PMI | Africa IRS (AIRS) Project

Indoor Residual Spraying (IRS 2) Task Order Six

AIRS NIGERIA
FINAL ENTOMOLOGY REPORT

NOVEMBER 2014 - DECEMBER 2015

Recommended Citation: *AIRS Nigeria Final Entomology Report. November 2014 – December 2015,* Africa Indoor Residual Spraying Project, Abt Associates Inc.

Contract: GHN-I-00-09-00013-00

Task Order: AID-OAA-TO-11-00039

Submitted to: United States Agency for International Development/PMI

Abt Associates Inc. | 4550 Montgomery Avenue | Suite 800 North | Bethesda, Maryland 20814 | T. 301.347.5000 | F. 301.913.9061 | www.abtassociates.com

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ACRONYMS

CDC	Centers for Disease Control and Prevention
CDC LT	CDC Light Trap
ELISA	Enzyme-Linked Immunosorbent Assay
HLC	Human Landing Catch
IRD	Indoor Resting Density
IRS	Indoor Residual Spraying
ITN	Insecticide-treated Net
IVM	Integrated Vector Management
Kdr	Knock down resistance
LGA	Local Government Area
NMEP	National Malaria Elimination Program
PBO	Piperonyl butoxide
PCR	Polymerase Chain Reaction
PMI	President's Malaria Initiative
PSC	Pyrethrum Spray Catch
WHO	World Health Organization

EXECUTIVE SUMMARY

Background

The President's Malaria Initiative (PMI) supported entomological surveillance in six sentinel sites namely Enugu, Lagos, Nasarawa, Plateau, Rivers and Sokoto States from November 2014 – October 2015. AIRS Nigeria captured PMI entomological indicators from all sentinel sites. Additionally, information the collected from these sites was for the purpose of supporting the National Malaria Elimination Program (NMEP) in making data-driven decisions for programming vector control activities. All teams across the six sentinel sites of Nigeria used PSC and Human - baited CDC light trap collections (indoor and outdoor) to sample mosquitoes and determine key entomological indicators. To measure insecticide resistance, all teams conducted WHO tube and CDC tests, and insecticide resistance intensity assays. Molecular characterization included identification of *An. gambiae* M and S sibling species, *kdr* and metabolic resistance mechanisms.

Methods

Entomological surveillance activities were carried out using human- baited CDC light trap methods (placed indoors and outdoors) in two houses for three nights per sentinel site to measure mosquito biting time. All teams systematically sampled 32 houses per sentinel site per month using the PSC method to sample indoor-resting mosquitoes. Parity rates were determined by dissecting the ovaries from randomly selected unfed female *An. gambiae* s.l. specimens collected using human- baited CDC light traps. Molecular identification of Anopheles mosquitoes collected from the six vector surveillance sites were conducted using the Polymerase Chain Reaction (PCR). Plasmodium infection rate in the mosquito population was estimated through Enzyme-Linked Immunosorbent Assay (ELISA) tests for Plasmodium falciparum. Insecticide susceptibility tests were carried out using the standard WHO protocol and CDC bottle bioassay (to determine phenotypic resistance of An. gambiae s.l. to the four classes of WHOPES) – approved IRS insecticides which include alphacypermethrin, deltamethrin, permethrin and lambda-cyhalothrin (all pyrethroids), bendiocarb and propoxur (carbamates), pirimiphos-methyl (organophosphate) and DDT (organochlorine). Resistance intensity was determined through intensity assays carried out following the Center for Disease Control and Prevention (CDC) protocol with three to four day old adult Anopheles mosquitoes using four different concentrations of deltamethrin (x1, x2, x5 and x10). Resistance mechanism analysis was conducted to identify underlying resistance mechanism(s) and to estimate the frequency of the knock down resistance (kdr) gene in the mosquito population in Nasarawa Eggon (Nasarawa) and Epe (Lagos) sentinel sites. Synergist test was conducted to investigate the plausible role of metabolic enzymes in insecticide detoxification in the resistant mosquito population from Nasarawa and Lagos sentinel sites. Synergist assay was done using piperonyl butoxide (PBO) an inhibitor of mixed function oxidase on *Anopheles gambiae* from each site. The presence of the knockdown resistance (kdr) mutation using allele-specific PCR diagnostic tests designed for the West African *kdr* mutation was used. The proportion of the molecular M and S form of

Anopheles gambiae from samples collected at Nasarawa and Lagos sentinel sites were also determined using established protocols.

