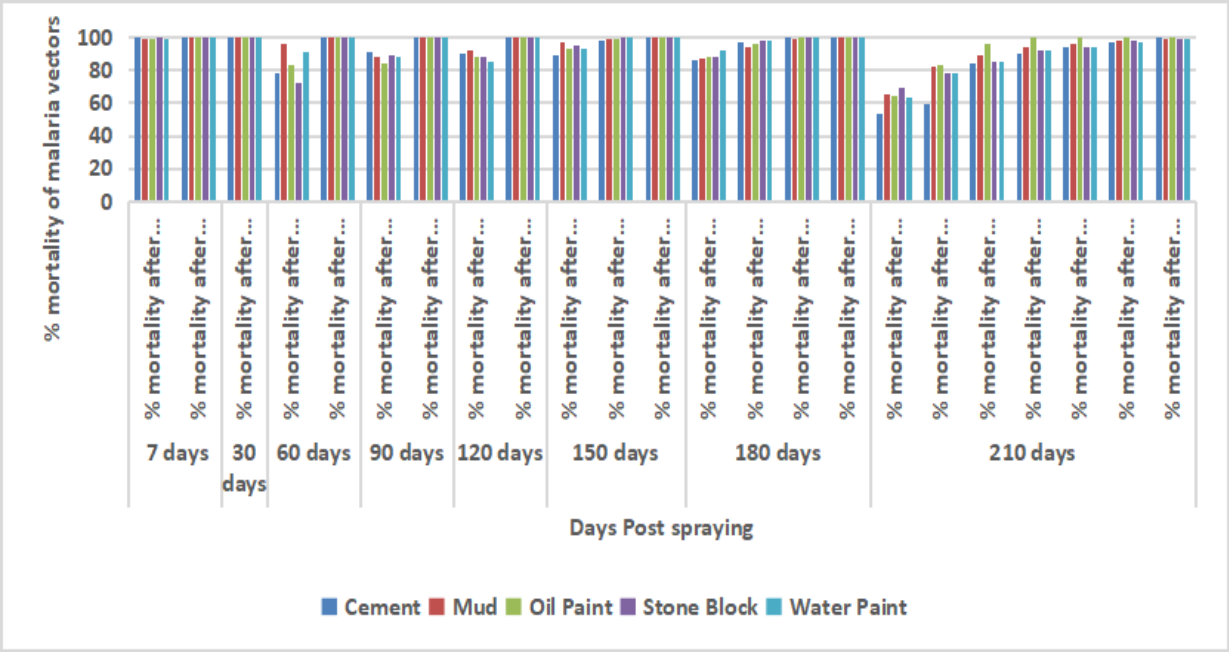
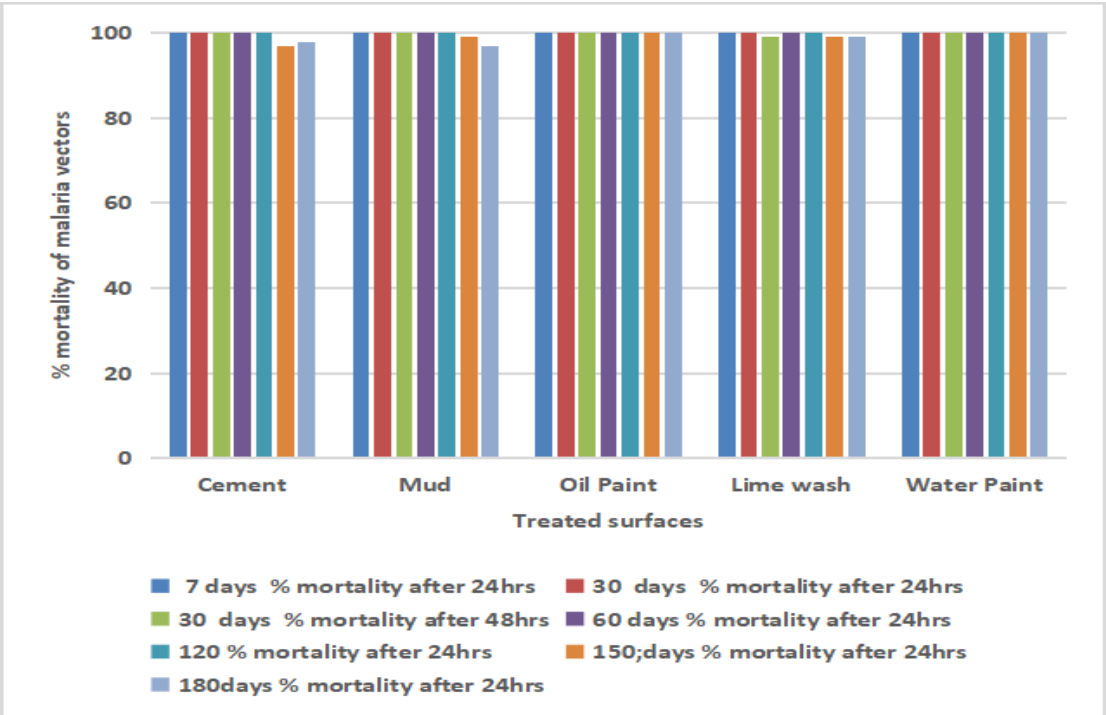


