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THE PMI VECTORLINK PROJECT

ZIMBABWE ANNUAL ENTOMOLOGICAL REPORT

MARCH 2021 – FEBRUARY 2022

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ACRONYMS

AChE	Acetylcholinesterase
iAChE	Insensitive Acetylcholinesterase
AU	Africa University
CDC	U.S. Centers for Disease Control and Prevention
CSP	Circumsporozite protein
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FF	Fludora®Fusion
HLC	Human Landing Catch
IRS	Indoor Residual Spraying
ITN	Insecticide-treated Nets
kdr	knockdown resistance
NIHR	National Institute of Health Research
NMCP	National Malaria Control Program
PCR	Polymerase Chain Reaction
PMI	President's Malaria Initiative
PPA	Prokopack Aspirator
PSC	Pyrethrum Spray Catch
s.l.	*Sensu lato* (in the broad sense)
s.s.	*Sensu stricto* (in the narrow sense)
SOP	Standard Operating Procedure
USAID	United States Agency for International Development
VGSC	Voltage-Gated Sodium Channel
WHO	World Health Organization

EXECUTIVE SUMMARY

With technical support from the U.S. President's Malaria Initiative (PMI), Zimbabwe's National Malaria Control Program (NMCP) implemented indoor residual spraying (IRS) with Fludora® Fusion in Mudzi District and with DDT in Mutoko District in Mashonaland East Province in 2021. During the 2021 IRS campaign, the province sprayed the sentinel site areas in October in Mudzi and November in Mutoko.

PMI VectorLink is implementing entomological monitoring for malaria vector control in Zimbabwe, in partnership with the National Institute of Health Research, NMCP, and Provincial Medical Directorates. Monthly longitudinal vector surveillance was conducted at three sites in Mashonaland East Province, namely Dendera, Kawere, and Makarara, and monitored briefly at Burma Valley in Manicaland Province. The project stopped routine entomological surveillance at Burma Valley after March 2021 due to the limited budget for the financial year. The residual efficacy of Fludora® Fusion and DDT was monitored at the PMI-supported districts in Mashonaland East. In addition, insecticide resistance tests were conducted at Dendera, Kawere, and Makarara in Mashonaland East (deltamethrin, clothianidin, pirimiphos-methyl, permethrin, alpha-cypermethrin, chlorfenapyr, and DDT) and at Burma Valley in Manicaland (deltamethrin and clothianidin). The project prioritized alpha-cypermethrin in resistance monitoring at Makarara (non-IRS site), where NMCP distributed pyrethroid-only long-lasting insecticidal nets treated with this insecticide.

Anopheles funestus s.l. and *An. gambiae* s.l. are the main malaria vectors in Zimbabwe. *An. funestus* s.l. was the predominant species at two of the three longitudinal monitoring sites, Kawere and Makarara, whereas *An. rufipes* was predominant at Dendera, followed by *An. pretoriensis*. *An. rufipes* is a potential secondary malaria vector whereas *An. pretoriensis* is a non-vector. *An. gambiae* s.l. and *An. rufipes* were the second most abundant species at Kawere and Makarara, respectively. All species were found in low densities possibly due to the vector control interventions in place and erratic rains that affect mosquito breeding sites. Routine monitoring was disrupted briefly in July 2021 due to COVID-19.

The presence of *An. funestus* s.l. sibling species varied at the three sites as follows: only *An. parensis* at Dendera; *An. funestus* s.s., *An. leesoni*, *An. parensis,* and *An. rivulorum*-like at Makarara; and *An. leesoni, An. parensis,* and *An. rivulorum*-like at Kawere. Two *An. gambiae* s.l. sibling species were recorded at all three longitudinal monitoring sites: *An. arabiensis* and *An. quadriannulatus*. Potential secondary vectors found occurring in low numbers included *An. rufipes, An. maculipalpis, An. coustani,* and *An. squamosus*. However, their propensity to feed on humans was low. *An. pretoriensis,* found at all sites and considered a non-vector, showed no tendency to feed on humans except for one specimen from Makarara.

An. gambiae s.l. (*An. arabiensis* and *An. quadriannulatus*) was the predominant species complex at Burma Valley, constituting 51% of the 39 specimens collected in March. *An. funestus* s.l. (*An. leesoni* and *An. parensis*) and *An. rufipes* were also present in March.

Mosquito densities at all sentinel sites in Mashonaland East and Manicaland using all collection methods were generally low, which did not allow for definitive conclusions to be made about vector behavior. However, U.S. Centers for Disease Control and Prevention (CDC) light trap collections and pit shelters indicated higher numbers of malaria vectors (both *An. funestus* s.l. and *An. gambiae* s.l.) collected outdoors compared to indoors at all sites and might indicate a preference to feed and rest outdoors. The low human blood index in both the two main vectors and in other species suggests an opportunistic feeding behavior. It is significant to note that the only two specimens infected with *Plasmodium* parasites—*An. funestus* s.s. and *An. parensis*-query —were collected resting outdoors. Note that all reference to *An. parensis* in the report means it the specimens amplified as 2-banded (rather than the diagnostic single-band). CDC is investigating.

Wall bioassays conducted monthly following the 2020 IRS campaign at two sites in Mashonaland East showed a residual efficacy of Fludora® Fusion of at least 10 months. DDT (sprayed by the NMCP) was monitored at

\Burma Valley in Manicaland and showed residual efficacy of up to nine months. Residual efficacy varied for DDT by wall surface type, with greater residual efficacy on mud and brick walls than on cement and painted walls. Residual efficacy of Fludora® Fusion was less varied on the four wall types in the observations completed.

The results from the 2021 spray campaign showed that the residual efficacy of Fludora® Fusion at Dendera and of DDT at Kawere was still above the 80% cut-off point four and three months after spraying, respectively. The team will continue monitoring the two insecticides until mosquito mortality drops below 80% for two consecutive months.

The primary vector *An. gambiae* s.l. remains susceptible to deltamethrin, clothianidin, alpha-cypermethrin, chlorfenapyr, and DDT at the sites tested. In Mashonaland East, *An. gambiae* s.l. was susceptible to clothianidin, DDT, pirimiphos-methyl, and deltamethrin in Mudzi and Mutoko districts, with possible resistance to permethrin in Mudzi, and pirimiphos-methyl in Mutoko. The observations apply to *An. gambiae* s.l. prior to species identification in the laboratory. Laboratory tests indicated absence of knockdown resistance (*kdr*) East alleles, but low frequency (1.1%; n=550) of *kdr* West resistant heterozygotes in *An. gambiae* s.l., specifically *An. quadriannulatus* from Kawere. Low frequency (2.0%; n=583) insensitive acetylcholinesterase (*Ace*-1) mutation was observed in *An. gambiae* s.l. (also *An. quadriannulatus*) from Dendera and Kawere. Molecular species identification of the *An. gambiae* s.l. included in the susceptibility tests, indicates that the proportion of the vector *An. arabiensis* was low, ranging from 0 to 15% while the non-vector *An. quadriannulatus* was dominant. *An. arabiensis* was absent at Marange. This observation underlines the value of laboratory analysis when monitoring insecticide resistance in local malaria vectors. Susceptibility tests will be extended to localities where *An. arabiensis* is predominant and to *An. rufipes* since this potential vector species is abundant in some areas such as Dendera. There is need to test the insecticide resistance status of *An. funestus* s.l. in its geographical range including Kawere where it is now the predominant species.

I. INTRODUCTION

Malaria is heterogeneously distributed in Zimbabwe, with most cases reported from three of the eight rural provinces: Manicaland, Mashonaland Central, and Mashonaland East. In Zimbabwe, malaria is transmitted by *Anopheles arabiensis*, *An. gambiae* s.s., and *An. funestus* s.s. More than 98% of cases are caused by *Plasmodium falciparum*, while *Plasmodium malariae* and *Plasmodium ovale* are responsible for the remainder. Following concerted efforts by stakeholders to prevent and control malaria transmission, the disease burden decreased from 32/1000 population in 2020 to 9/1000 in 2021 (NMCP 2022). Malaria remains one of the most important public health challenges in some parts of the country. To control malaria, Zimbabwe's National Malaria Control Program (NMCP) relies on two core vector control strategies: deployment of indoor residual house-spraying (IRS) and distribution of insecticide-treated nets (ITNs). Though these two strategies are known to contribute immensely to the prevention, control, and elimination of malaria in most settings, their effectiveness depends greatly on monitoring the behavior and insecticide resistance status of local primary vectors as well as human behavior. The country's Insecticide Resistance Management Plan guides the program to rotate insecticides for IRS every two years although compliance is a challenge often.

Regular entomological monitoring is important for evidenced-based selection and deployment of insecticides for IRS and ITNs because monitoring provides timely key information on vector species composition and their distribution, resting and feeding behavior, and susceptibility to insecticides. Additionally, these investigations are helpful for monitoring emerging and re-emerging primary and secondary malaria vectors and assessing the role they play in disease transmission. Information collected through entomological monitoring helps program managers and implementers understand the spatial and temporal changes in vector species, quality of IRS application, residual efficacy of insecticides on sprayed surfaces, and effectiveness of vector control interventions deployed to interrupt malaria transmission.

The U.S. President's Malaria Initiative (PMI) supported IRS and entomological surveillance under the Africa Indoor Residual Spraying Project from 2013 to February 2018. This support continues as the PMI VectorLink project, which started in March 2018. Prior to 2018, PMI supported IRS in four districts in Manicaland Province (Chimanimani, Mutare, Mutasa, and Nyanga), but in 2018 transitioned support to two districts in Mashonaland East Province (Mudzi and Mutoko). This report focuses on activities completed from March 2021 to February 2022 under the PMI VectorLink Project. The objectives included the following:

1. Monitor spray quality and residual efficacy of Fludora® Fusion (clothianidin and deltamethrin combination for IRS) used in Mashonaland East in the 2020 IRS campaign and of Fludora® Fusion and dichlorodiphenyltrichloroethane (DDT) IRS used in the 2021 campaign.
2. Monitor spray quality and residual efficacy of DDT in Manicaland in the 2020 spray campaign implemented by the NMCP.
3. Perform annual insecticide susceptibility testing at four sites in Mashonaland East (three sites) and in Manicaland (one site) to inform vector control decision making.
4. Continue monthly vector bionomics monitoring at three sites in Mashonaland East to monitor the impact of IRS.

The project continued the collaboration with Africa University (AU), which provided support in analyzing mosquitoes to determine species identification, parasite infection (sporozoite rate), host choice, and target site resistance mechanisms. AU also provided mosquitoes from the susceptible colony of *An. arabiensis* KGB strain for bioassay tests to monitor the residual efficacy of insecticides. The National Institute of Health Research (NIHR) also provided the same strain of colony mosquitoes for the bioassay tests to a limited extent.

2. MATERIALS AND METHODS

2.1 SITES

Entomological surveillance was conducted initially in March 2021 at three sites in Mashonaland East Province (IRS sites of Dendera, and Kawere, and control site of Makarara) and one site in Manicaland Province (IRS site of Burma Valley). The Burma Valley site was dropped from April 2021 onward, following budget cuts in 2021. Because of the COVID-19-related national lockdown, routine activities were disrupted at Dendera and Kawere sites, and in February and July 2021 activities were not conducted at any of the three sites.

Insecticide susceptibility tests were conducted in both provinces. Wall bioassay tests were conducted to monitor residual efficacy of Fludora® Fusion in Mashonaland East, sprayed during the 2020 IRS campaign, and of Fludora® Fusion in Mudzi and DDT in Mutoko, following the 2021 IRS campaign. Activities accomplished are outlined in Table 1 at sites shown in the map in Figure 1.

