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THE PMI VECTORLINK MADAGASCAR ADDENDUM TO THE 2020-2021 FINAL ENTOMOLOGICAL MONITORING REPORT

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Abt Associates Inc. | 6130 Executive Boulevard | Rockville, MD 20852
| T. 301.347.5000 | F. 301.913.9061 | www.abtassociates.com

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Acronyms

EIR	Entomological Inoculation Rate
ELISA	Enzyme-Linked Immunosorbent Assay
HBR	Human Biting Rate
HLC	Human Landing Catch
IPM	Institut Pasteur of Madagascar
KD	Knockdown
NMCP	National Malaria Control Program
ORC	Outdoor Resting Collection
PCR	Polymerase Chain Reaction
PMI	President's Malaria Initiative
RFLP	Restriction Fragment Length Polymorphism
WHO	World Health Organization

1. INTRODUCTION

This addendum report presents results from the laboratory analysis of entomological samples, which were not included in the approved PMI VectorLink Madagascar 2020-2021 Final Entomological Monitoring Report. The molecular analysis work, initially scheduled to be conducted by Institut Pasteur of Madagascar (IPM) or the National Malaria Control Program (NMCP), was later transferred to the Entomological Research Center of Cotonou / Centre de Recherche Entomologique de Cotonou (CREC) laboratory in Benin for completion as IPM switched their priorities to focus more on the COVID-19 pandemic at the time and NMCP was out of reagent. The report, which covers the period of September 2020 to July 2021, includes the following data:

- Molecular species identification of *An. gambiae* s.l. and *An. funestus* s.l. mosquitoes collected during monthly adult collections and those used in insecticide susceptibility tests.
- *Plasmodium* falciparum sporozoite rates and entomological inoculation rates (EIRs).
- Allelic frequencies of genetic markers of insecticide resistance.

2. METHODOLOGY

2.1. VECTOR SPECIES COMPOSITION

A total of 1,218 *Anopheles gambiae* s.l. collected through both collection methods, including human landing catch (HLC) indoors (497) and outdoors (613), and 108 collected using Prokopack indoors (44) and outdoors (64) from all the vector surveillance sites, were identified to the species level. The DNA of each individual *An. gambiae* s.l. mosquito was extracted using the protocol designed by Collins et al. (1987) and analyzed using Polymerase Chain Reaction (PCR) following the protocol described by Scott et al. (1993) to differentiate the subspecies of the complex and the Short-Interspersed Element (SINE) protocol described by Santolamazza et al. (2008) for *An. gambiae* and *An. coluzzii*. A total of 90 *An. funestus* s.l. samples collected from four sites using HLC method and in larger numbers, were further identified at the subspecies level using the protocol of Koekemoer et al. (2002) from DNA extracted using the protocol of Collins et al. (1987).

In addition, 200 randomly selected *An. gambiae* s.l. mosquitoes among the dead per site and the surviving mosquitoes from the WHO susceptibility tests (all pyrethroids) conducted in Morombe, Kiliarivo, Vavatenina, Marofatika and Anamakia were further analyzed for species identification.

2.2. ENTOMOLOGICAL INOCULATION RATES

Plasmodium falciparum infection rates of *An. gambiae* s.l. collected using HLC were determined using enzyme-linked immunosorbent assay (ELISA) method described by Burkot et al and modified by Wirtz et al., for sporozoite detection in the head and thorax of mosquitoes. This method uses a monoclonal antibody that recognizes a repetitive epitope on the circumsporozoite protein *of P. falciparum*. *Plasmodium falciparum* sporozoite ELISA Reagent Kits (MRA-890) were obtained from BIE Resources (NIAID, NIH, USA). Diluted *P. falciparum* sporozoite proteins supplied by the Center for Disease Control (CDC, Atlanta, USA) were used as positive controls, while ground male mosquitoes were used as negative controls. Determination of positive samples were done after reading optical densities (OD) at 405 nm on an ELISA plate reader. Positive samples were determined by OD readings 2-fold greater than the negative controls. The sporozoite rate was calculated as the ratio of the number of circumsporozoite-positive mosquitoes over the total number of mosquitoes analyzed by site. The monthly entomological inoculation rate (EIR) is calculated as the product of the sporozoite rate and the human biting rate per month (HBR). The EIR measures exposure to infectious bites and represents the intensity of malaria transmission. The mean EIR was calculated per month of collection using the HBR and the sporozoite rate recorded after analysis. The mean EIR per sentinel site was later calculated by multiplying the EIR recorded per month by the number of collection months.