Figure 2 : Residual Efficacy of Sumishield 50WG in Treated Walls in Unguja



3.2 Malaria Vector ecology in Zanzibar

Malaria vector abundance, distribution, Seasonality and species composition

The Pemba team collected 1,652 female *Anopheles mosquitoes* from October 2020 to September 2021 across four sentinel sites. Of the total females' *Anopheles* vectors collected,1430(87%) were morphologically identified as *Anopheles gambiae s.l*, 205(12%) were *Anopheles funestus* group, and 1% (n = 17) were a combination of *An. coustani, An. rufipes and An. pretoriensis* (see tables below). The team in Unguja collected 522 female *Anopheles mosquitoes* across the six sentinel sites with 100% morphologically identified as *Anopheles gambiae s.l*
Anopheles gambiae s.l was the most abundant species sampled by all collection methods at all ten (10) sentinel sites in Zanzibar.
Human landing catch was the most efficient method by collecting 53.5% (n = 884) and 49%(n= 248) of the total mosquitoes in Pemba and Unguja relatively.

Table 3 : Number of *An gambiae s.l* and *An funestus s.l* by location and collection methods

Location			Human landing Catch		Pyrethrum spray catch		CDC Light trap		Pit trap		Total	
Zone	**District**	**Sentinel site**	***An gambiae s.l(n)***	***An funestus s.l(n)***	***An gambiae s.l(n)***	***An funestus s.l(n)***	***An gambiae s.l(n)***	***An funestus s.l(n)***	***An gambiae s.l(n)***	***An funestus s.l(n)***	***An gambiae s.l(n)***	***An funestus s.l(n)***
Sprayed sites												
Pemba	Micheweni	Tumbe	204	43	198	25	4	1	194	56	600	125
	Mkoani	Wamba a	122	4	3	1	1	0	32	12	158	17
Total			**326**	**47**	**201**	**26**	**5**	**1**	**226**	**68**	**758**	**142**
Unsprayed sites												
Pemba	Wete	Bopwe	451	30	11	2	5	0	61	11	528	43
	Chake	Uwandani	25	2	9	3	1	0	109	15	144	20
Total			**476**	**32**	**20**	**5**	**6**	**0**	**170**	**26**	**672**	**63**
Sprayed site												
Unguja	Kaskazini B	Bumbwini	13	0	0	0	0	0	0	0	13	0
Total			**13**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**13**	**0**
Unsprayed sites												
Unguja	Kati	Cheju	180	0	0	0	0	0	0	0	180	0
	Kusini	Muyuni	1	0	0	0	0	0	0	0	1	0
	Mjini	Stone town	3	0	0	0	0	0	0	0	3	0
	Kaskazini A	Donge	41	0	1	0	0	0	0	0	42	0
	Magharibi	Mwera	33	0	2	0	0	0	248	0	283	0
Total			**258**	**0**	**3**	**0**	**0**	**0**	**248**	**0**	**509**	**0**

Table 4 : Number of other females *Anopheles mosquitoes* collected from Pemba sentinel sites

Location	District	Sentinel site	An coustani	An rufipes	An pretoriensis
Pemba	Micheweni	Tumbe	9	4	1
	Wete	Bopwe	2	0	0
	Mkoani	Wambaa	0	0	0
	Chake Chake	Uwandani	1	0	0
Total			**12**	**4**	**1**

Molecular identification of An gambiae s.l and An funestus group

ZAMEP PCR facility conducted molecular identification of 1434 samples from Pemba sentinel sites. Of these, *An arabiensis* was predominant by accounting *82%(* n = 1171) followed by *An leesoni* 11% (n = 157*), An merus* 5% (n = 75) *and An. parensis and An rivulorum* 25% (n = 31).
In Unguja,434 mosquito samples were analysed to molecular species level. Of these,76%(n = 328) were *Anopheles arabiensis,* 13(n = 57) *Anopheles leesoni*,3.4% (n =15) *Anopheles merus*,2% (n = 9) *Anopheles rivulorum*, 0.23% (n = 1) *Anopheles gambiae s.s* and 5.2% (n = 23) samples did not amplify.