TABLE 1. SENTINEL SITES BY GEOGRAPHIC LOCATIONS AND ACTIVITIES MARCH 2021-APRIL 2022*

Province	District	Site	VC** intervention	Ento activity	M	A	M	Jn	Ju	A	S	O	N	D	J	F	M	A
Manicaland	Mutare	Burma Valley***	2020 & 2021: IRS with DDT	VB	X													
				CB	X													
				IR												X	X	X
Mashonaland East	Mudzi	Dendera****	2020 & 2021: IRS with Fludora® Fusion	VB	X	X	X	X		X	X	X	X	X	X	X		
				CB	X	X	X	X		X	X	X		X	X	X		
				IR	X		X										X	X
	Mutoko	Kawere****	2020 IRS with Fludora® Fusion 2021: IRS with DDT	VB	X	X	X	X		X	X	X	X	X	X	X		
				CB	X	X	X	X		X	X	X		X	X	X		
				IR	X	X				X						X	X	X
	Hwedza	Makarara	2020 & 2021: ITNs	VB	X	X	X	X		X	X	X	X	X	X	X		
				IR	X	X	X											

LEGEND

Gray shading	Activity not done because of COVID-19	Orange shading	Activity terminated with new work plan	Blue shading	Challenge with availability of colony mosquitoes	X	Activity completed

Note:

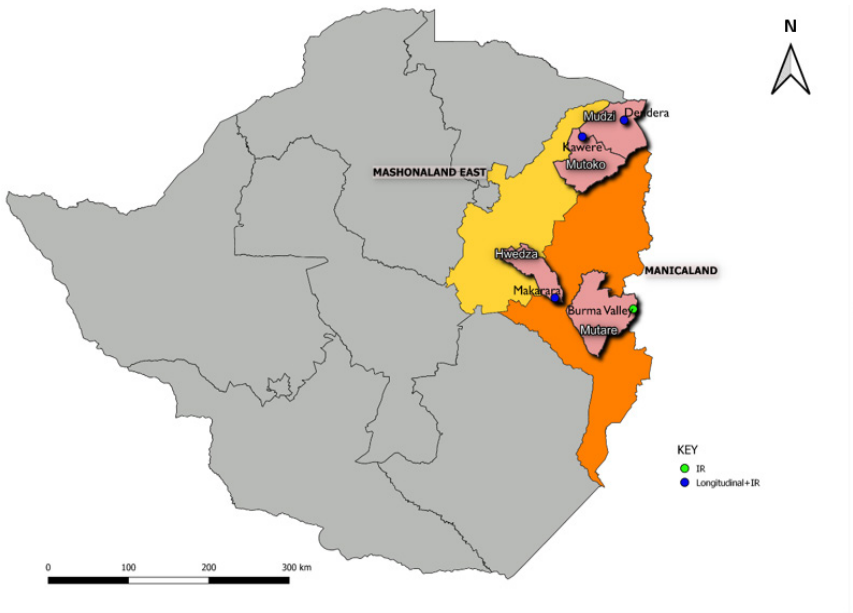
* IR tests are reported up to April 2022 (i.e. beyond the reporting period) to provide up-to-date information on resistance status to guide choice of insecticide for VC

**VC=vector control, IR=insecticide resistance testing, VB=vector bionomics, CB=cone bioassays

***Non-PMI supported spray district

****PMI-supported spray district in 2020 and 2021

FIGURE 1. MAP OF ZIMBABWE SENTINEL SITES, MARCH 2021-FEBRUARY 2022



2.2 ROUTINE VECTOR BIONOMICS MONITORING

Mosquito collections were done to monitor vector bionomics at sites in Mashonaland East Province (Dendera, Kawere, and Makarara) and in Manicaland (Burma Valley) for a single month (March 2021) within this reporting period. Pyrethrum spray catches (PSCs) or Prokopack aspirator collections (PPA) and pit shelters were used to assess indoor and outdoor resting site densities. U.S. Centers for Disease Control and Prevention (CDC) light traps were used as a proxy for human landing catches (HLCs) to assess indoor and outdoor human biting rates. Mosquitoes from all collections were used to assess sporozoite infection rates (Table 2).

TABLE 2. SUMMARY OF SAMPLING METHODS

Collection method	Collection time	Frequency	Sample
PSC*	6:00 am to 9:00 am	Two days per site per month	25 houses per site
PPA*	6:00 am to 9:00am	Two days per site per month	25 houses per site
CDC light trap as HLC proxy	6:00 pm to 6:00 am, hourly	Two nights per site per month	Two houses per site per night, using four CDC light traps: two indoor, two outdoor
CDC light trap	6:00 pm to 6:00 am collection	Two nights per site per month	Two houses per site (two houses per night), using four CDC light traps: two indoor, two outdoor
Pit shelters	6:00 am to 9:00 am	Two days per site per month	Five pits at each site

*PSC - method used from Mar to Jun 2021; PPA – method used from Aug 2021 to Feb 2022

All entomological monitoring at each sentinel site was conducted by teams consisting of staff from NIHR, the Provincial Medical Directorates, and PMI/VectorLink.

2.2.1 Estimating Indoor Resting Densities Using PSCs and PPAs

Indoor resting mosquitoes were sampled from 25 houses (1 sleeping room per house) per month at each of the vector bionomics monitoring collection sites following Standard Operating Procedures (SOP) #3 (PSC) and # 11 (PPA).[1] Mosquitoes collected from the different rooms were transferred to separate petri dishes. Each petri dish was labeled with the following information: location, household name, method of collection, and date. The abdominal stage of all female *Anopheles* was recorded as unfed, blood-fed, half-gravid, or gravid. Data on the number of people who slept in the house the previous night, the type of house and walls, and the number of ITNs present were recorded on appropriate forms. All *Anopheles* mosquitoes collected were identified morphologically and preserved individually in silica gel for laboratory analysis. The team used PSCs from March 2021 to June 2021, and PPAs from August 2021 to February 2022 following guidance from CDC. Routine activities were suspended in July due to COVID-19-related lockdown.

2.2.2 Estimating Outdoor Resting Densities Using Pit Shelter Collections

Outdoor resting mosquitoes were sampled using five pit shelters per site. The pit shelters were dug at least 10m away from nearest household and fenced for human and animal safety. Each pit shelter had a depth of 2m, a 1.5m x 1.5m opening, and at least eight holes (2 holes x 4 sides) 12-15 cm deep and about 10 cm wide on its walls for mosquitoes to rest. Mosquito sampling was conducted following SOP #13.[1] The abdominal stage of all female *Anopheles* mosquitoes was recorded. All *Anopheles* mosquitoes collected were identified morphologically and preserved in silica gel for laboratory analysis.

2.2.3 Estimating Indoor and Outdoor Densities Using CDC Light Traps

A total of four battery-operated CDC light traps per site per month were used to collect mosquitoes from 6:00 p.m. to 6:00 a.m. for two consecutive nights following SOP #1.[1] Two sentinel houses were randomly selected, with one trap placed indoors and one trap placed outdoors at each house. Households selected for PSC collections were excluded from the sampling pool. The same houses were used for collections throughout the reporting period. Both indoor and outdoor CDC light traps were suspended 1m above the ground next to a person sleeping under an untreated mosquito net. Outdoor CDC light traps were about 10m away from the house and, when possible, in a shaded area with a person sleeping under an untreated mosquito net. Traps were set at 6:00 p.m. and mosquitoes were collected from each of the traps at around 6:00 a.m. the following morning. The persons did not swap positions, from indoor to outdoor or vice versa, at hourly intervals due to COVID-19 regulations. All *Anopheles* mosquitoes collected were identified morphologically and preserved in silica gel for laboratory analysis.

2.2.4 Estimating Biting Time and Behavior Using CDC Light Traps

PMI VectorLink used CDC light traps with human bait as a proxy for HLCs to evaluate human-vector contact including the place, time, and seasonal activity of the vectors estimated through hourly mosquito collections from CDC light traps set indoor and outdoor alongside a human bait protected by a mosquito net. The procedure is a modification of SOP #1. Houses used for PSCs and CDC light traps for density estimation were excluded from the sample. Collections were done over two consecutive nights at each site. In both indoor and outdoor collections, the light trap was set at the feet of a volunteer sleeping under an untreated mosquito net. For outdoor placement, light traps were set about 10m from the house. The persons did not swap positions, from indoors to outdoors or vice versa, at hourly intervals due to COVID-19 regulations. Mosquitoes were collected from each trap hourly from 6:00 p.m. to 6:00 a.m. Mosquito collections were conducted indoors and outdoors simultaneously, using the same set-up, to compare vectors host-seeking activity inside and outside houses. The teams recorded temperature, relative humidity, wind status, and precipitation hourly during the

[1] Complete SOPs can be found here: https://pmivectorlink.org/resources/tools-and-innovations/

night. All *Anopheles* mosquitoes collected were identified morphologically and preserved in silica gel for laboratory analysis.

2.3 Measuring Quality of Spray and Residual Efficacy

The quality of IRS application and insecticide residual efficacy of clothianidin-deltamethrin combination IRS (Fludora® Fusion) in Mudzi and DDT in Mutoko was measured using SOP #9[1] following IRS at the two sites in October and November 2021, respectively. Bioassays were conducted within a week after spraying to assess the spray quality of the IRS operation and then monthly until the mean mortality rates fell below 80% for two consecutive months. Susceptible *An. arabiensis* (KGB strain), from insectaries at AU in Mutare and some from NIHR in Harare, were used to conduct the cone bioassays. Ten rooms were tested at each site per month. Additionally, the final monthly bioassays from the 2020 spray campaign were completed in this reporting period (before the 2021 IRS campaign started), and full results are presented in this report. The number of houses from each sentinel site by wall surface types, and insecticide sprayed are summarized in Table 3.

Table 3. Summary of Wall Types Tested with Cone Bioassays, March 2021-February 2022

Province	District	Sentinel site	Month Year sprayed	Insecticide sprayed	Type of wall	Number of houses
Mashonaland East	Mutoko	Kawere	November 2020	Fludora® Fusion	Mud	4
					Brick	1
					Cement	4
					Painted	1
			November 2021	DDT	Mud	1
					Brick	1
					Cement	5
					Painted	3
	Mudzi	Dendera	November 2020	Fludora® Fusion	Mud	2
					Brick	2
					Cement	3
					Painted	3
			October 2021	Fludora® Fusion	Mud	2
					Brick	2
					Cement	4
					Painted	2

2.3.1 Wall Cone Bioassay Tests

For the wall cone bioassays, 10 unfed, two- to five-day-old female susceptible *An. arabiensis* (KGB strain) mosquitoes were exposed on the treated walls per cone following SOP #9. Three cones were randomly positioned per room at 0.5, 1.0, and 1.5 meters above the floor, all on one wall but with different spray swaths. These positions were marked and used in all subsequent tests. Mosquitoes were exposed for 30 minutes, after which they were aspirated to a holding paper cup and provided with 10% sugar solution. Knockdown rates were also recorded at 30 minutes (inside the cones) and 60 minutes (30 minutes after removal from the cones). Mortality was recorded at 24 hours after exposure for DDT and up to 120 hours for Fludora® Fusion. Controls were run concurrently with the wall cone bioassays with mosquitoes exposed to unsprayed surfaces in an unsprayed room. Temperature and relative humidity were recorded hourly during the exposure and three times per day during the subsequent 24-hour holding period for DDT, and up to the 120-hour holding period for Fludora® Fusion. Clothianidin (a constituent active ingredient in Fludora® Fusion) is a slow-acting insecticide, hence the extended observation period.

2.3.2 Bioassay Tests to Assess Fumigant Effect of Insecticide

Bioassays to assess the fumigant effect of Fludora® Fusion were conducted in each room where wall cone bioassay tests were done. Ten two- to-five-day-old unfed female mosquitoes placed in one paper cup per room were exposed for 30 minutes at the same time as the wall bioassay tests. The paper cup was held by a wire support, designed so it was 10 cm away from a sprayed wall and 1m above the floor. Mosquitoes were removed after 30 minutes, and knockdown recorded. They were then transferred to holding paper cups using a clean aspirator and provided with 10% sugar solution during the holding period. Mortality was recorded up to 120 hours. Controls for the bioassays were conducted simultaneously using a similar set-up, but in an unsprayed room. The fumigant effect was not determined for DDT since the insecticide is not known to have a pronounced airborne effect.