2.3. CHARACTERIZATION OF INSECTICIDE RESISTANCE MARKERS

The insecticide resistance markers of 483 mosquitoes identified after insecticide susceptibility tests were assessed to determine the frequency of the mutations associated with resistance among the population tested from Vavatenina and Mahambo. The presence of knock down resistance (*Kdr)*-West and East was characterized using the conventional Polymerase Chain Reaction (PCR) restriction fragment length polymorphism (RFLP) method as described by Martinez-Torres et al. (1999). The protocol described by Weill et al. (2004) was used for the determination of the (acetylcholinesterase) *Ace-1* mutation within the population of each site.

3. RESULTS

3.1. SPECIES COMPOSITION OF *AN. GAMBIAE* COMPLEX

A subset of 1,218 *An. gambiae* s.l. were sampled from the 12 sites surveyed. *Anopheles gambiae* and *An. arabiensis* were the two species found in the different sites with *An. arabiensis* being the predominant species in eight of the sites surveyed. The proportion of *An. arabiensis* varied from 63.4% percent in the locality of Ranotsara Nord in the Iakora district to 100 percent in the three sites of Atsimo Andrefana region (Behaza, Marofatika and Tsaragiso), Anamakia, Irina and Ankilivalo. The lowest proportion of *An. arabiensis* was recorded in Vavatenina (0.9%). Both species were living in sympatry in Mahambo, Vavatenia, Mahasoa, Ranotsara North, Ampasipotsy and Marofarihy with *An. gambiae* predominant in all these sites except in Mahasoa and Ranotsara North (Table 1).

Table 1: Distribution of *An. gambiae* s.l. Species

			An. gambiae s.l. species		
Regions	**Districts**	**Localities**	**# Tested**	*An. gambiae*	*An. arabiensis*
Analanjirofo	Fenerive Est	Mahambo	43	36 (83.7%)	7 (16.3%)
	Vavatenina	Vavatenina	350	347 (99.1%)	3 (0.9%)
Atsimo Andrefana	Betioky	Behaza	60	0 (0.0%)	60 (100%)
	Tuléar II	Marofatika	67	0 (0.0%)	67 (100%)
		Tsaragiso	46	0 (0.0%)	46 (100%)
Diana	Antsiranana I	Anamakia	58	0 (0.0%)	58 (100%)
Haute Matsiatra	Amabalavao	Mahasoa	47	6 (12.8%)	41 (87.2%)
Ihorombe	Ihosy	Irina	42	0 (0.0%)	42 (100%)
	Iakora	Ranotsara North	71	26 (36.6%)	45 (63.4%)
Menabe	Mahabo	Ankilivalo	120	0 (0.0%)	120 (100%)
Vatovavy Fitovinany	Manakara	Ampasipotsy	182	163 (89.6%)	19 (10.4%)
		Marofarihy	132	77 (58.3%)	55 (41.7%)
Total			**1218**	**655 (53.8%)**	**563 (46.2%)**

3.2. SPECIES COMPOSITION OF *AN. FUNESTUS* GROUP

A total of 90 *An. funestus* s.l. were identified to sibling species in four sites. *An. funestus* s.s. was the only species of the group found in the different sites tested (Table 2).