Table 5 : PCR Species identification of *An gambiae s.l and An funestus s.l* in Zanzibar

Location			# tested	An gambiae s.s(n) %	An arabiensis (n) %	An merus(n)%	An funestus s.s(n) %	An leesoni (n)%	An rivuloru m(n)%	An parens is(n)%	An veenedin (n)%	Unampl ified
Zone	District	Sentinel site										
Sprayed sites												
Pemba	Micheweni	Tumbe	599	0	446 (74)	44 (7)	0(0)	85(14)	21(3.5)	3(0.5)	0(0)	0
	Mkoani	Wambaa	154	0	118(77)	19(12)	0(0)	15(10)	2(1.3)	0(0)	0(0)	0
Total			753	0	604(80)	113(15)	0(0)	100(13)	23(3.0)	3(0.4)	0(0)	0
Unsprayed sites												
Pemba	Wete	Bopwe	537	0	486(91)	9(1.5)	0(0)	40(7)	1(1.2)	1(1.2)	0(0)	0
	Chake	Uwandani	144	0	121(84)	3(2)	0(0)	17(12)	2 (1.4)	1(0.7)	0(0)	0
Total			681	0	607(89)	12(1.8)	0(0)	57(8.4)	3(0.4)	2(0.3)	0(0)	0
Sprayed site												
Unguja	Kaskazini B	Bumbwini	12	0	7(58)	1(8)	0(0)	3(25)	0	0		1(8)
Total			12	0	7(58)	1(8)	0(0)	3(25)	0	0		1(8)
Unsprayed sites												
Unguja	Kati	Cheju	141	0	130(92)	5(3.5)	0(0)	6(4)	0	0	0	0
	Kusini	Muyuni	1	0	0	0	0(0)	1(100)	0	0	0	0
	Mjini	Stone town	2	0	2(100)	0	0(0)	0	0	0	0	0
	Kaskazini A	Donge	41	1(2.4)	30(73)	6(15)	0(0)	4(10)	0	0	0	0
	Magharibi	Mwera	236	0	159 (67)	3(1.3)	0(0)	43(18)	9 (4)	0	0	22(9)
Total			421	1(0.2)	294(70)	9(2)	0(0)	54(13)	9(2)	0	0	22(5)

Mosquito sporozoite rate

Mosquito infectivity was estimated by calculating the sporozoite rate (i.e., the proportion of mosquitoes in a population harbouring sporozoite in their salivary glands). PCR facility screened 1,582 *Anopheles* vectors (521 from Unguja and 1099 from Pemba) for the presence of sporozoite. The overall sporozoite rate was 0.38%(n=2) in Unguja and 0.9%(n =10) in Pemba, varying from sentinel sites. In Unguja, the vector infection rate ranged from 0% to 0.65%, with Cheju leading (0.65%) followed by Mwera with 0.37%. The other four sentinel sites reported zero infection rate.

In Pemba sentinel sites, the sporozoite rate ranged from 0% to 1.4%. Bopwe had a higher mosquito infection rate (1.4%) followed by Wambaa (1.4%), Tumbe (0.69%) and Uwandani with zero per cent. (Note that Bopwe did not receive IRS in last operation, February 2021).

Table 6 : Malaria vector infection rate per sentinel site in Zanzibar 2020/2021

Location	Sentinel site	# of vectors tested	# of positive sporozoite	sporozoite rate %
Pemba	Tumbe	582	4	0.69
	Bopwe	277	4	1.44
	Wambaa	141	2	1.42
	Uwandani	99	0	0.00
Unguja	Bumbwini	12	0	0.00
	Donge	42	0	0.00
	Mwera	273	1	0.37
	Muyuni	1	0	0.00
	stone town	3	0	0.00
	Cheju	153	1	0.65

Further analysis in Pemba indicated that *Anopheles merus* had higher infection rate 7.5%(n = 3) followed by *Anopheles leesoni* (n = 1) and *Anopheles arabiensis 0.6%* (n = 6). In Unguja, *Anopheles leesoni* had higher infection rate 1.75%(n = 1) followed by *Anopheles arabiensis* 0.33% (n = 1)