2.4 Insecticide Resistance Monitoring

Insecticide susceptibility testing was conducted at Dendera, Kawere, Makarara, and Burma Valley across several months from March 2021 to April 2022 (see Table 1). The insecticides tested were:

1. Alpha-cypermethrin (1X)
2. Deltamethrin (1X)
3. Permethrin (1X)
4. DDT (1X)
5. Clothianidin (4µg)
6. Pirimiphos-methyl (1X)
7. Chlorfenapyr (1X)

Insecticide susceptibility tests were performed using *An. gambiae* s.l. raised from larvae for all sites (Table 4). At the time of conducting the tests, the proportion of the sibling species included in the assays is unknown. Previously the team has tested *An. funestus* s.l. susceptibility but the adult population of this species has since dwindled in Burma Valley following IRS with pirimiphos-methyl. Alpha-cypermethrin was the only insecticide tested on mosquitoes from Makarara because DuraNet is the most common ITN brand in Makarara (and there is no IRS). Going forward, other pyrethroids used in public health will be tested if the availability of local mosquitoes is sufficient to include these extra tests. The number of insecticides tested at any given site was determined by the availability of mosquitoes.

Table 4. Summary of Insecticides Tested in Monitoring Sites, March 2021-April 2022

Province	District	Sentinel site	Anopheles species	Deltamethrin	DDT	Clothianidin	Alpha-cypermethrin	Pirimiphos-methyl	Permethin	Chlorfenapyr
Mashonaland East	Mutoko	Kawere	*An. gambiae* s.l.	X	X	X	X	X	X	X
	Mudzi	Dendera		X	X	X		X	X	
	Hwedza	Makarara					X			
Manicaland	Mutare	Burma Valley	*An. gambiae* s.l.	X		X				

CDC bottle assays (SOP #4) were used to test all insecticides including clothianidin (mixed with acetone and 800ppm Mero). Four replicates of 25 female *An. gambiae* s.l. were exposed. Mortality was recorded at the

diagnostic time: 30 minutes for pyrethroids, 45 minutes for DDT, and 60 minutes for pirimiphos-methyl. Clothianidin mortality was recorded 24 hours after exposure. Chlorfenapyr is a slow-acting insecticides, hence mosquito mortalities were monitored every 24 hours up to three days after exposure. *An. funestus* s.l. could not be collected as larvae from Makarara because it is difficult to collect this species as larvae.

2.5 LABORATORY ANALYSES

All laboratory analyses of mosquito specimens were conducted following established protocols at the AU molecular laboratory.

2.5.1 MOLECULAR IDENTIFICATION OF *ANOPHELES* SPECIES

Anopheles mosquitoes collected from all four sentinel sites (from all collection methods and from resistance tests) were analyzed for species identification using polymerase chain reaction (PCR) methods. Briefly, DNA was extracted from either single whole mosquitoes or available parts of single mosquitoes using standard extraction protocols and amplified through PCR. The selection of the PCR protocol was based on morphological identification of the mosquito specimen done initially by the PMI VectorLink team and verified by the AU team. The protocol for *An. gambiae* s.l. is described by Wilkins et al. (2006), while the protocol for *An. funestus* s.l. is described by Koekemoer et al. (2002). For *An. rufipes*, *An. maculipalpis,* and *An. pretoriensis,* AU is using a protocol developed by CDC.

2.5.2 IDENTIFICATION OF BLOOD MEALS

Mosquitoes collected and recorded as freshly fed or half-gravid from all adult collection methods were tested for the blood meal source using PCR (Kent and Norris 2005).

2.5.3 SPOROZOITE RATE

Mosquitoes collected from all adult collection methods during the reporting period were tested for sporozoite rate using enzyme-linked immunosorbent assay (ELISA). Specimens positive for circumsporozoite protein (CSP) with ELISA were subsequently processed by the boiling method (Durnez et al. 2011) and further analyzed by PCR to confirm *Plasmodium* infection.

2.5.4 KDR ASSAYS

Target-site mutations encoding the voltage-gated sodium channel (VGSC) cause pyrethroid resistance and confer cross-resistance to the organochlorine DDT. The VGSC mutations are referred to as 'knockdown resistance' (*kdr*). The MR4[2] protocol was used for detecting *kdr* in *An. gambiae* s.l., specifically allele L1014F (*kdr* West) and L1014S (*kdr* East).

2.5.5 ACE-1 ASSAYS

The presence of insensitive acetylcholinesterase (AChE) was determined in *An. gambiae* s.l. mosquitoes. The analysis detects the G119S mutation in the acetylcholinesterase (Ace-1) gene, a target site mutation that is associated with resistance to carbamates and organophosphates. The MR4 protocol was followed for the Ace-1 analysis.

[2] MR4: The Malaria Research and Reference Reagent Resource Center

3. RESULTS

3.1 ROUTINE VECTOR BIONOMICS LONGITUDINAL MONITORING

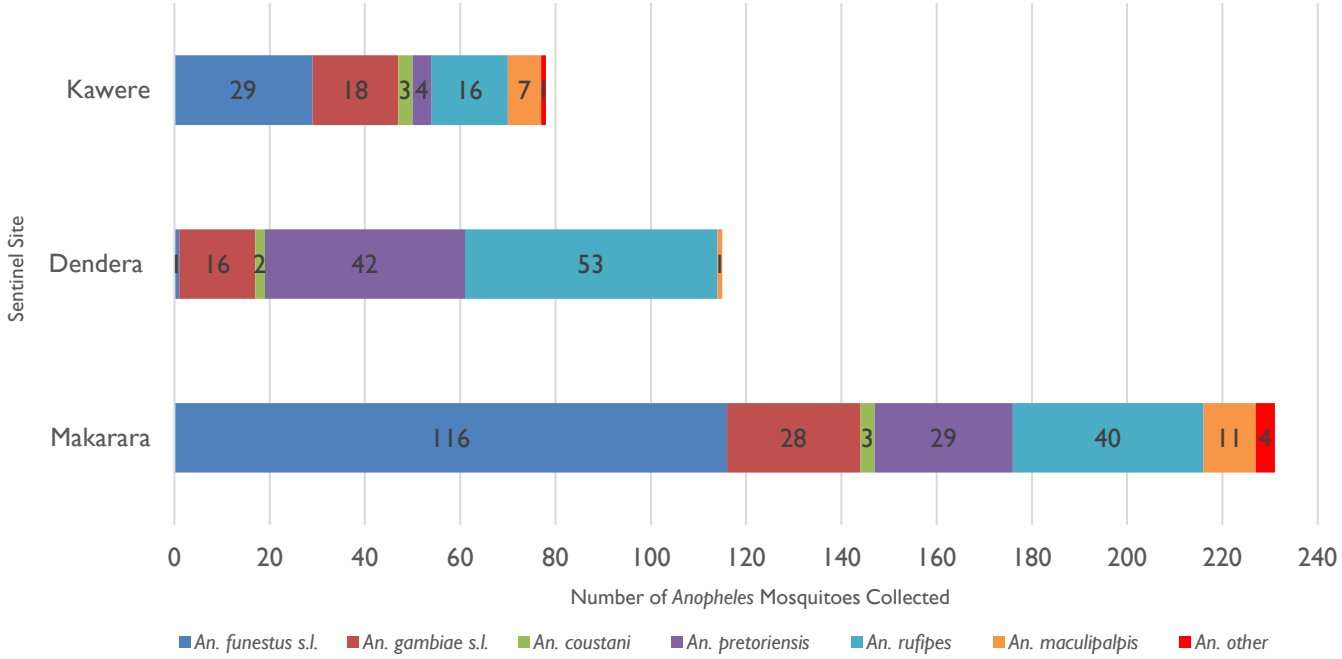
Dendera (Mudzi District) was sprayed with Fludora® Fusion during the 2020 and 2021 IRS campaign. Kawere (Mutoko District) was sprayed with Fludora® Fusion during 2020 and with DDT during the 2021 IRS campaign. Burma Valley (Mutare District) was sprayed with DDT during the 2020 IRS campaign, for which the site was monitored for the last time. Makarara (Hwedza District) was not sprayed and serves as the control site. Longitudinal monitoring was suspended in July 2021, during the lockdown resulting from the coronavirus pandemic.

3.1.1 VECTOR COMPOSITION

A total of 424 female *Anopheles* mosquitoes were collected from all methods combined, between March 2021 and February 2022 at the three sites. At all sites, six or more *Anopheles* species were collected but the species composition varied between sites. *An. rufipes* was the predominant species at Dendera (Figure 2A). *An. funestus* s.l. was the predominant species at Kawere and Makarara (Figure 2B and 2C). *An. gambiae* s.l. and *An. funestus* s.l. are the major malaria vectors in Zimbabwe, whereas the role of *An. rufipes* as a vector remains uncertain. Two species, *An. coustani* and *An. rufipes,* considered secondary malaria vectors, and *An. pretoriensis,* a non-vector, were caught at Dendera, Kawere, and Makarara.

Thirty-nine mosquitoes were collected from Burma Valley, but mosquito collections were conducted in March only at that site and thereafter excluded from routine surveillance during the reporting period (and therefore not included in Figure 2). *An. gambiae* s.l. was the most common species at Burma Valley in March, followed by *An. funestus* s.l. and *An. rufipes.*

FIGURE 2. *ANOPHELES* SPECIES MORPHOLOGICAL COMPOSITION AT SENTINEL SITES (DENDERA, KAWERE, MAKARARA) FROM ALL COLLECTION METHODS COMBINED WHERE N = NUMBER OF *ANOPHELES* MOSQUITOES PER SITE, MARCH 2021-FEBRUARY 2022.



3.1.2 INDOOR RESTING DENSITIES

PSC and PPA collections caught no *Anopheles* mosquitoes throughout the monitoring period, except at Makarara, where a mean of 0.01 *An. gambiae* s.l. mosquitoes were collected per house per night from using PPA.

3.1.3 OUTDOOR RESTING DENSITIES

The mean number of *Anopheles* mosquitoes collected outdoors with the pit shelter collection (Table 5) was more than from indoors with PSC and PPA where no mosquitoes were collected (except for Makarara). Some *An. funestus* s.l. were collected resting outdoors at all sites except Dendera. The species was collected at Kawere and Makarara with a mean of 0.2 and 0.5 mosquitoes per trap per day at these sites, respectively. Fewer *An. gambiae* s.l. per trap per day were collected than *An. funestus* s.l. at Dendera, Kawere, and Makarara. The higher outdoor resting densities of both *An. gambiae* s.l. and *An. funestus* s.l. from the pit shelter collections than the indoor resting collections from the PSCs and PPAs might indicate that these vectors tend to rest outdoors. Other species collected outdoors from pit shelters included *An. rufipes* at Dendera and Makarara, and *An. pretoriensis* at Dendera. Pit shelters in Burma Valley caught *An. funestus* s.l. and *An. rufipes* in low numbers but these collections were only performed in March 2021.

TABLE 5. OUTDOOR VECTOR MEAN DENSITIES (BASED ON PIT SHELTER COLLECTIONS) IN SPRAYED (FF = DENDERA, FF /DDT= KAWERE, DDT = BURMA VALLEY) AND UNSPRAYED

(ITNs = Makarara) Sites in Mashonaland East and Manicaland Provinces, March 2021-February 2022

Site	Total number of days sampled per pit	*An. gambiae* s.l.	*An. funestus* s.l.	*An. pretoriensis*	*An. coustani*	*An. maculipalpis*	*An. rufipes*	Other species
Dendera*	25	0.03	0	0.1	0	0	0.3	0
Kawere	23	0.12	0.2	0	0	0	0	0
Makarara	33	0.06	0.5	0	0	0	0.1	0
Burma Valley	2	0	0.2	0	0	0	0.1	0

Note: FF=Fludora® Fusion

*For Dendera, the team sometimes sampled from more than five pits to increase the yield of mosquitoes. These mosquitoes were analyzed.

3.1.4 Indoor and Outdoor Densities from CDC Light Trap Collections

CDC light traps set outdoors collected a higher mean number of mosquitoes per trap per night of mosquitoes than traps set indoors for all species at Dendera, Kawere, and Makarara, with an exception of *An. rufipes* at Makarara (Table 6). These data indicate that these vectors tend to feed outdoors. At Burma Valley, the mean number of mosquitoes per trap was greater (up to eight times more) outdoors than indoors for *An. funestus* s.l., *An. coustani,* and *An. rufipes*. but these collections were only performed in March 2021 since routine monitoring was discontinued as the project focus changed to monitoring insecticide resistance only.