Results

Vector seasonality

Overall, significantly higher numbers of *An. gambiae* s.l. were collected indoors than outdoors across all sentinel sites. The primary vector across all six sentinel sites was *An. gambiae* s.l.. The presence of *An. funestus* was also observed markedly in two sentinel sites of Plateau and Enugu states. Other secondary vectors collected were *An. pharoensis* and *An. coustani.* Although overall, consistently higher numbers of indoor resting mosquitoes were observed in Enugu and Plateau States as compared to the other sentinel sites, indoor biting peaks were observed to be highest in Sokoto.

Of the six sentinel sites the highest indoor collections from PSC were recorded in Sokoto in the Sahel/Sudan savannah. Peak collections were recorded in the months of August, September and October with mean IRD ranging from 0.3-38.8. In Plateau state, which is located in the Guinea savannah, peak collections from PSC were recorded in the months of June and August with mean IRD ranging from 0.6 in the month of February increasing to a peak of 23.1in the month of August.

Nasarwa Eggon recorded higher numbers in November, January, and February with mean IRD ranging from 3.4-13.9.

In both ecozones peak IRD was in the month of August . In River state located in the mangrove the mean IRD ranged from 0.6 in December to 3.2 in August while in Enugu (Rain forest) the mean IRD ranged from 0.2 in January to 6.9 June . In Lagos (coastal mangrove) the mean IRD ranged from 0.1 in February to 1.4 in June .

Parity rate

Parity rate was determined at two sites where there is capacity for dissection. *An. gambiae* s.l. caught at Enugu and Nasarawa sentinel sites were dissected for parity. Between the months of November 2014 and October 2015, significantly higher rates of parity were observed in both Doma and Nasarawa Eggon in the Guinea savannah than Enugu in the rainforest (Fishers exact test $p<0.0001$). Parity rates were both high and similar in August, September and October in both ecozones. Between April and June 2015, a significant increase in parity was observed in Enugu while parity in both Doma and Nasarawa Eggon state remained low. This could be due partly to seasonal variations as the rains peaked.

Insecticide resistance

Both WHO tube tests and CDC bottle bioassay methods were used to determine the susceptibility level of the vector population across the different ecological zones. Findings indicated that local mosquitoes (*An. gambiae* s.l) were found to show resistance to DDT (organochlorine) across all six sentinel sites. *An. gambiae* s.l. was found also to be resistant to the pyrethroids lamdacyhalothrin, deltamethrin, and permethrin across all sites with the exception of alphacypermethrin to which local mosquitoes showed susceptibility in Rivers. In the carbamate class susceptibility to bendiocarb was observed across all sentinel sites except Sokoto state while propoxur and pirimiphos -methyl (organophosphate) showed susceptibility in Lagos. The low mortality of mosquitoes exposed to pirimiphos-methyl in other sites may be associated with issues of stability of insecticide used for the tests.

Resistance intensity assays

Resistance intensity assays showed variations in intensity across the six sentinel sites. There was high intensity resistance to deltamethrin (survival at x5 and x10 dosage in three sentinel sites of Lagos, Plateau and Rivers), while susceptibility was at x2 was observed in Enugu, Nasarawa and Sokoto.

Molecular results

PCR analysis showed that *Anopheles gambiae* s.s. was the predominant member of the *Anopheles gambiae* complex representing 78 to 100% of the population at the different sites. *Anopheles arabiensis* was the other member of the complex identified by PCR but absent in Enugu and Rivers sentinel sites. Overall, 85.7% of mosquitoes that were PCR positive were *An. gambiae* s.s. while *An. arabiensis* represented 14.3%. PCR analyses to determine the proportion of the Molecular M (*Anopheles coluzzii*) and S-form (*Anopheles gambiae* s.s) at each site indicated that the molecular M and S form of *Anopheles gambiae* occurred in sympatry at both Nasarawa and Lagos sites with the S form being predominant and representing 79.2 and 68. 2% in Nasarawa and Lagos respectively. ELISA analysis for sporozoite infection indicated that infection rate was highest (7.8%) in Sokoto followed by Enugu (6.6) and Lagos (5.5). In Nasarawa sentinel site only 1.8% of the samples were positive for *Plasmodium* infection. Aside from Sokoto with 0.6% infected *An. arabiensis* none of the *Anopheles arabiensis* from the others 5 sites were infected with *P. falciparum*. Kdr PCR assay shows the presence of the West kdr mutation (*kdr-w*) in 3.3% of samples collected indoor at Nasarawa but in 27.0 % of samples that survived insecticide exposure. Similar findings were found in samples from the Lagos site with the kdr present in 10.0 % of sample collected indoor as against 28.8% in sample that survived the insecticide exposure. At each site the homozygote resistant state (RR) predominated the allelic frequency. The overall frequency of the *kdr-w* was 0.263 and 0.326 in Nasarawa and Lagos respectively. Interestingl,y none of the molecular M form in Nasarawa or Lagos was positive for the kdr mutation. PCR tests using primers designed for the East African kdr mutation did not show any positive case of the *kdr-e* in all the samples from both sites.