Table 7 : Sporozoite results by mosquito species in Zanzibar 2020/2021

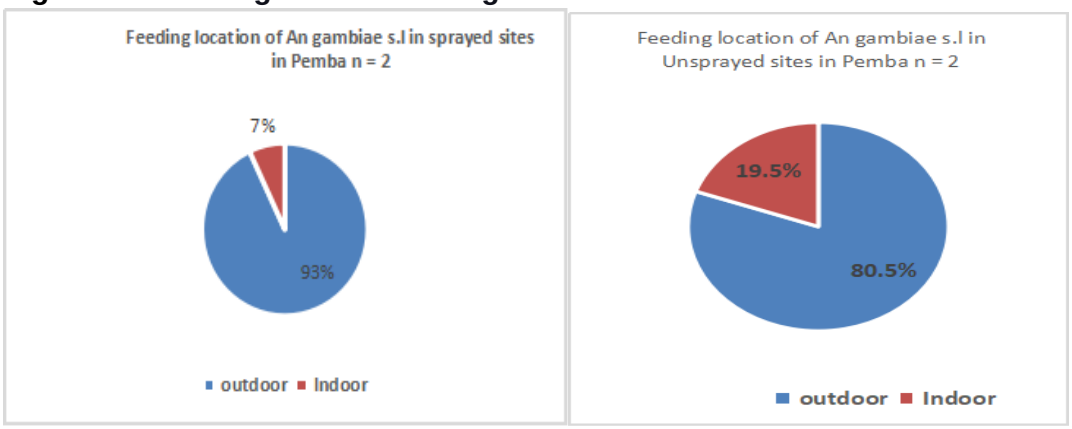
Location	Mosquito species	# tested	# of positive sporozoite	sporozoite rate %
Pemba	*Anopheles merus*	40	3	7.50
	Anopheles leesoni	76	1	1.32
	Anopheles arabiensis	970	6	0.62
	Anopheles rivulorum	8	0	0.00
	An parensis	3	0	0.00
	Under process	198	0	0.00
Unguja	*Anopheles arabiensis*	304	1	0.33

	Anopheles leesoni	57	1	1.75
	Anopheles merus	15	0	0.00
	Anopheles rivulorum	9	0	0.00
	Under process	74	0	0.00
	Not amplified	23	0	0.00
	Anopheles gambiae s.s	1	0	0.00

Feeding location of Anopheles gambiae s.l as expressed by Human Landing catch

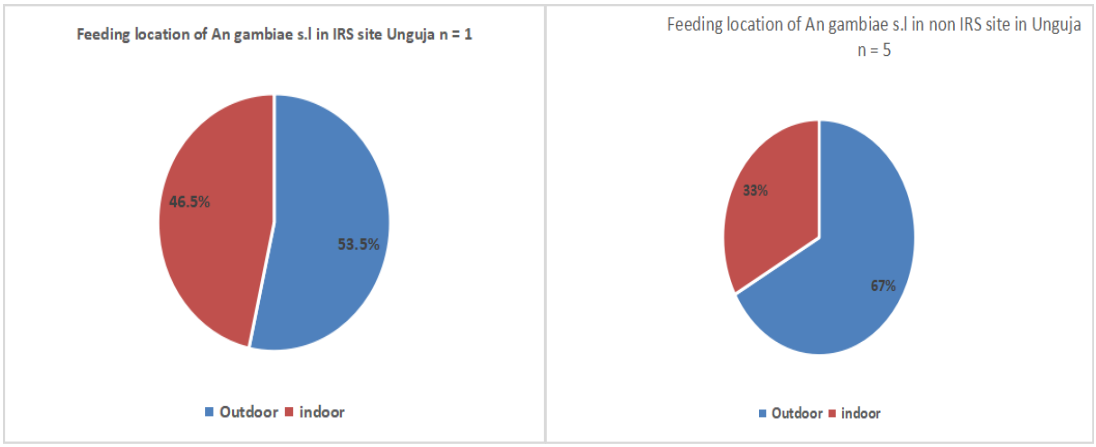
The general feeding location of *An gambiae s.l* was out of the door in Unguja and Pemba, regardless of the spraying status of sentinel sites. In Tumbe and wambaa IRS treated shehia, The team recorded 93% (n = 262) of *An gambiae s.l* to feed out of the door with many of them before midnight (n = 188). In Unsprayed sites, Uwandani and Bopwe 80.5%(n = 188) of *An gambiae s.l* fed out of the door. However, the indoor biting density was significantly higher compared to IRS received sites.

Figure 3 : Feeding location of An gambiae s.l in Pemba



In IRS received site Bumbwini, the team recorded 53.5%(n = 5) of *An gambiae s.l* feeding outdoor with many of them before midnight n = 4). In unsprayed sites(stone town, Cheju, mwera, Donge and Muyuni), the feeding location of *An gambiae s.l* was 67%(n =151) and 73% (n = 71) out of the door.

Figure 4 : Feeding location of An gambiae s.l in Unguja



Anopheles gambiae s.l biting rates as expressed by Human landing catch

The bites of *An gambiae s.l* was higher outdoor in both unsprayed and sprayed sites. We observed the peak bites during the wet season(March-May and October - December). In comparing, the unsprayed vs IRS sentinel sites in Pemba. The Unsprayed sites had almost one (1) indoor bite/person/night in April - June than the sprayed which had 0.2bites. In Unguja, the situation was different, the indoor biting of *An gambiae s.l* was higher in IRS compared to the non-IRS sites

Figure 5 : *An gambiae s.l biting rate in* Sprayed areas vs Unsprayed sites in Unguja