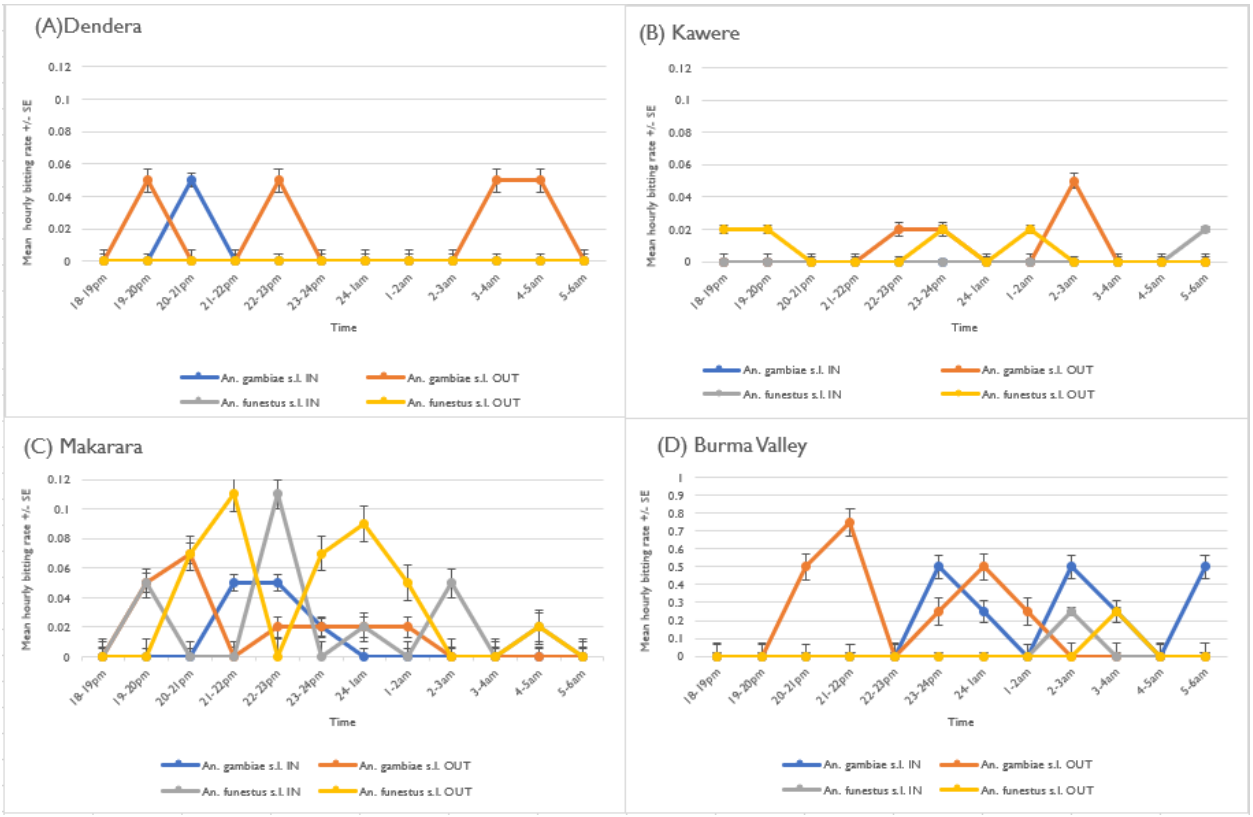
Table 6. Indoor and Outdoor Mean Densities of Anopheles Mosquito Vectors as Collected by the CDC Light Traps at Three Sentinel Sites in Mashonaland East and One Sentinel Sites in Manicaland, March 2021-February 2022

Site	In/Out	No. of months sampled	*An. gambiae* s.l.	*An. funestus* s.l.	*An. pretoriensis*	*An. coustani*	*An. maculipalpis*	*An. rufipes*	Other species
Dendera	In	11	0	0	0	0	0	0	0
	Out	11	0.05	0	0.3	0	0	0.1	0
Kawere	In	11	0	0	0	0	0	0	0
	Out	11	0.02	0.1	0	0	0.1	0.2	0
Makarara	In	11	0.02	0	0	0	0	0.1	0
	Out	11	0.05	0.1	0.3	0	0	0	0
Burma Valley	In	1	0.75	0	0	0	0	0	0
	Out	1	0	0.8	0	0.5	0	0.3	0

Hourly Biting Rates of *An. funestus* s.l. and *An. gambiae* s.l.

Too few mosquitoes were collected during the night to determine a clear pattern in hourly biting behavior (Figure 3 and Table 7). However, there is predominantly more biting outdoors compared with indoors for both *An. gambiae* s.l. (at Dendera and Makarara) and *An. funestus* s.l. (at Kawere and Makarara). Crude endophagic indices are generally low – ranging from nil to 0.5 - for both *An. gambiae* s.l. and *An. funestus* s.l. At Burma Valley, the biting of the predominant *An. gambiae* s.l. occurred pre-midnight and early morning outdoors and midnight to dawn indoors (Table 7). The predominant biting times varied by sentinel site from early night outdoors for *An. funestus* s.l. at Dendera, pre-midnight outdoors to early morning for *An. gambiae* s.l. at Dendera.

FIGURE 3. AVERAGE INDOOR AND OUTDOOR HOURLY BITING RATES OF *AN. FUNESTUS* S.L. AND *AN. GAMBIAE* S.L. AS DETERMINED BY CDC LIGHT TRAP COLLECTIONS AS PROXY FOR HLCS AT DENDERA (A), KAWERE (B), AND MAKARARA (C) IN MASHONALAND EAST AND BURMA VALLEY (D) IN MANICALAND, MARCH 2021-FEBRUARY 2022



Different scales were deliberately used for the y-axis for Burma Valley because the site data was much greater than for the other sites (Dendera, Kawere and Makarara). The graphs for the three sites would be enormously dwarfed using the Burma Valley scale.

TABLE 7. BITING PATTERN FOR *AN. GAMBIAE* S.L. AND *AN. FUNESTUS* S.L. ACROSS ALL SITES

Site	*An. gambiae* s.l.		*An. funestus* s.l.	
	Indoor	Outdoor	Indoor	Outdoor
Burma Valley	23-24 pm, 24-1 am, 2-3 am, 3-4 am, 5-5 am	20-21 pm, 21-22 pm, 23-24 pm, 24-1 am, 1-2 am	2-3 am	3-4 am
Dendera	20-21 pm	19-20 pm, 22-23 pm, 3-4 am, 4-5 am		
Kawere		22-23pm, 2-3am	5-6 am	18-19 pm, 19-20 pm, 22-23 pm, 1-2 am
Makarara	21-22 pm	19-20 pm, 20-21 pm 22-23 pm	19-20 pm, 22-23 pm, 2-3 am	20-21 pm, 21-22 pm, 23-24 pm, 24-1 am,

				4-5 am

3.2 IRS Spray Quality and Residual Efficacy

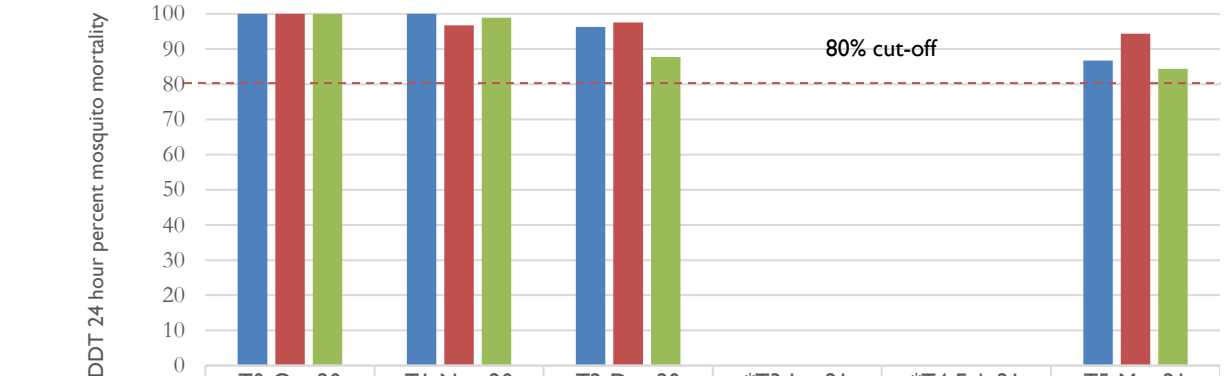
For the 2020 and 2021 IRS campaigns, the team monitored the spray quality, the residual efficacy, and the fumigant effect of the insecticides sprayed. In 2020, these were pirimiphos-methyl at Dendera (November 2020 until August 2021), Fludora® Fusion at Kawere (November 2020 until September 2021), and DDT at Burma Valley (October 2020 until March 2021). For the 2021 IRS campaign, the team monitored Fludora® Fusion at Dendera from October 2021 and DDT from November 2021 at Kawere. Cone assays were not performed at Dendera in November 2021 due to unavailability of susceptible colony *An. arabiensis* (KGB strain) mosquitoes from the insectary at AU. Monthly monitoring is ongoing.

3.2.1 Cone Bioassay Tests

2020 IRS Campaign

Quality of spray was acceptable at the two sites sprayed with Fludora® Fusion: Dendera (Tizora Village) and Kawere (Katiyo and Machona villages) and at the site sprayed with DDT at Burma Valley (Brandhill Farm Compound). Residual efficacy of DDT at Burma Valley was good with mosquito mortality still above the 80% cut-off point for painted brick, cement, and painted cement five months after spraying (Figure 4). Cone bioassay tests were discontinued after March 2021 at Burma Valley as the project shifted focus from longitudinal surveillance to monitoring insecticide resistance due to budgetary constraints. Residual efficacy of Fludora® Fusion at Dendera and Kawere was good with mosquito mortality above 80% on all four wall surface types ten months after spray (Figures 5 and 6) and in January at Dendera. Bioassay tests were not done in January in Dendera because of a lack of colony mosquitoes compounded by the pandemic-related lockdown and not done in February and July at Dendera or Kawere due to the lockdown.

Figure 4. Residual Efficacy of DDT in Burma Valley, Mutare District, Reported as *An. arabiensis* (KGB strain) Mean Mortality After 24-hour Holding Period in WHO Cone Bioassays, October 2020-March 2021

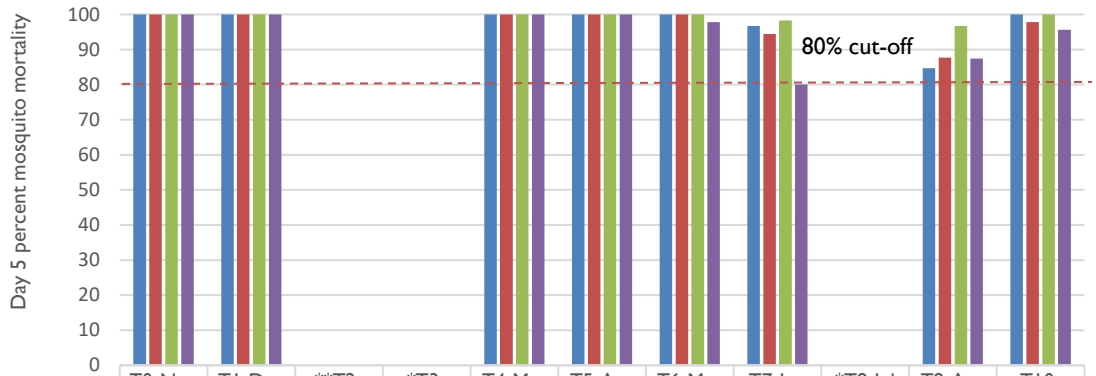


	T0-Oct 20	T1-Nov-20	T2-Dec 20	*T3-Jan-21	*T4-Feb 21	T5-Mar 21
Cement	100	100	96.3	0	0	86.7
Painted Cement	100	96.7	97.6	0	0	94.4
Other Wall Type	100	98.9	87.7	0	0	84.4