Table 2: Distribution of *An. funestus* Group Species

		***An. funestus* group species**		
Regions	**Districts**	**Localities**	**# Tested**	***An. funestus* s.s**
Analanjirofo	Fenerive Est	Mahambo	27	27
	Vavatenina	Vavatenina	35	35
Atsimo Andrefana	Betioky	Behaza	9	9
Vatovavy Fitovinany	Manakara	Marofarihy	19	19
Total			**90**	**90**

3.3. ELISA CIRCUMSPOROZOITE DETECTION

ELISA CSP tests for *Plasmodium falciparum* only were carried out on the 1,218 *An. gambiae* s.l. including the 655 *An. gambiae* and 563 *An. arabiensis* that were identified above. Three *An. gambiae* s.l. from three different sites (Vavatenina, Mahasoa and Ankilivalo) were found positive for *P. falciparum*. *Anopheles gambiae* s.l. of the nine remaining sites out of the 12 surveyed did not record any mosquito carrying the sporozoite infections. The sporozoite rates recorded in the three sites were 0.0029 (1/350) for Vavatenina within outdoor HLC *An. gambiae* collected mosquitoes, 0.0213 (1/47) indoor HLC *An. arabiensis* collected for Mahasoa and 0.0083% (1/120) for Ankilivalo among the outdoor resting collection (ORC) *An. arabiensis* collected (Table 3).

For *An. funestus* group, the 90 specimens identified as *An. funestus* s.s. and analyzed for ELISA CSP yielded a single infected mosquito (1/35) and recorded in the site of Vavatenina within the indoor HLC collected *An. funestus* s.s. (Table 4).

Table 3: *Anopheles gambiae* s.l. Circumsporozoite Rate per Locality

			Anopheles gambiae s.l.		
Regions	**Districts**	**Localities**	**# Tested**	**CSP+**	**SR**
Analanjirofo	Fenerive Est	Mahambo	43	0	0
	Vavatenina	Vavatenina	350	1	0.0029
Atsimo Andrefana	Betioky	Behaza	60	0	0
	Tuléar II	Marofatika	67	0	0
		Tsaragiso	46	0	0
Diana	Antsiranana I	Anamakia	58	0	0
Haute Matsiatra	Amabalavao	Mahasoa	47	1	0.0213
Ihorombe	Ihosy	Irina	42	0	0
	Iakora	Ranotsara Nord	71	0	0
Menabe	Mahabo	Ankilivalo	120	1	0.0083
Vatovavy Fitovinany	Manakara	Ampasipotsy	182	0	0
		Marofarihy	132	0	0
Total			**1218**	**3**	**0.25**

CSP+ = circumsporozoite positive; SR = sporozoite rate

Table 4: *Anopheles funestus* Group Circumsporozoite Rate per Locality

			Anopheles funestus s.s.		
Regions	**Districts**	**Localities**	**# Tested**	**CS+**	**SR**
Analanjirofo	Fenerive Est	Mahambo	27	0	0.0
	Vavatenina	Vavatenina	35	1	0.0286
Atsimo Andrefana	Betioky	Behaza	9	0	0.0
Vatovavy Fitovinany	Manakara	Marofarihy	19	0	0.0
Total			**90**	**1**	**0.0111**

CSP+ = circumsporozoite positive; SR = sporozoite rate

3.4. Entomological Inoculation Rate (EIR)

The mean EIR representing the product of the HBR and the sporozoite rate was calculated only for the sites where infection was recorded using HLC method (Vavatenina and Mahasoa). The mean EIR was 0.767 and 1.271 infected bites (ib)/person/month in Mahasoa (outdoor) and Vavatenina (indoor) respectively for *An. gambiae* s.l. With the single infected *An. funestus* s.s. recorded in Vavatenina, the mean indoor EIR was 0.343 ib/p/m (Table 5).