Conclusions

A total of 85.7% of mosquitoes that were PCR positive were *An. gambiae* s.s. while *An. arabiensis* represented 14.3% across the six sentinel sites. PCR analyses was conducted to determine the proportion of the molecular M and S-form at each site indicated that the molecular M and S form of *Anopheles gambiae* occurred in sympatry at both Nasarawa and Lagos sites with the S form predominantly representing 79.2 and 68. 2% in Nassarawa and Lagos respectively. Insecticide resistance data shows very high pyrethroid resistance across all sentinel sites, only the molecular S form in Nasarawa and Lagos were positive for the kdr mutation. Interestingly none of the molecular M form in Nasarawa or Lagos were positive for the kdr mutation.

1. INTRODUCTION

In September 2014, Abt Associates was awarded a new contract to implement indoor residual spraying (IRS) under IQC IRS 2 Task Order Six (TO6) in up to 17 African countries. The Nigeria program is included to continue entomological activities started under the TO4 contract. In 2015, PMI supported entomological surveillance in six sentinel sites namely Enugu, Lagos, Nasarawa, Plateau, Rivers and Sokoto States. AIRS Nigeria captured PMI entomological indicators in all sentinel sites and information collected from these sites are meant to support the National Malaria Elimination Program (NMEP) in making data-driven decisions for programming vector control activities. This report provides information on the entomological monitoring activities completed between November 2014-October 2015.

The Africa Indoor Residual Spraying (AIRS) Nigeria program, funded by the President's Malaria Initiative (PMI) supported entomological surveillance in six sentinel sites across a geographic transection of all five ecological zones. The sites, in South West, South East, South, North West, and two in North Central geopolitical zones, were selected from 18 sites proposed by the National Malaria Control Program. The objectives for work in the sentinel sites were to:

- Identify malaria vectors in the sites, (using both morphological identification keys and molecular assays.
- Determine Sporozoite rates
- Establish vector density and seasonality,
- Monitor vector feeding period and time in the sentinel sites,
- Determine vector susceptibility and mechanism of resistance
- Determine intensity of resistance among local malaria vectors.

2. MONITORING VECTOR BEHAVIOR AND DENSITY

2.1 OVERVIEW

Nigeria's National Malaria Elimination Program (NMEP) of the Federal Ministry of Health in collaboration with PMI - AIRS Nigeria, established pioneer malaria vector surveillance sentinel sites in six states (Figure 1 and Table 1). The sentinel sites are linked to universities/research institutions located in the same states. Some of the institutions were selected based on their proximity to the Drug Therapeutic and Efficacy Trial sentinel sites that the NMEP established in the early 1990s. They were also selected because they have the human capacity, facilities, and basic equipment for entomological work.

Each sentinel site had a team made up of a Principal Investigator and eight technicians, who carried out the surveillance work, including the determination of indoor resting densities (IRDs) with Pyrethrum Spray Catches (PSC), mosquito biting time and location (indoor/outdoor) using CDC light traps, and mosquito identification and preservation in Eppendorf tubes. Protocols for the mosquito collections are as approved in the 2015 work plan and described below. In 2015, a sentinel site was established in Sokoto to replace Jigawa which was dropped due to security concerns as well as for underperformance by the Principal Investigator. Furthermore, the teams produced monthly data - and submitted them to the AIRS Project alongside all mosquito samples collected. The PMI AIRS Nigeria team monitored and supervised the data collection procedures in the field during the months of surveillance, ensuring that the surveillance was carried out as stipulated in the work plan.