Time Points

* Not done due to lockdown

Figure 5. Residual Efficacy of Fludora® Fusion in Dendera (Tizora Village), Mudzi District, Reported as *An. arabiensis* (KGB Strain) Mean Mortality After Five-Day Holding Period in WHO Cone Bioassays, November 2020-September 2021

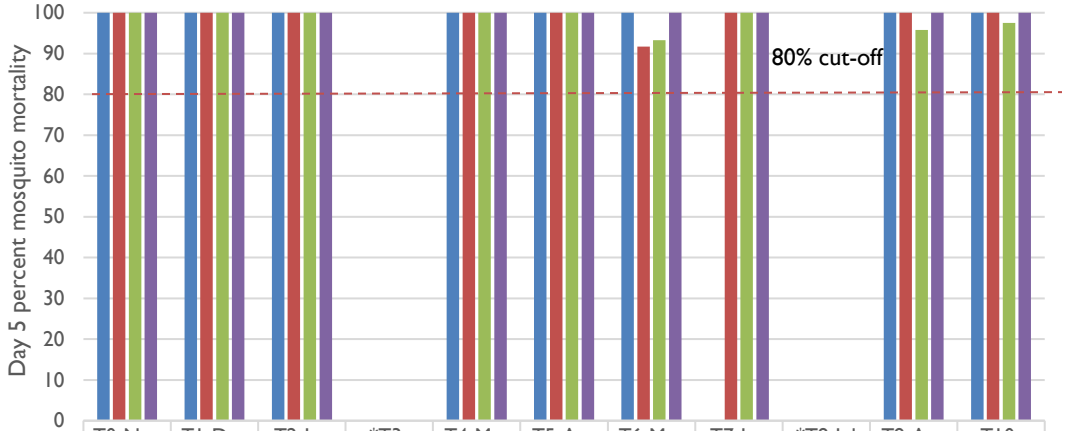


	T0-Nov 20	T1-Dec 20	**T2-Jan 21	*T3-Feb 21	T4-Mar 21	T5-Apr 21	T6-May 21	T7-Jun 21	*T8-Jul 21	T9-Aug 21	T10-Sep 21
Brick	100	100			100	100	100	96.7		84.7	100
Cement	100	100			100	100	100	94.4	0	87.7	97.8
Mud	100	100			100	100	100	98.3	0	96.7	100
Painted Cement	100	100			100	100	97.8	80	0	87.4	95.6

Time Points

*Not done due to lockdown
**Not done due to lack of mosquitoes and lockdown

Figure 6. Residual Efficacy of Fludora® Fusion in Kawere (Chitiyo/Machona Villages), Mutoko District, Reported as *An. arabiensis* (KGB Strain) Mean Mortality After Five-Day Holding Period in WHO Cone Bioassays, November 2020-September 2021



	T0-Nov 20	T1-Dec 20	T2-Jan 21	*T3-Feb 21	T4-Mar 21	T5-Apr 21	T6-May 21	T7-Jun 21	*T8-Jul 21	T9-Aug 21	T10-Sep 21
Brick	100	100	100	0	100	100	100	0	0	100	100
Cement	100	100	100	0	100	100	91.7	100	0	100	100
Mud	100	100	100	0	100	100	93.3	100	0	95.8	97.5
Painted Cement	100	100	100	0	100	100	100	100	0	100	100

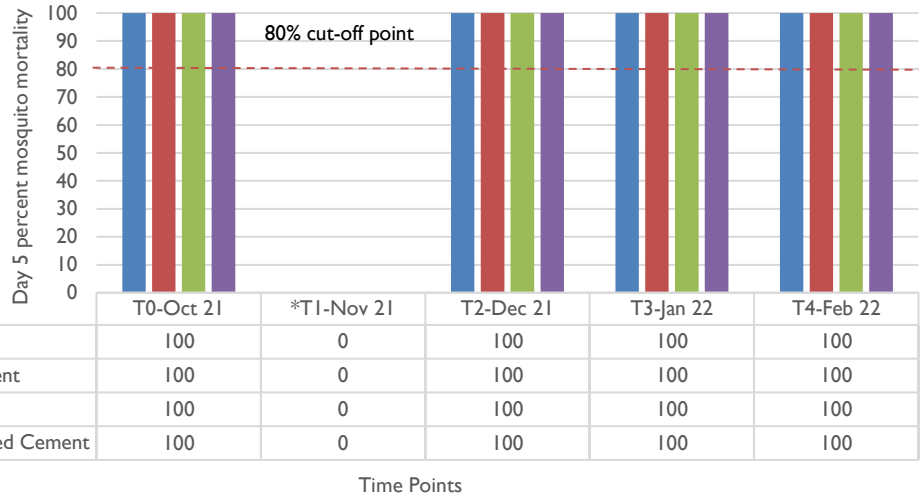
Time Points

*Not done due to lockdown

2021 IRS Campaign

The team monitored the IRS performance in Dendera and Kawere following the 2021 IRS campaign. Quality of spray was acceptable at the two sites although the mortality on the single room with brick walls at Kawere was 90% for unclear reasons. As of the writing of this report, Fludora® Fusion at Dendera retains efficacy after four months: mean mosquito mortality is 100% (Figure 7) for all surface types. Likewise, DDT at Kawere retains efficacy after three months; mean mosquito mortality is 100% for three surfaces and 94.7% for cement (Figure 8). The team will continue monitoring residual efficacy until mosquito mortality falls below the 80% cut-off point for two consecutive months for each wall surface type.

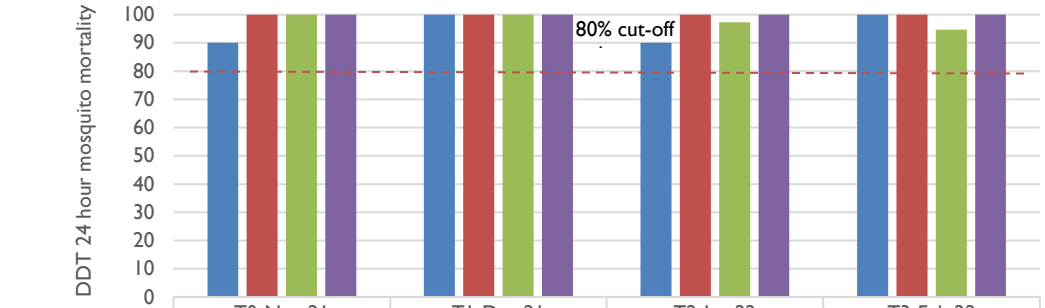
FIGURE 7. RESIDUAL EFFICACY OF FLUDORA® FUSION IN DENDERA (ZHUWAU VILLAGES), MUDZI DISTRICT, REPORTED AS *AN. ARABIENSIS* (KGB STRAIN) MEAN MORTALITY AFTER FIVE-DAY HOLDING PERIOD IN WHO CONE BIOASSAYS, OCTOBER 2021-FEBRUARY 2022



	T0-Oct 21	*T1-Nov 21	T2-Dec 21	T3-Jan 22	T4-Feb 22
Brick	100	0	100	100	100
Cement	100	0	100	100	100
Mud	100	0	100	100	100
Painted Cement	100	0	100	100	100

*Not done due to inadequate mosquitoes

FIGURE 8. RESIDUAL EFFICACY OF DDT AT KAWERE (BOTSANZIRA, SIXPENCE, KAWERE VILLAGES), MUTOKO DISTRICT, REPORTED AS *AN. ARABIENSIS* (KGB STRAIN) MEAN MORTALITY AFTER 24-HOUR HOLDING PERIOD IN WHO CONE BIOASSAYS, NOVEMBER 2021-FEBRUARY 2022



	T0-Nov 21	T1-Dec 21	T2-Jan 22	T3-Feb 22
Brick	90	100	90	100
Mud	100	100	100	100
Cement	100	100	97.3	94.7
Painted Cement	100	100	100	100

Time Points

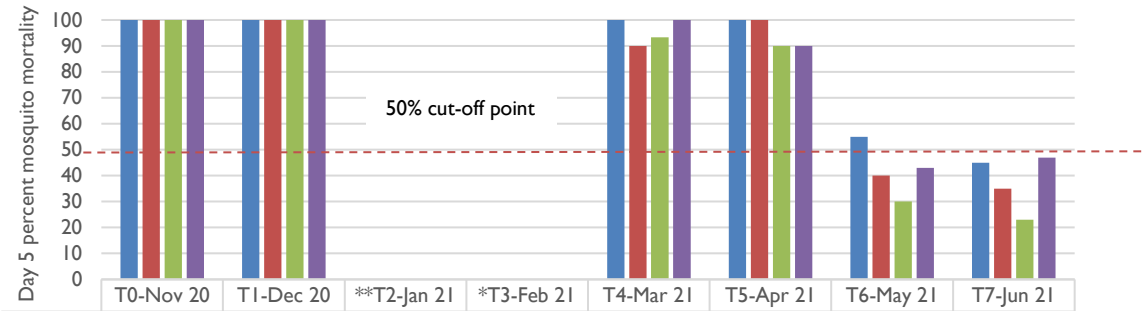
3.2.2 FUMIGANT EFFECT

The fumigant effect for Fludora® Fusion was assessed for the 2020 IRS campaign at Dendera and Kawere, whereas for the 2021 IRS campaign it was done for Fludora® Fusion at Dendera and not for DDT at Kawere. The fumigant effect assays were done irregularly because the team prioritized cone bioassays when availability of colony mosquitoes from AU was a challenge.

2020 IRS Campaign

The tests for fumigant effect at Dendera were not done in January and February 2021, partly due to the COVID-19 national lockdown (January and February) and partly due to lack of colony mosquitoes. Tests were stopped seven months after spray when mosquito mortality fell below 50% on all wall surface types (mud, brick, cement, and paint) (Figure 9). Mosquito mortality was also below 50% seven months after IRS at Kawere, after which tests were discontinued (Figure 10).

FIGURE 9. FUMIGANT EFFECT OF FLUDORA® FUSION IN DENDERA (TIZORA VILLAGE), MUDZI DISTRICT, REPORTED AS *AN. ARABIENSIS* (KGB STRAIN) MEAN MORTALITY AFTER FIVE-DAY HOLDING PERIOD, NOVEMBER 2020- JUNE 2021

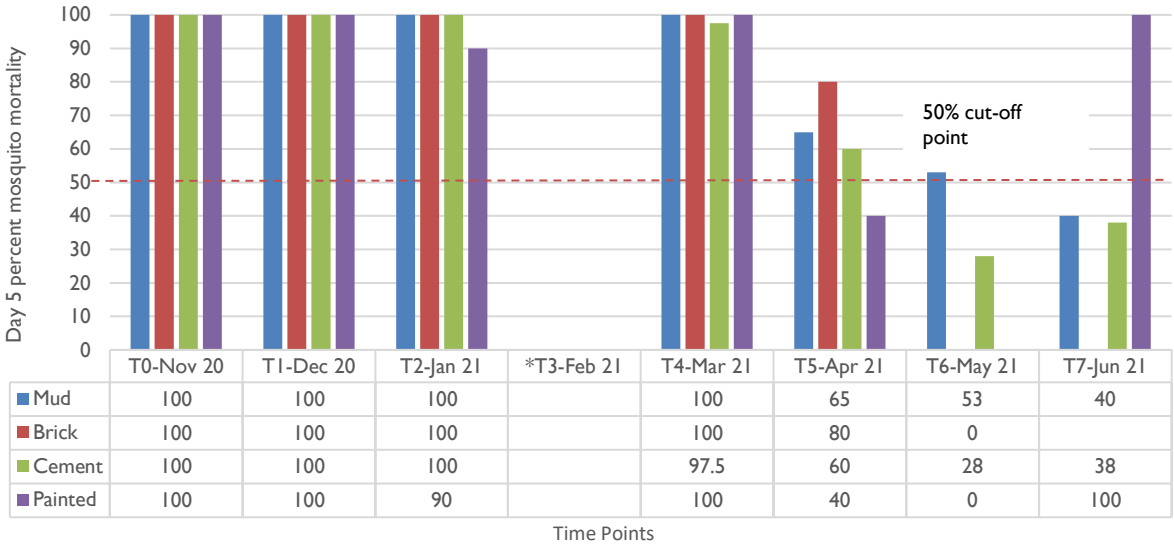


	T0-Nov 20	T1-Dec 20	**T2-Jan 21	*T3-Feb 21	T4-Mar 21	T5-Apr 21	T6-May 21	T7-Jun 21
Mud	100	100			100	100	55	45
Brick	100	100			90	100	40	35
Cement	100	100			93.33	90	30	23
Painted	100	100			100	90	43	47