Table 5: EIR of Malaria Vectors Collected Using HLC

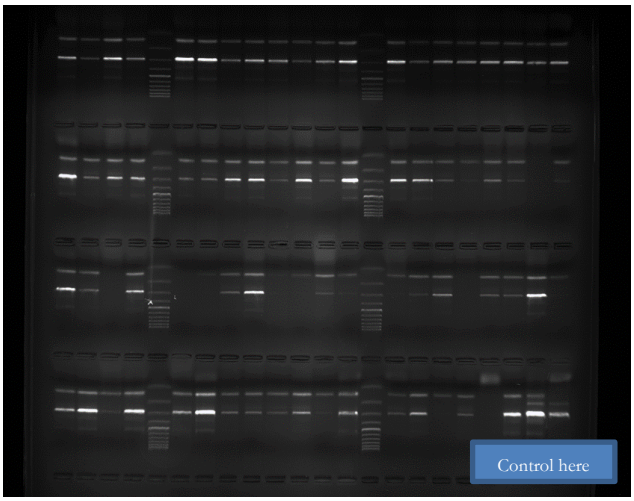
			An. arabiensis				*An. gambiae*		
	Sites	**Area**	**Mean HBR (*An. gambiae* s.l.)**	**Sporozoite Rate**	**EIR (ib/p/n)**	**EIR (ib/p/m)**	**Sporozoite Rate**	**EIR (ib/p/n)**	**EIR (ib/p/m)**
***An. gambiae* s.l.**	**Vavatenina**	Indoor	9.35	0.0	0.0	0.0	0.0	0.0	0.0
		Outdoor	14.61	0.0	0.0	0.0	0.0029	0.0424	1.271
	Mahasoa	Indoor	1.20	0.0213	0.0256	0.767	0.0	0.0	0.0
		Outdoor	1.28	0.0	0.0	0.0	0.0	0.0	0.0
			***An. funestus* s.s.**	**Sporozoite Rate**	**EIR (ib/p/n)**	**EIR (ib/p/m)**			
***An. funestus* s.l.**	**Vavatenina**	Indoor	0.40	0.0286	0.0114	0.343			
		Outdoor	0.78	0.0	0.0	0.0			

3.5. Molecular Detection of Resistance Markers

Molecular markers of resistance were determined among samples of *An. gambiae* ss and *Anopheles arabiensis*, showing phenotypic resistance to pyrethroids (deltamethrin, permethrin or alpha-cypermethrin), from WHO tube test in five localities (Morombe, Kiliarivo, Vavatenina, Marofatika and Anamakia).

Two hundred (200) *An. gambiae* s.l. including 77 *An. gambiae* and 123 *An. arabiensis* from the five localities were analyzed for *kdr*-west and east and *Ace*-1. All mosquitoes were susceptible (SS) in all localities for all three mutations showing that the mutation does not exist in the country (Table 6).