Table 1: Sentinel Sites Supported by PMI in 2014 and 2015

	Sentinel Sites	Zone	Ecozone
1	Lagos	South West	Coastal/mangrove
2	Enugu	South East	Rainforest
3	Rivers	South	Mangrove/forest
4	Sokoto	North West	Sahel/Sudan savannah
5	Plateau	North Central	Guinea savannah
6	Nasarawa	North Central	Guinea savannah

Table 2: Number of months suitable for Malaria Transmission in Nigeria

Zone	Eco zone	Number of Months suitable for Malaria Transmission	Months suitable for Malaria Transmission	Sentinel Sites within the zone
South	Mangrove/forest	8 1/2 Months	March, April, May, June, July, August, September, October, November	Rivers
South West	Coastal/mangrove	7 1/2 Months	April, May, June, July, August, September, October, November	Lagos
South East	Rainforest	7 1/2 Months	April, May, June, July, August, September, October, November	Enugu
North Central	Guinea savannah	6 1/2 Months	May, June, July, August, September, October, November	Nasarawa
North Central	Guinea savannah	5 Months	May, June, July, August, September	Plateau
North West	Sahel/Sudan savannah	3 Months	July, August, September	Sokoto

Source : Ayanlade *et al*. (2010)

2.2 CLIMATIC CONDITIONS AND VECTOR CONTROL INTERVENTIONS AT THE SENTINEL SITES

Nigeria is a tropical country which lies on the southern coast of West Africa between latitudes 4^0 and 14^0N of the equator and longitudes 2^0 45' and 15^0 30'E of the Greenwich meridian. According to Ayanlade *et al*. (2010) two climatic regimes are associated with the Inter-Tropical Discontinuity experienced in Nigeria: the wet and the dry seasons. These two seasons are highly influenced by the two prevailing air masses blowing over the country at different times of the year. These include the warm and moist tropical maritime air mass (Southwesterlies) that originate in the Atlantic Ocean and bring rainfall, while the other is the cool dry and dust-laden continental air mass (North Easterly winds) that originates from the Sahara Desert.

Table 3: Nigeria LLIN Replacement Campaign 2014 (PHASE 1 and 2)

CAMPAIGN DATA SUMMARY (as at 25/02/2015)							
SN	State	Campaign Date	LLIN Distributed	Net Retention In Households	End Process Hang Up	End Process Use (PM)	End Process Use (CU5)
1	Enugu	Apr-11	1,367,506	98.00%	58%	70%	69%
3 2	Sokoto	Dec-13	2,490,061	NA	NA	NA	NA
3	Nasarawa	Oct-14	1,617,399	98.10%	66%	75%	66%
4	Rivers	Oct-14	2,784,319	93.00%	63%	85%	82%
5	Plateau	Mar-15	2,065,653	98.50%	44.80%	52.60%	52.80%
6	Lagos	2011	4,194,464	94.7%	41%	58.3%	45.3%
	TOTALS		14,519,402				

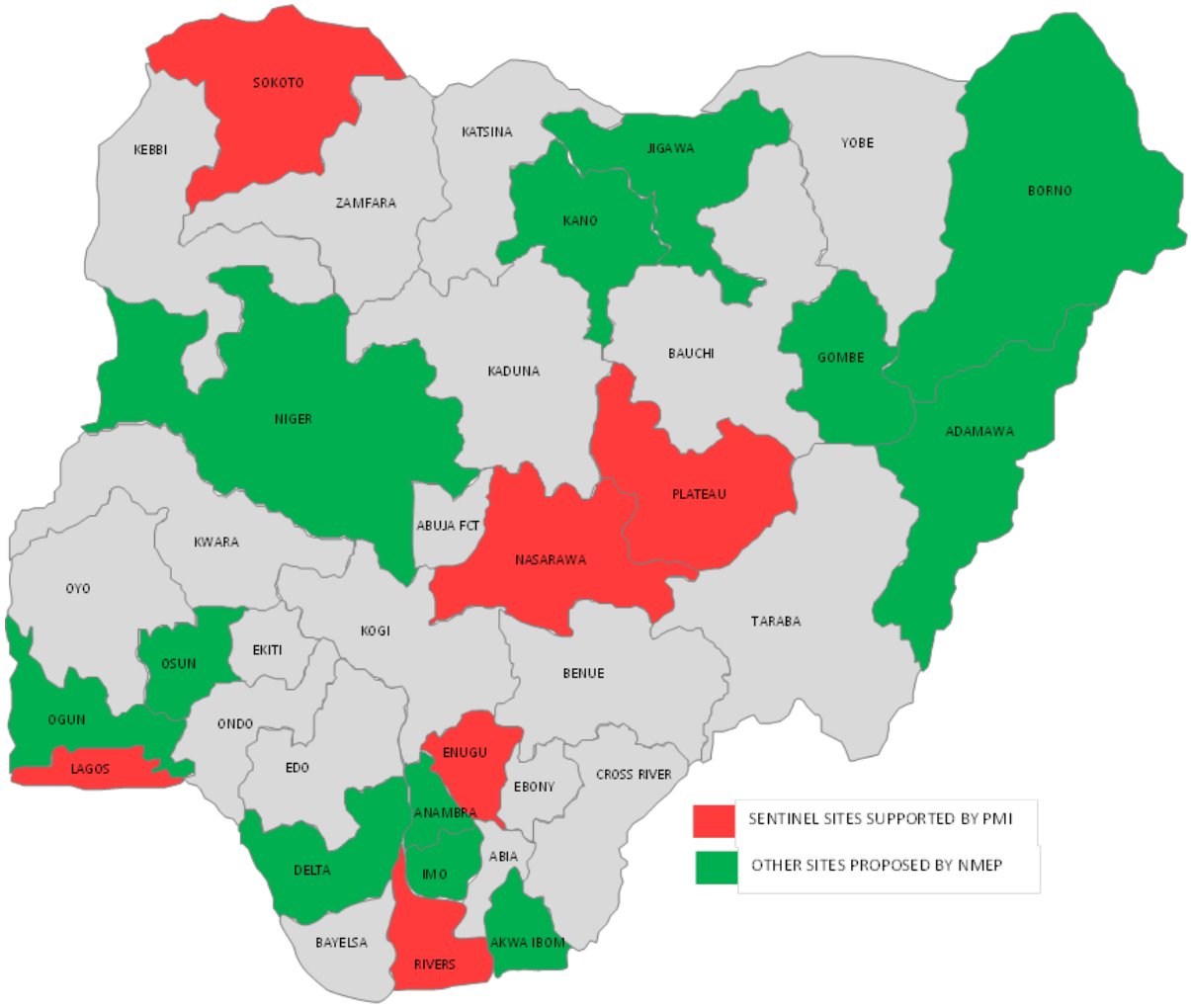
(Source: NMEP unpublished data)