Time Points

*Not done due to lockdown
**Not done due to lack of mosquitoes and lockdown

FIGURE 10. FUMIGANT EFFECT OF FLUDORA® FUSION IN KAWERE (CHITIYO AND MACHONA VILLAGES), MUTOKO DISTRICT, REPORTED AS *AN. ARABIENSIS* (KGB STRAIN) MEAN MORTALITY AFTER FIVE-DAY HOLDING PERIOD, NOVEMBER 2020-JUNE 2021



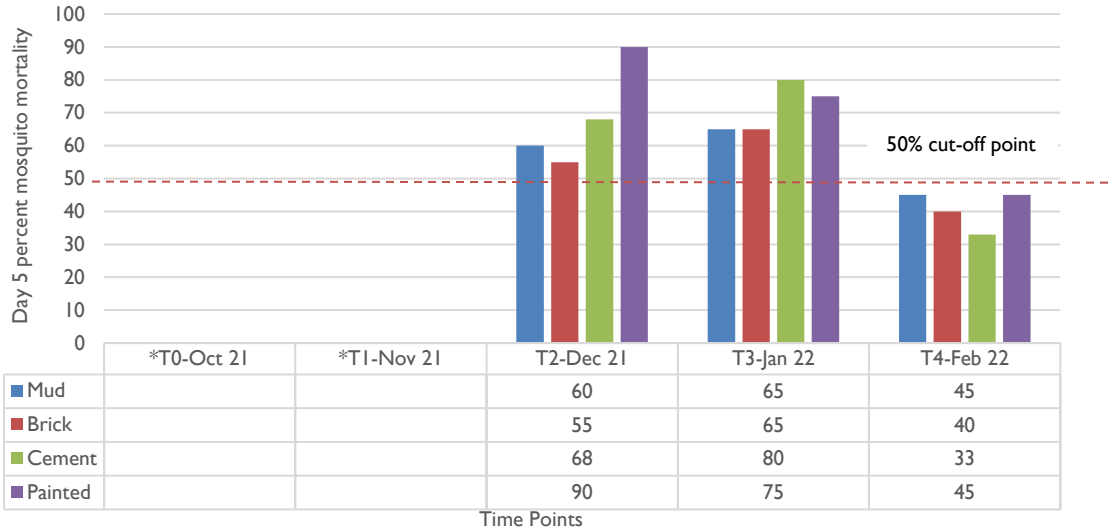
	T0-Nov 20	T1-Dec 20	T2-Jan 21	*T3-Feb 21	T4-Mar 21	T5-Apr 21	T6-May 21	T7-Jun 21
Mud	100	100	100		100	65	53	40
Brick	100	100	100		100	80	0	
Cement	100	100	100		97.5	60	28	38
Painted	100	100	90		100	40	0	100

*****Not done due to lockdown

2021 IRS Campaign

The fumigant effect was monitored irregularly for Fludora® Fusion at Dendera following the 2021 IRS campaign. Mosquito mortality was below the 50% cut-off point on all four wall types (mud, brick, cement, and paint) four months post IRS at Dendera (Figure 11).

FIGURE 11. FUMIGANT EFFECT OF FLUDORA® FUSION IN DENDERA (ZHUWAU VILLAGE 1 AND 2), MUDZI DISTRICT, REPORTED AS *AN. ARABIENSIS* (KGB STRAIN) MEAN MORTALITY AFTER FIVE-DAY HOLDING PERIOD, NOVEMBER 2021-FEBRUARY 2022



	*T0-Oct 21	*T1-Nov 21	T2-Dec 21	T3-Jan 22	T4-Feb 22
Mud			60	65	45
Brick			55	65	40
Cement			68	80	33
Painted			90	75	45

*Not done due to lack of mosquitoes

3.3 INSECTICIDE RESISTANCE MONITORING

The insecticide susceptibility of *An. gambiae* s.l. collected from localities in Mashonaland East and Manicaland Provinces was done using the CDC bottle bioassay method for all insecticides tested. The seven insecticides tested were alpha-cypermethrin, deltamethrin, permethrin, clothianidin, pirimiphos-methyl, DDT, and chlorfenapyr (Table 8). Few mosquitoes were collected from all sites mostly due to erratic rains, and a drought at Makarara.

An. gambiae s.l. was susceptible (100% mortality) to most of the diagnostic doses of the insecticides tested at the three sites. *An. gambiae* s.l. collected from Makarara (Samuriwo and Chikomba in Hwedza District) were susceptible to alpha-cypermethrin but caution should be applied with this interpretation because a very low number of mosquitoes were tested. There was limited mosquito breeding due to the dry weather conditions.

TABLE 8. RESULTS OF INSECTICIDE SUSCEPTIBILITY TESTS ON *AN. GAMBIAE* S.L. CONDUCTED AT THREE SITES IN MASHONALAND EAST PROVINCE

District	Sentinel site	Larval collection locality	Date of test	Insecticide tested (Dose)	Total # mosquitoes tested	Resistance status (mosquito mortality)*
Mudzi	Dendera	Meza	May 2021	Pirimiphos-methyl	101	S (100%)
		Meza	Mar 2021	DDT(1X)	30	S (100%)
		Zhuwau Meza	March, Apr 2021, Mar, Apr 2022	Deltamethrin (1X)	217	S (100%)
		Meza	Mar 2021	Permethrin	100	PR (95%)
		Gatakata, Chiunye,	May 2021, Apr 2022	Clothianidin	187	S (100%)
Mutoko	Kawere	Hunda	Mar, Apr 2021, Feb, 2022	DDT (1X)	100	S (100%)
		Hunda	Mar 2022	Alpha-cypermethrin	75	S (99%)
		Hunda	Feb 2022	Permethrin(1X)	100	S (100%)
		Hunda	Feb 2022	Pirimiphos-methyl (2%)	79	PR (90%)
		Hunda	Mar 2022	Chlorfenapyr	78	S (100%)
		Hunda	Aug 2021, Mar, Apr 22	Deltamethrin (1X)	163	S (100%)
		Kawere	May, Jun 2021, Apr 2022	Clothianidin	43	S (100%)
Hwedza	Makarara	Chikomba, Samuriwo	Mar, Apr, May 2021	Alpha-cypermethrin (1X)	22	S (100%)

Note: shaded rows indicate sample sizes below 100
*Where S – susceptible; PR – possible resistance; R – resistant

An. gambiae s.l. from Burma Valley (collected from Marange area) in Mutare District were susceptible to deltamethrin and clothianidin but fewer than 100 mosquitoes were tested (Table 9).

TABLE 9. RESULTS OF INSECTICIDE SUSCEPTIBILITY TESTS ON *AN. GAMBIAE* S.L. CONDUCTED AT BURMA VALLEY IN MANICALAND PROVINCE

District	Sentinel site	Collection locality	Date of test	Insecticide tested (Dose)	Test	Total number of mosquitoes tested	Resistance status (mosquito mortality)
Mutare	Burma Valley	Mafararikwa, Marange	Feb 2022	Deltamethrin (1X)	CDC	31	S (100%)
			April 2022	Clothianidin (1X)	CDC	64	S (100%)

Note: shaded rows indicate sample sizes below 100

3.4 RESULTS OF LABORATORY ANALYSIS

3.4.1 MOLECULAR IDENTIFICATION OF *ANOPHELES* SPECIES FROM LONGITUDINAL MONITORING

A total of 463 *Anopheles* mosquitoes collected in 2021-2022 were assayed for species identification in the AU laboratory as follows: 39 from Burma Valley, 115 from Dendera, 78 from Kawere, and 231 from Makarara (Figure 12). Pit shelters provided the most mosquitoes for lab analysis from the three main sites. All the 39 *Anopheles* from Burma Valley were collected in March 2021 only and so are likely to be less representative of the species complex composition than the other sites.

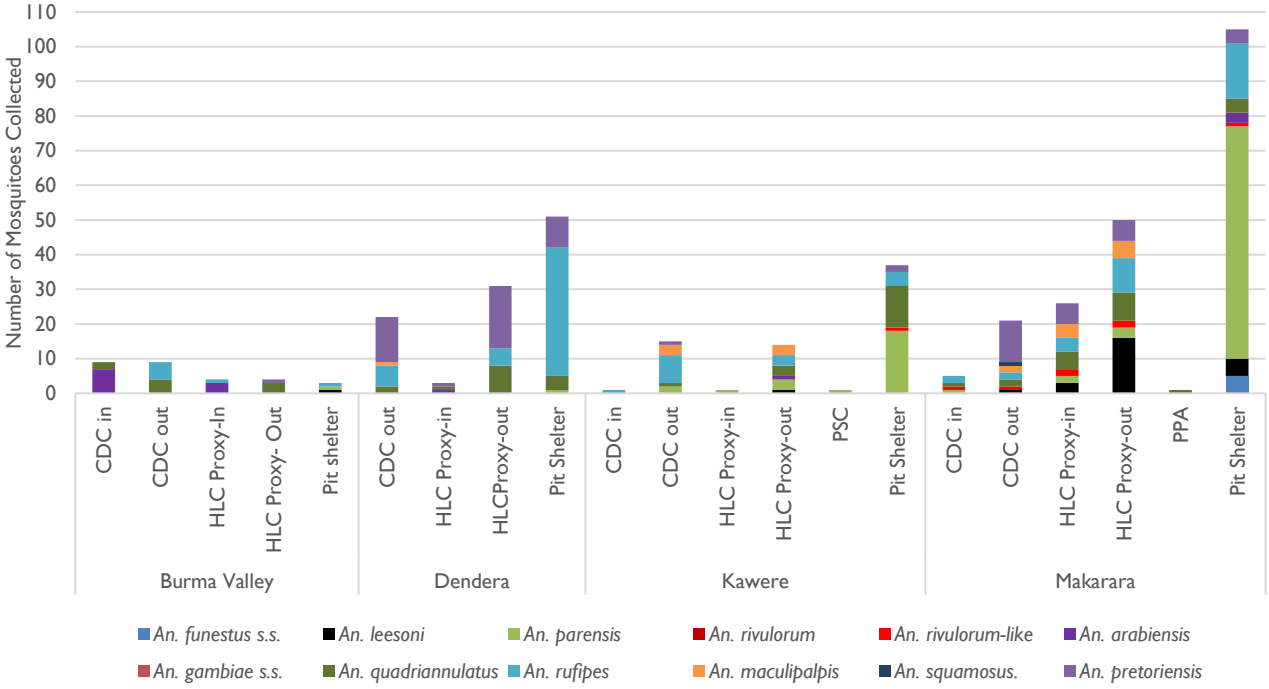
At Burma Valley, only 2/39 were *An. funestus* s.l. (5.1%), which were identified to two sibling species, one *An. leesoni* and one *An. parensis*. All reference to *An. parensis* in this report is subject to confirmation as 2 bands were visible on the gel electrophoresis post-PCR. CDC is investigating the 2-banded specimens to guide subsequent data analysis. *An. gambiae* s.l. constituted 48.7% (19/39) of the total *Anopheles* collected from Burma Valley that were identified as *An. arabiensis* (10/19) and *An. quadriannulatus* (9/19). The other species were mainly *An. rufipes* (7/39) and *An. pretoriensis* (1/39) constituting 17.9% and 2.6%, respectively. A total of 8 (20.5%) *Anopheles* mosquitoes from Burma Valley did not amplify, while 2/39 specimens were not subjected to the molecular analysis as there is no appropriate protocol.

At Dendera 0.9% (1/115) was *An. funestus* s.l., which was identified as *An. parensis*. The other species found were *An. rufipes* (48/115) and *An. pretoriensis* (41/115). Sixteen *An. gambiae* s.l. were collected: 6.2.% (1/16) was identified as *An. arabiensis* and the remaining 93.8% was *An. quadriannulatus* (15/16). Six out of 115 specimens did not amplify.