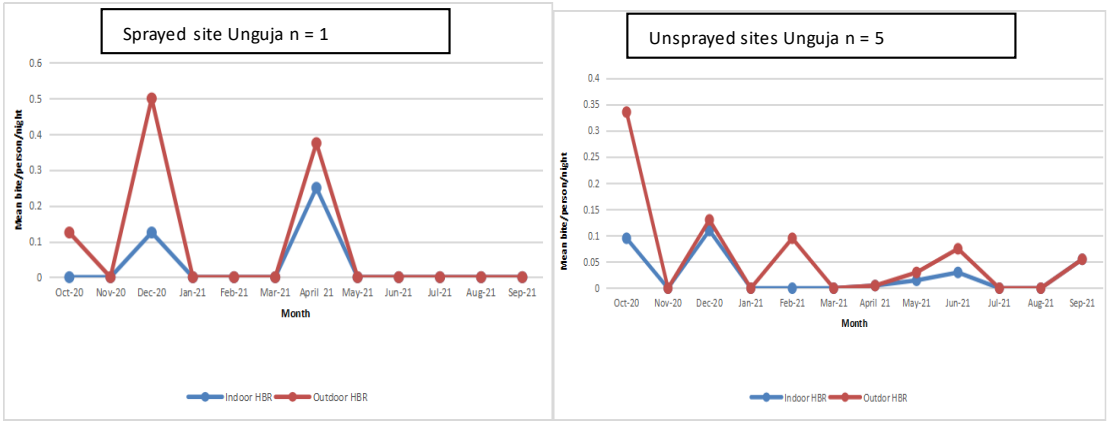
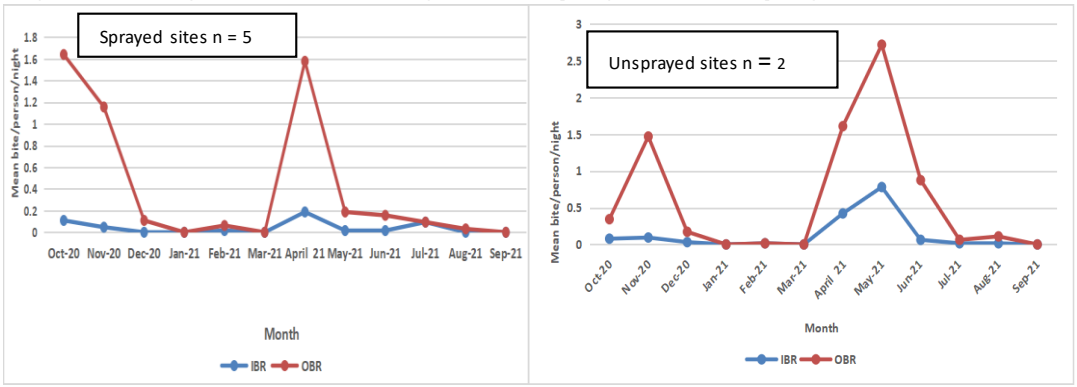


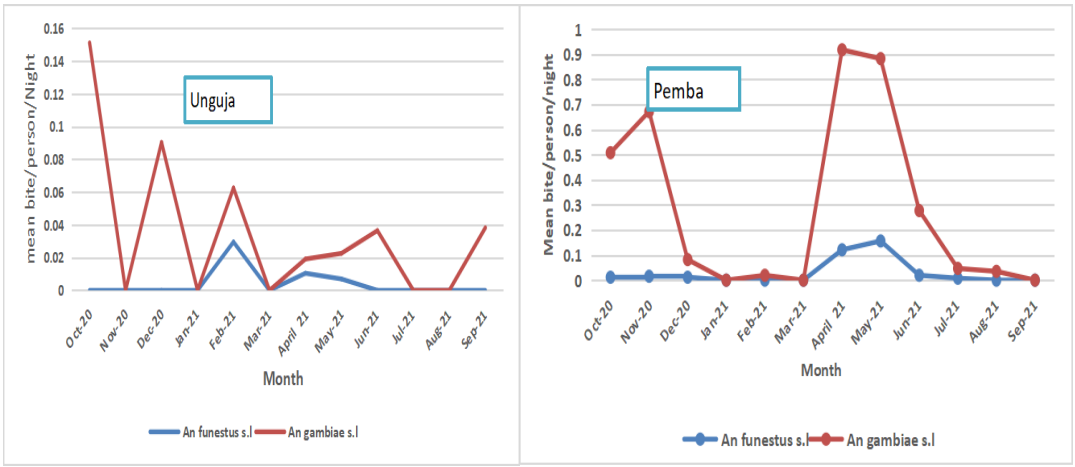
Figure 6 :*An gambiae s.l* biting rate in Sprayed Vs Unsprayed sites in Pemba



Malaria vector seasonality in Zanzibar

This indicator describes how the vector population changes over time might be due to interventions or climatic changes. These population changes were measured using human landing catch to assess the average bites/man/night. *Anopheles gambiae s.l* was dominant in both dry and wet season. However, *Anopheles funestus s.l* bites was also available in small percentage in rain seasons.