Kdr gel



Ace-1 gel

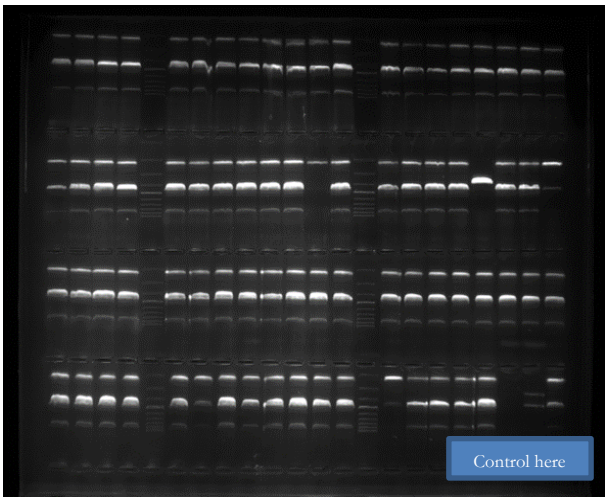


Table 6: Frequency of *Kdr* (West and East) and *Ace-1* of all Tested Localities

						Kdr-West				*Kdr-East*				*Ace-1*			
Regions	**Districts**	**Localities**	***# An. gambiae* s.l.**	**Species**	**#/species and (%)**	RR	RS	SS	*Freq*	RR	RS	SS	*Freq*	RR	RS	SS	*Freq*
Analanjirofo	Fenerive Est	Morombé	50	*An. gambiae*	50 (100.0%)	0	0	50	0	0	0	50	0	0	0	50	0
	Vavatenina	Vavatenina	25	*An. gambiae*	24 (96.0%)	0	0	24	0	0	0	24	0	0	0	24	0
				An. arabiensis	1 (4.0%)	0	0	1	0	0	0	1	0	0	0	1	0
Atsimo Andrefana	Sakaraha	Kiliarivo	25	*An. arabiensis*	25 (100.0%)	0	0	25	0	0	0	25	0	0	0	25	0
	Tuléar II	Marofatika	75	*An. gambiae*	3 (4.0%)	0	0	3	0	0	0	3	0	0	0	3	0
				An. arabiensis	72 (96.0%)	0	0	72	0	0	0	72	0	0	0	72	0
Diana	Antsiranana I	Anamakia	25	*An. arabiensis*	25 (100.0%)	0	0	25	0	0	0	25	0	0	0	25	0
Total			**200**		**200**	**0**	**0**	**200**	**0**	**0**	**0**	**200**	**0**	**0**	**0**	**200**	**0**

RR: Homozygous Resistant, RS: Heterozygous Resistant, SS: Homozygous Susceptible, Freq: Frequency

4. Conclusions

Molecular analysis of entomological samples collected from September 2020 to July 2021 under the PMI VectorLink Madagascar project, including bionomic and susceptibility monitoring in the sentinel sites, showed that *An. gambiae* s.s. was the predominant malaria vector in only four of the twelve sites surveyed while *An. arabiensis* represented the main vector in all the other eight remaining sites and was the only species recorded in five sites.

Anopheles funestus s.s was the only species of the group characterized in the four sites tested with a single mosquito recorded carrying *P. falciparum* circumsporozoite infection in Vavatenina.

Both *An. arabiensis* and *An. gambiae* species were positive for sporozoite infection, but in different sites (Mahasoa, Vavatenina and Ankilivalo), while no infection was recorded in the other sites. *An. gambiae* s.l. represents the main vector species found in the surveyed sites. The *An. gambiae* and *An. arabiensis* entomological inoculation rate was low in all sites where *Plasmodium* infection was recorded and ranged between 0.0256 ib/p/n indoors in Mahasoa for *An. arabiensis* and 0.0424 ib/p/n outdoor in Vavatenina with *An. gambiae*. This trend may be discordant with malaria endemicity recorded in Madagascar, where malaria is considered to be an endemic disease affecting most of the population, because it does not represent a complete picture of vector diversity and sporozoite rates and is based on few collections.

The *kdr*-east, *kdr*-west and the *Ace*-1 mutations were absent within the vector populations of all localities tested and correlate with the phenotypic status of the mosquitoes recorded during the WHO susceptibility test. However, caution will need to be taken and close follow-up to be considered as few sites are now showing possible resistance to deltamethrin and confirmed resistance to either alpha-cypermethrin or permethrin, as described in the annual report. However, the outcomes could still support the deployment of any recommended vector control interventions using any of the tested insecticides.

The findings of the species composition, sporozoite rates and insecticide resistance allele detections of the 2020-2021 samples analyzed by CREC in Benin were similar to those reported for 2019-2020 samples that were run by the NMCP molecular laboratory in Madagascar. This is a good sign and encouraging to boost the country's capacity to sustain the analyses of the samples locally. This will help improve the number of mosquitoes that could potentially be analyzed molecularly for recording data that could better explain the malaria transmission in Madagascar.

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