At Kawere, 34.6% (27/78) of the *Anopheles* collected were *An. funestus* s.l. that were identified as *An. leesoni* (1/27; 3.7%), *An. parensis* (25/27; 92.6%), and *An. rivulorum*-like (1/27; 3.7%). There was fewer *An. gambiae* s.l. (17/78) than *An. funestus* s.l. (27/78); most were *An. quadriannulatus* (16/17; 94.1%), with *An. arabiensis* constituting a mere 5.9% (1/17).

Out of 231 *Anopheles* mosquitoes from Makarara, 47.6% (110/231) were *An. funestus* s.l. and 10.4% (24/231) *An. gambiae* s.l. Most of the *An. funestus* s.l. were identified as *An. parensis* 66.4% (73/110), *An. leesoni* (22.7%; 25/110), *An. funestus* s.s. (4.5%; 5/110), and *An. rivulorum*-like (6.4%; 7/110). Most of the *An. gambiae* s.l. from Makarara were *An. quadriannulatus* (87.5%; 21/24) with *An. arabiensis* constituting only 12.5% (3/24). Seventy-four of the *Anopheles* from Makarara were other species, namely, *An. rufipes* (14.7%; 34/231), *An. maculipalpis* (4.8%; 11/231), *An. squamosus* (0.4%; 1/231), and *An. pretoriensis* (12.1%; 28/231). Sixteen of the 231 (6.9%) did not amplify, while seven (3.0%) were not subjected to molecular analysis.

FIGURE 12. MAIN ANOPHELES MOSQUITOES COLLECTED AT BURMA VALLEY, DENDERA, MAKARARA, AND KAWERE IDENTIFIED WITH PCR, 2021, EXCLUDING SPECIMENS NOT IDENTIFIED



Footnote on species: Vectors: *An. funestus* s.s.; *An. leesoni*; *An. parensis*; *An. arabiensis; An. gambiae* s.s.
Potential Vectors: *An. rivulorum; An. rivulorum*-like; *An. rufipes; An. squamosus*
None Vectors: *An. quadriannulatus; An. maculipalpis; An. pretoriensis*
The *An. parensis* is *An. parensis*, pending sequencing
CDC: CDC light trap set indoors or outdoors 'IN' or 'OUT'
HLC Proxy: human landing collection set indoors or outdoors 'IN' and 'OUT'
PSC: Pyrethrum Spray Catch
PPA: Prokopack Aspirator

3.4.2 MOSQUITOES COLLECTED FOR INSECTICIDE RESISTANCE MONITORING

Laboratory analysis of the *An. gambiae* s.l. specimens tested for insecticide susceptibility showed a low proportion of the malaria vector *An. arabiensis* as its occurrence ranged from 0% at Burma Valley (Marange samples) to 15% at Kawere (Table 10). Most of the *An. gambiae* s.l. were *An. quadriannulatus*, a species regarded as a non-vector in the preliminary results. It is difficult to make inferences about the resistance status of known vector species from these sparse data. Getting adequate proportions of known vectors (i.e. *An.* arabiensis and *An. gambiae* s.s.) in wild-caught *An. gambiae* s.l. is an ongoing challenge as the vector population continues to change. Other mosquito specimens included in susceptibility tests are yet to have species identification determined by AU.

TABLE 10. SIBLINGS SPECIES OF *AN. GAMBIAE* S.L. FOLLOWING INSECTICIDE SUSCEPTIBILITY TESTS

Site	Insecticide	*An. arabiensis*	*An. quadri*annulatus	*An. gambiae s.s.*
Dendera	DDT	1/30 (3%)	29/30 (97%)	0
	Deltamethrin	3/61 (5%)	58/61 (95%)	0
	Pirimiphos-methyl	3/42 (7%)	39/42(93%)	0
Kawere	Clothianidin	5/34 (15%)	29/34 (85%)	0
	Deltamethrin	1/115 (1%)	113/115 (98%)	1/115 (1%)
	Pirimiphos-methyl	3/79 (4%)	76/79 (96%)	0

Makarara	Alpha-cypermethrin	1/22 (5%)	21/22 (96%)	0
Burma Valley	Deltamethrin	0	11/11 (100%)	0

One *An. gambiae* s.s. was classified by molecular method as *An. gambiae* (former 'S' molecular form), while *An. coluzzii* was absent. The *An. gambiae* s.s. was collected as larvae from Kawere (Hunda locality) in August 2021. Two *An. gambiae* s.s. reported previously were collected one from Makarara and another from Burma Valley.

Results of Blood Meal Analysis

A total of 222 blood-fed mosquitoes collected from the four sites were analyzed by PCR to determine the blood meal sources (Table 11). Cows were the most or joint-most common sources of bloodmeals at all sites. A single *An. arabiensis* from Burma Valley, one *An. pretoriensis* at Makarara, and another unidentified specimen from Dendera had fed solely on human blood. Other specimens were identified with human blood mixed with goat: one *An. rufipes* at Dendera, two *An. funestus* s.s. and one *An. rufipes* at Makarara, and one *An. parensis* with human-goat-cow blood at Makarara. No change in mosquito feeding behavior over time is discernable from these data.

TABLE 11. RESULTS OF THE BLOOD MEAL ANALYSES OF ANOPHELES SPECIES FROM BURMA VALLEY, DENDERA, KAWERE, AND MAKARARA

Site	Blood meal source	Species											Total
		An. funestus s.l.				*An. gambiae* s.l.			Other species			Unidentified specimen	
		An. funestus s.s.	*An. leesoni*	*An. parensis*	*An. rivolum-like*	*An. arabiensis*	*An. quadriannulatus*	*An. rufipes*	*An. macullipalpis*	*An. squamous*	*An. pretoriensis*		
Burma Valley	Cow	0	1	1	0	0	0	1	0	0	0	0	3
	Human	0	0	0	0	1	0	0	0	0	0	0	1
	No amplification	0	0	0	0	0	1	0	0	0	0	1	2
	Total	**0**	**1**	**1**	**0**	**1**	**1**	**1**	**0**	**0**	**0**	**1**	**6**
Dendera	Cow	0	0	1	0	0	3	10	0	0	4	1	19
	Human	0	0	0	0	0	0	0	0	0	0	1	1
	Goat	0	0	0	0	0	0	15	0	0	2	2	19
	Human & goat	0	0	0	0	0	0	1	0	0	0	0	1
	No amplication	0	0	0	0	0	0	10	1	0	1	1	13
	Total	**0**	**0**	**1**	**0**	**0**	**3**	**36**	**1**	**0**	**7**	**5**	**53**
Kawere	Cow	0	0	7	0	0	2	5	3	0	0	2	19
	Goat	0	0	0	0	0	1	0	1	0	0	0	2
	Human	0	0	0	0	0	0	0	0	0	0	0	0
	No amplification	0	0	2	0	1	5	0	1	0	2	0	11
	Total	**0**	**0**	**9**	**0**	**1**	**8**	**5**	**5**	**0**	**2**	**2**	**33**
Makarara	Cow	3	13	55	2	0	3	16	0	0	8	9	109
	Human	0	0	0	0	0	0	0	0	0	1	0	1
	Human & goat	2	0	0	0	1	0	1	0	0	0	0	4
	Human, goat & cow	0	0	1	0	0	0	0	0	0	0	0	1
	No amplification	0	0	8	1	0	2	2	1	0	0	1	15
	Total	**5**	**13**	**64**	**3**	**1**	**5**	**19**	**1**	**0**	**9**	**10**	**130**

Footnote on species: Vectors: *An. funestus* s.s.; *An. leesoni*; *An. parensis*; *An. arabiensis; An. gambiae* s.s.
Potential Vectors: *An. rivulorum; An. rivulorum*-like; *An. rufipes; An. squamosus*

None Vectors: *An. quadriannulatus; An. maculipalpis; An. pretoriensis*
The *An. parensis* is actually *An. parensis*, pending sequencing

3.4.3 Sporozoite Infection Rate

A total of 379 *Anopheles* mosquitoes collected from three sentinel sites by various methods were analyzed by ELISA for CSP for *Plasmodium falciparum*: *An. funestus* s.l. (n=137), *An. gambiae* s.l. (n=56), and other *Anopheles* (n=186) consisting mainly of *An. maculipalpis* (n=18), *An. rufipes* (n=98), *An. pretoriensis* (n=69), and *An. squamosus* (n=1). Only 20 specimens were positive in the preliminary test), of which only two confirmed positive by the boiling procedure. The confirmed specimens were collected outdoors from pit shelters at Makarara site: *An. parensis* – 1/98 of this species, and *An. funestus* s.s. – 1/5 of this species (Table 12).

Table 12. Specimens Confirmed Sporozoite From Makarara

Attributes of specimens tested for infection	Species confirmed CSP Positive	
	An. funestus s.s.	*An. parensis*
Period of collection	Feb 2022	May 2021
Number confirmed positive*	1/5	1/98

*The numerator denotes the number of specimens positive by ELISA out of the total number tested (denominator).

3.4.4 Results of KDR Assays

A total of 550 *An. gambiae* s.l. were tested for the kdr mutation; Leu – Ser (kdr East) and Leu – Phe (*kdr* West). Only 1.1% (6/550) had the heterozygote allele for *kdr* West, whereas most (98.9%; 544/550) were susceptible homozygous (Table 13). All the six specimens with *kdr* West heterozygote allele were *An. quadriannulatus*, a non-vector. All 550 specimens were susceptible homozygous for *kdr* East gene. The *An. gambiae* s.l. analyzed were collected mainly by larval collection for insecticide resistance (483/550) and others from longitudinal monitoring. Six specimens collected by larval collection for insecticide resistance from Kawere were resistant homozygous for *kdr* West.

Table 13. Results of *KDR* Assays

Province	District	Site	Number of species tested	Kdr East			Kdr West		
				SS	RS	RR	SS	RS	RR
Manicaland	Mutare	Burma Valley	25 (11 LC-Mafararikwa+14 routine surve)y	25	0	0	25	0	0
Mashonaland East	Mudzi	Dendera	291 (276 LC+15 LM)	291	0	0	291	0	0
	Mutoko	Kawere	189 (171 LC+18 LM)	189	0	0	183	6	0
	Hwedza	Makarara	45 (25 LC+ 20 LM)	45	0	0	45	0	0
Total			550	550	0	0	544	6	0

Note: SS=Susceptible homozygous, RS=Resistant heterozygous, RR=Resistant homozygous
LC = larval collection; LM = longitudinal monitoring

3.4.5 Results of Ace-1 Assays

A total of 595 *An. gambiae* s.l. were analyzed for insensitive AChE (acetylcholinesterase) gene by molecular method. Twelve of the 595 were homogeneous for the resistant gene for Ace-1: three were from Dendera and nine from Kawere (Table 14). All the 12 specimens with Ace-1 resistance allele were *An. quadriannulatus*, a non-vector.