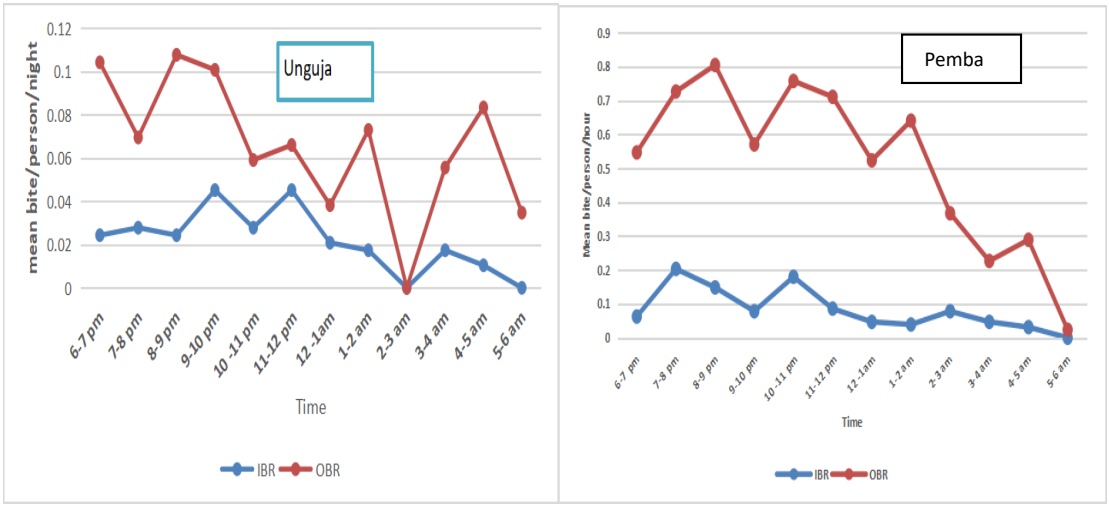
Figure 7 : Malaria vector seasonality in Zanzibar



Hourly biting rate of An gambiae s.l in Zanzibar

In Zanzibar, the team noted the peak biting rate *of An gambiae s.l* at the earlier time between 18hrs to 24hhrs both indoor and outdoor. However, the outdoor biting of the vectors was significantly higher compared to indoor. The average bites/man in Pemba was over five times higher than in Unguja.

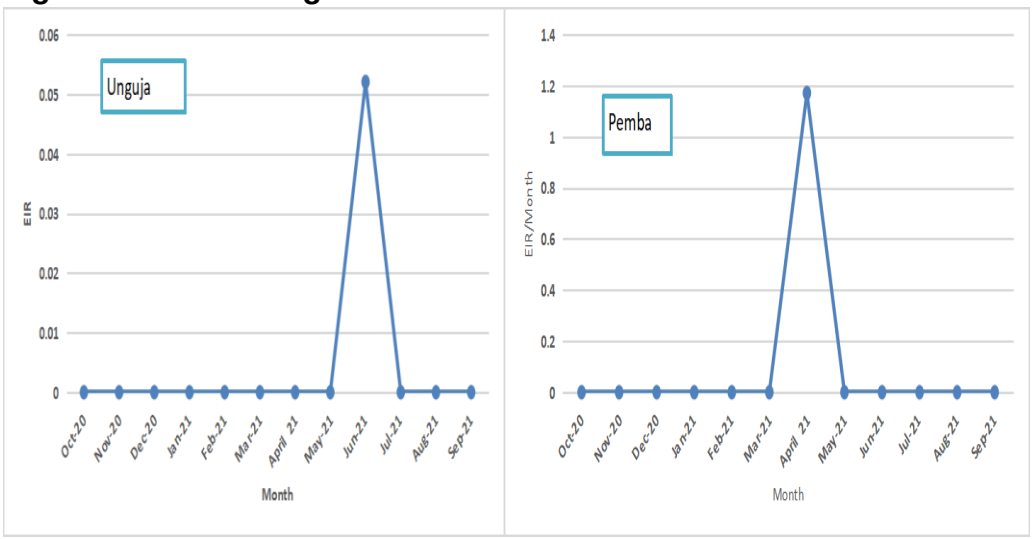
Figure 8 : Hourly biting rate rate of *An gambiae* s.l in Zanzibar



Entomological inoculation rate in Zanzibar 2020/2021

The purpose was to determine mosquito infectivity by calculating the sporozoite rate (i.e., the proportion of mosquitoes in a population harbouring infective sporozoites in their salivary glands). sporozoite detection is necessary to determine the entomological inoculation rate (EIR), which describes the number of infectious bites an individual is exposed to in a given time period (typically a year or transmission season). The entomological inoculation rate in Unguja and Pemba ranges from 0.38 to 1 relatively.

Figure 9 : Entomological inoculation rate in Zanzibar



3.3 Insecticide resistance monitoring of malaria vectors in Zanzibar 2021

Susceptibility test of An gambiae s.l in Zanzibar

The results of the WHO susceptibility tests indicated that *Anopheles gambiae* s.l. from sprayed and non-sprayed sentinel sites in Pemba and Unguja were fully susceptible to Pirimiphos-methyl, clothianidin and Bendiocarb. However, the vectors were found to be resistant to Permethrin, deltamethrin, and alpha cypermethrin across all the tested sites.(see the table below)

Table 8 : Susceptibility status(Percentage Mortality) of *Anopheles gambiae s.l* to WHO discriminating concentration of insecticide

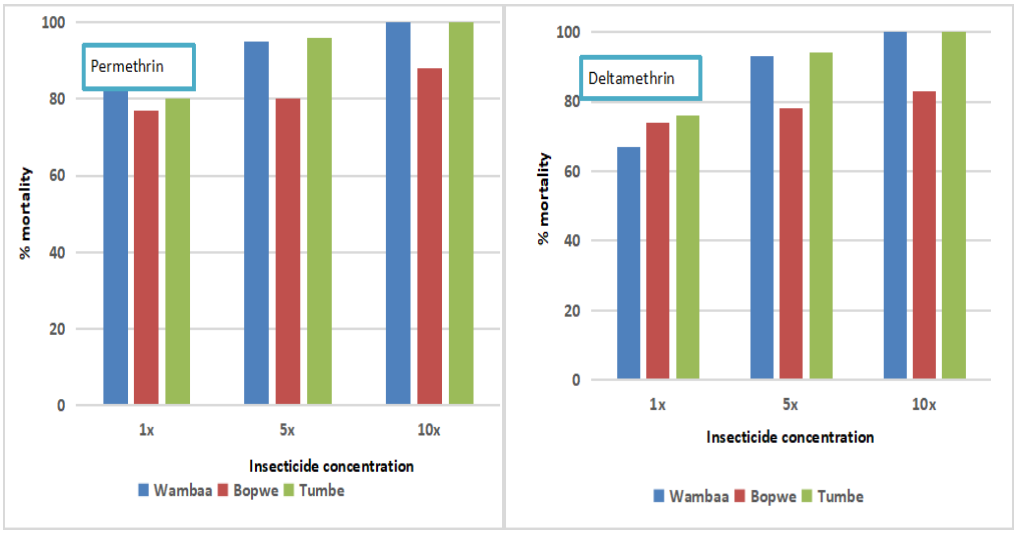
Location	District	sentinel site	Permethrin	Alpha cypermethrin	Deltamethrin	L/cyhalothrin	Bendiocarb	Pirimiphos methyl	Clothianidin
Pemba	Wete	Bopwe	74	9	77	56	100	100	100
	Chake	Uwandani	72	49	62	51	100	100	100
	Mkaoni	Wambaa	86	30	69	68	100	100	100
	Michweni	Tumbe	76	56	80	66	100	100	100
Unguja	Kati	Cheju	85	81	Not tested	Not tested	100	100	100
	Kusini	Muyuni	82	74	Not tested	Not tested	100	100	100
	Mjini	Stone town	94	93	Not tested	Not tested	100	100	100
	Kaskazini A	Donge	87	74	Not tested	Not tested	100	100	100
	Magharibi	Mwera	98	86	Not tested	Not tested	100	99	100
	Kaskazini B	Bumbwini	85	83	Not tested	Not tested	100	100	100

Resistance | Possible resistance | Susceptible

The strength of Pyrethroid resistance against An gambiae s.l in Pemba

Due to the shortage of *Anopheles* larvae during the experiment, the team prioritize the Permethrin and Deltamethrin testing as they are widely used in LLINs(Olyset and Permanet). The results indicated that the strength of Pyrethroid resistance varies across the sites, and the vectors are capable of surviving up to 10x concentration (see the figure below). The intensity of resistance ranges from low, moderate and high.