TABLE 14. RESULTS OF ACE-1 ASSAYS

Province	District	Site	Insecticide	Ace-1 Status		
				*SS	RS	RR
Manicaland	Mutare	Burma Valley	Deltamethrin	11	0	0
Mashonaland East	Mudzi	Dendera	Clothianidin	142	0	3
			Pirimiphos-methyl	42	0	0
			DDT	30	0	0
			Deltamethrin	59	0	0
	Mutoko	Kawere	Clothianidin	31	0	3
			Pirimiphos-methyl	84	0	0
			DDT	53	0	0
			Deltamethrin	106	0	6
	Hwedza	Makarara	Alpha-cypermethrin	25	0	0
Total				583	0	12

Note: SS=Susceptible homozygous, RS=Resistant heterozygous, RR=Resistant homozygous

4. DISCUSSION

Entomological monitoring results from March 2021 to February 2022 show variation in the species composition at the three main sites. Overall *An. funestus* s.l. is the major malaria vector species compared with *An. gambiae* s.l. with variation by site. *An. funestus* s.l. was dominant at Kawere and Makarara, but at Dendera, the dominant species were *An. rufipes* and *An. pretorienis*. The relative proportion for *An. rufipes* at Dendera increased to 46% (n=53) from 29% (n=9) in the previous report. The predominance of *An. rufipes* in comparison to the major malaria vectors at Dendera is noted with interest as this species is considered a secondary vector. At the fourth site, Burma Valley, *An. gambiae* s.l. was the predominant vector species but collections were done only in one month. The importance of *An. funestus* s.l. at Makarara is substantiated as mosquitoes from two sibling species, *An. funestus* s.s. and *An. parensis,* from that site were confirmed CSP-positive during this reporting period. *An. funestus* s.s. is a known efficient malaria vector whereas *An. parensis* is considered a secondary vector. None of the *An. gambiae* s.l. were CSP-positive. This is perhaps not surprising given the low proportion of *An. arabiensis* relative to *An. quadriannulatus* from that species complex. The relative proportion of *An. funestus* s.l. to *An. gambiae* s.l. has gone up at Kawere and Makarara compared to the last reporting period, whereas the reverse has happened at Dendera. Whereas the relative proportion of *An. funestus* s.l.: *An. gambiae* s.l. at Kawere was 1:7 in 2020, this was 1.6:1 in 2021. At Makarara, *An. funestus* s.l.: *An. gambiae* s.l. was 1:1.6 in 2020 but 4.2:1 in 2021. At Dendera, the *An. funestus* s.l.: *An. gambiae* s.l. ratio changed from 1.2:1 in 2020 to 1:14 in 2021. The low mosquito densities are attributed to the erratic rains that affected *An. gambiae* s.l., which typically breeds in temporary rainwater pools, as compared to *An. funestus* s.l., which breeds in semi-permanent water bodies that are available at Kawere and Makarara. The longitudinal monitoring was conducted mostly under drought conditions, especially for Makarara, and hence the low number of mosquitoes collected. Longitudinal entomological surveillance was affected by the COVID-19 lockdown in January and February and July 2021.

Virtually no *Anopheles* mosquitoes were collected indoors (by HLC proxy and light trap) at the four sites except at Makarara, the control site, which is not under routine spraying. Some *An. funestus* s.l. and *An. gambiae* s.l. were collected resting outdoors (in pits) and from light traps and HLC proxy outdoors. This suggests a vector population that prefers resting outdoors and/or the impact of insecticide from routine IRS on indoor resting mosquitoes. However, this observation is based on very low mosquito densities in the areas. Other species, *An. pretoriensis* and *An. rufipes,* were also relatively more abundant outdoors than indoors. CDC light traps set outdoors generally collected more mosquitoes than those set indoors.

Too few mosquitoes were collected to determine indoor and outdoor hourly biting rates. Fewer *An. gambiae* s.l. were collected than *An. funestus* s.l. except at Burma Valley in March 2021. Most *An. gambiae* s.l. and *An. funestus* s.l., were observed biting outdoors before and after midnight with limited biting during the early morning, between 4-5 a.m. The outdoor biting behavior presents challenges for vector control using traditional strategies such as IRS and ITNs. However, these data need to be interpretated along with human population behavior (i.e. the time at which people retreat indoors and the time at which they are in bed under an ITN) at each given locality to get a better estimate of exposure

Laboratory analysis provided insights on species occurrence at all four sites. *An. parensis* was the predominant sibling species of *An. funestus* s.l. at Kawere and Makarara followed by *An. leesoni* at Makarara. *An. gambiae* s.l. was represented by the major malaria vector *An. arabiensis*. s.s but overshadowed by *An. quadriannulatus* at Dendera, Kawere, and Makarara. *An. merus* was conspicuous by its absence at the four sites among the species identified by PCR during this reporting period. Data from Burma Valley show the occurrence of two sibling species of *An. funestus* s.l. and for *An. gambiae* s.l. albeit for a short observation period. *An. leesoni* and *An. parensis* were found at Burma Valley in low densities compared with *An. arabiensis* and *An. quadriannulatus*.

Mosquitoes that had fed solely on humans were few, consisting of *An. arabiensis* from Burma Valley and one *An. pretoriensis* from Makarara, and one unidentified mosquito from Dendera. Mixed human-animal blood meals were found at Dendera for *An. rufipes* and Makarara for *An. funestus* s.s. and *An. arabiensis* (human-goat), and for at Makarara for *An. parensis* (human-goat-cow). This indicates an opportunistic feeding tendency in these species.

Other species found include *An. rufipes*, *An. maculipalpis*, *An. squamosus,* and *An. pretoriensis*. While *An. rufipes* and *An. squamosus* are considered potential malaria vectors, none of the other species were CSP-positive. *An. pretoriensis* is probably not a vector although the species has been reported positive for sporozoites in Eastern Zambia that borders with Mozambique. The low human blood index in most of the other species suggests it is unlikely they feed on humans often enough to be transmitting malaria.

Only two mosquitoes of the 379 tested were circumsporozoite-positive with *Plasmodium falciparum*. The two specimens that were confirmed by the additional step of boiling positive were all members of the *An. funestus* s.l. The two specimens, one *An. funestus* s.s. and one *An. parensis* were collected from pit shelters from Makarara, the control site. That these were collected outdoors does not augur well for IRS or ITNs. . This confirms *An. parensis* as a secondary malaria vector in its geographical range albeit based on a limited sample.

Entomological monitoring yielded low numbers of mosquitoes overall, limiting the ability to identify clear seasonal trends yet highlighting the need for an assessment and potential consideration around other mosquito collection methods such as using animal- or human-baited tent traps. The pit shelter has proven to be an affordable but productive outdoor collection method in vector surveillance.

Results of insecticide susceptibility tests on *An. gambiae* s.l. and *An. funestus* s.l. are encouraging since there was generally no resistance to clothianidin, deltamethrin, alpha-cypermethrin, chlorfenapyr, and DDT. However, there is need for caution in making inferences from the susceptibility tests since most mosquitoes tested are non-vector species.

Laboratory tests for insecticide resistance indicate the presence of heterozygous resistant *kdr* West gene and some Ace-1 resistant mutations. Only 1.1% of the specimens analyzed for *kdr* had the heterozygous allele for *kdr* West. None of the specimens had *kdr* East resistance. Six (2.0%) of the *An. gambiae* s.l. analyzed had Ace-1 resistance although these tests were based on limited sample sizes. This augurs well for insecticide use in vector control although monitoring should be done on a wider scope geographically.

For the 2020 IRS campaign, the residual efficacy of Fludora® Fusion was at least nine months at Dendera and Kawere. For the 2021 IRS campaign, Fludora® Fusion has had a residual life of at least four months at Dendera, and DDT residual efficacy at Kawere is still well above the 80% cut-off point four months after spray. Current monitoring following the 2021 IRS campaign shows the residual efficacy of Fludora® Fusion at Dendera and DDT at Kawere remains good four months after spray based on the 120-hour holding period for Fludora® Fusion, and three months after the 24-hour holding period for DDT. Fumigant effect remains high at both Dendera and Kawere. The team will continue monitoring residual efficacy of Fludora® Fusion at Dendera and DDT at Kawere until the mosquito mortality falls below 80% for consecutive months.

5. RECOMMENDATIONS

Based on the data presented and discussed in this report, the following recommendations and next steps should be considered going forward:

- *An. gambiae* s.l. is susceptible to clothianidin at the sites tested in Mashonaland East Province and Mutare in Manicaland Province, and therefore clothianidin-based products, such as Fludora® Fusion, continue to be appropriate for IRS in those areas in line with the rotation recommended in the National Insecticide Resistance Management Plan. Caution is needed to infer from these susceptibility results since most mosquitoes tested were non-vector.
- *An. gambiae* s.l. is susceptible to alpha-cypermethrin in Makarara so the distribution and use of long-lasting insecticidal nets treated with that pyrethroid is still appropriate. Caution is needed to infer from these susceptibility results since most mosquitoes tested were non-vector.
- Given the challenges in collecting *An. funestus* s.l. larvae from the field for insecticide resistance testing, it is recommended that susceptibility tests be done on F1 pooled from parents of the same sibling species following laboratory analysis. This will enhance sample sizes of *An. funestus* s.l. collections.
- PMI VectorLink and the NIHR should continue to inform NMCP and sensitize the Vector Control Technical Sub-Committee on insecticide resistance to guide policy and action.
- Because more mosquitoes are caught outdoors than indoors, PMI VectorLink in collaboration with the NIHR should evaluate alternative collection methods, such as the window trap, to determine mosquito behavior.
- The project should disseminate the observed outdoor mosquito biting behavior at the sentinel sites and urge NMCP and partners to investigate the trends in other geographical areas to guide targeted intervention
- In collaboration with the NMCP, PMI VectorLink should continue training staff at the NIHR and AU laboratories to improve capacity for morphological identification of *Anopheles* mosquitoes for focused molecular and immunodiagnostic analyses and build similar morphological identification capacity at sentinel sites for environmental health technicians.
- The NMCP and partners, in collaboration with CDC, AU, and NIHR, should establish *An. funestus* s.s. colonies for reference in bioassay and resistance tests for the 3rd and 4th quarter 2022.
- VectorLink in collaboration with the NMCP should determine the role of each sibling species of the now prevalent *An. funestus* s.l. and other Anopheline species (including *An. rufipes*) in malaria transmission and investigate approaches to control residual transmission.

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7. ANNEX.

Annex 1. Data on Main *Anopheles* Mosquitoes Collected at Burma Valley, Dendera, Makarara, and Kawere Identified with PCR, 2021 shown in Figure 12.

Site	Collection method	*An. funestus* s.l.					*An. gambiae* s.l.			*Other*						
		An. funestus s.s.	*An. leesoni*	*An. parensis*	*An. rivulorum*	*An. rivulorum*-like	*An. arabiensis*	*An. gambiae s.s.*	*An. quadriannulatus*	*An. rufipes*	*An. maculipalpis*	*An. squamosus.*	*An. pretoriensis*	No amplification	Not Applicable	Total
Burma Valley	CDC in	0	0	0	0	0	7	0	2	0	0	0	0	0	0	9
	CDC out	0	0	0	0	0	0	0	4	5	0	0	0	3	2	14
	HLC Proxy-In	0	0	0	0	0	3	0	0	1	0	0	0	2	0	6
	HLC Proxy- Out	0	0	0	0	0	0	0	3	0	0	0	1	3	0	7
	Pit shelter	0	1	1	0	0	0	0	0	1	0	0	0	0	0	3
	Total	**0**	**1**	**1**	**0**	**0**	**10**	**0**	**9**	**7**	**0**	**0**	**1**	**8**	**2**	**39**
Dendera	CDC out	0	0	0	0	0	0	0	2	6	1	0	13	1	1	24
	HLC Proxy-in	0	0	0	0	0	1	0	1	0	0	0	1	0	0	3
	HLCProxy-out	0	0	0	0	0	0	0	8	5	0	0	18	0	1	32
	Pit Shelter	0	0	1	0	0	0	0	4	37	0	0	9	5	0	56
	Total	**0**	**0**	**1**	**0**	**0**	**1**	**0**	**15**	**48**	**1**	**0**	**41**	**6**	**2**	**115**
Kawere	CDC in	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
	CDC out	0	0	2	0	0	0	0	1	8	3	0	1	3	1	19
	HLC Proxy-in	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
	HLC Proxy-out	0	1	3	0	0	1	0	3	3	3	0	0	0	2	16
	PSC	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
	Pit Shelter	0	0	18	0	1	0	0	12	4	0	0	2	1	2	40
	Total	**0**	**1**	**25**	**0**	**1**	**1**	**0**	**16**	**16**	**6**	**0**	**3**	**4**	**5**	**78**
Makarara	CDC in	0	0	1	0	1	0	0	1	2	0	0	0	1	0	6
	CDC out	0	1	0	0	1	0	0	2	2	2	1	12	2	1	24
	HLC Proxy-in	0	3	2	0	2	0	0	5	4	4	0	6	2	1	29
	HLC Proxy-out	0	16	3	0	2	0	0	8	10	5	0	6	3	1	54
	PSC	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
	PPA	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
	Pit Shelter	5	5	67	0	1	3	0	4	16	0	0	4	7	4	116
	Total	**5**	**25**	**73**	**0**	**7**	**3**	**0**	**21**	**34**	**11**	**1**	**28**	**16**	**7**	**231**