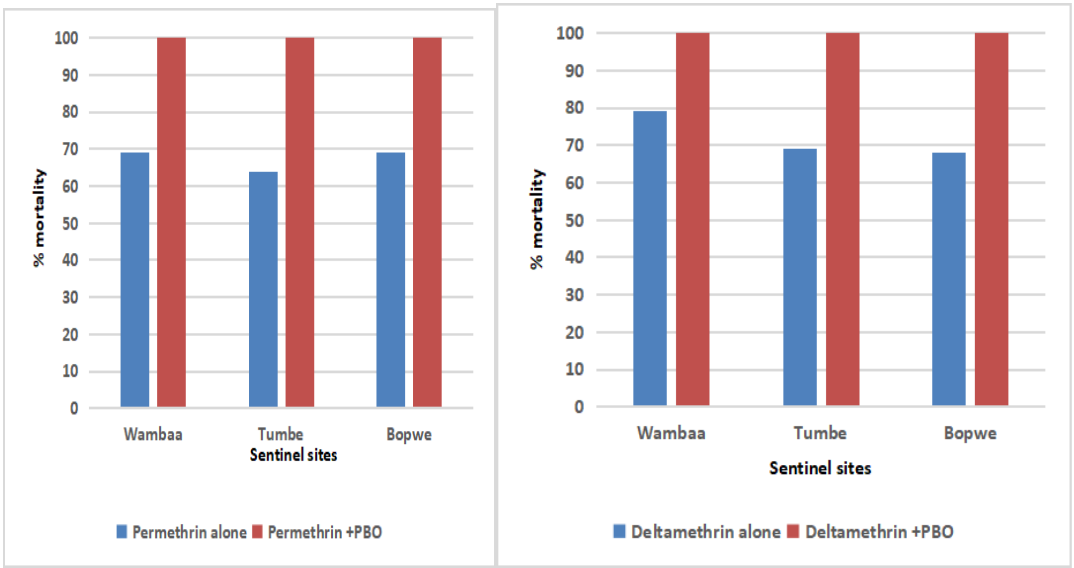
Figure 10: Intensity of Deltamethrin and Permethrin resistance in Pemba



PBO Synergist bioassay test in Pemba

Malaria vectors were resistant to 1x, 5x and even 10 x of permethrin and deltamethrin. However, the resistance level went to zero per cent at 1x when the mosquitoes were treated with PBO then exposed to the insecticides.

Figure 11: Response of *An. gambiae* s.l exposed to Deltamethrin and Permethrin after preexposure to PBO in Pemba



4.0 Discussion

4.1 Residual efficacy of Clothianidin in treated walls

The average mosquito mortality for the wall cone bioassays in tested areas is still effective (≥80% mortality) against susceptible *Anopheles gambiae s.s* R70 at the age of eight months. However, in Pemba, all wall surfaces reached equal to or above 80% mortality on days three to four, indicating that the residual efficacy of clothianidin is gradually decreasing compared to the first four months of spraying, whereby all exposed mosquitoes died within 48 hours following insecticide exposure.

The insecticide is promising for malaria vector control as long as the mosquitoes are endophagic and endophilic. However, lack of repellent effect and delaying mortality against vectors might bring poor community perception.

4.2 Species composition, behavior and vector distributions

The molecular identification of malaria vectors has identified *An. Arabiensis* as the predominant malaria vector during the wet and dry seasons in both islands. The An. *gambiae* s.l abundance is dependent on mean rainfall, particularly in October - December and April - June, suggesting that the risk of malaria transmission is dependent on rainfall patterns. This highlight the critical time for the programme to apply the vector control interventions, including IRS and, LLINs to interrupt malaria transmission and reduce receptivity.

The exophagic (outdoor feeding) behavior of *An gambiae s.l* has increased significantly in IRS received sites due to sumishield or high coverage of long-lasting insecticide nets. Indoor biting density/man of malaria vectors has increased in non received IRS sites. It gives a descriptive clue that once you stop IRS, endophagic and endophilic vectors will return.

Early biting behaviour of malaria vectors is likely to affect young people, particularly in urban and semi-urban following social interaction activities, including watching football.

We assume the outdoor biting behaviour of *An gambiae s.l* is due to IRS, genetic make up and LLINs. However, they reduce the effectiveness of vector control interventions and therefore, complementary methods to fight outdoor malaria vectors are necessary to increase efficiency towards elimination.

4.3 Sporozoite rate and Entomological inoculation rate

The overall sporozoite rate was 0.38%(n=2) in Unguja and 0.9%(n =10) in Pemba, varying from sentinel sites with an average of less than one (1) Entomological inoculation rate (Pemba = 1.2 and 0.38 in Unguja). EIR corresponds to a few indigenous cases in Zanzibar. Despite the higher EIR in

Pemba, it has few local malaria cases compared to Unguja. The findings suggest the presence of local malaria cases

4.4 Insecticide resistance

Pyrethroid resistance in Zanzibar is still stable, focusing on *An arabiensis*. However, they were full susceptible to clothianidin, pirimiphos-methyl and Bendiocarb. The strength of Deltamethrin and Permethrin resistance to *An gambiae s.l* is not homogeneous across the sites, and it ranges from low, moderate to high.

Complete restoration of Deltamethrin and Permethrin susceptibility (mitigation of resistance) after exposing mosquitoes to PBO implies that a monooxygenase-based resistance mechanism fully accounts in the vector population. This finding suggests that the PBO nets within the community could have additional value in malaria protection.

Despite the pyrethroid resistance being stable for a long time, we are confident that there is no indication of disease control failure. Possibly the resistance phenotype is less frequent among the old mosquitoes, which are mature enough to transmit malaria parasites, than among younger mosquitoes.