NB: Net Retention in Households: Percentage of nets retained by households after distribution
End process hang up: Percentage of nets hanging in the households after distribution
End process use (PM): Percentage of pregnant mothers who slept under the net the previous night.
End process use (CU5): Percentage of children under 5 years who slept under the net the previous night

3. COLLECTION METHODS

Bi- monthly collections were carried out in the various sentinel sites using PSCs and CDC Light trap methods. Prokopac aspirators were used in collecting adult indoor resting mosquitoes for resistance intensity assays. Anopheline larvae were collected using ladles.

Figure 1: Sentinel Sites Supported By PMI 2015 for Monitoring Mosquito Density and Behavior



3.1 CDC Light Trap Collection

CDC light trap methods (baited traps, one placed indoors and one outdoors) were used in two houses for three nights each month per sentinel site to measure mosquito biting time and location. The light trap bag was replaced every hour by two mosquito collectors from 18:00 to 06:00 per house per night in order to have proxy estimate on the peak biting time. One collector worked from 18:00 to 24:00 and was replaced by a second collector both indoor and outdoor from 24:00 to 06:00 following the methods of Yohannes and Boelee (2012). The trap was placed close to the leg of a person sleeping under an untreated bed net both indoors and outdoors with paper cups changed hourly. The mosquitoes were kept in separate labeled paper cups for identification and further analysis.

3.2 Pyrethrum Spray Catches

The teams systematically sampled 32 houses per sentinel site per month using the PSC method as described by the WHO (1975) to sample indoor-resting mosquitoes. The houses were sampled by two people, one inside and the other one outside, using an aerosol insecticide (Raid) containing the active ingredients of 0.250 percent Allethrin, 0.150 percent Tetramethrin, 0.015 percent Deltamethrin and 99.585 percent inert ingredients. The two sprayers began spraying at the same time as they moved in opposite directions spraying inside the room as well as the eaves outside of the house. The door was then closed for 15 minutes, and opened so the technicians could enter and collect mosquitoes. Mosquitoes that were knocked down were collected from the white cloth sheets that the technicians had laid down prior to spraying. They collected the mosquitoes using forceps and placed them in petri dishes containing damp filter paper. Anopheline mosquitoes were kept on damp absorbent paper in a cool box and later identified to the species level by morphological criteria (Gillies and De Meillon 1968; Gillet 1972; Gillies and Coetzee 1987; Kent, 2006). All samples collected from the field were sent to the centrally located insectary at Nasarawa State University Keffi for further processing and analysis.

3.3 Identification of Malaria Vectors

The *Anopheles* mosquitoes collected using human - baited CDC light traps and PSC were preliminarily identified to the species level morphologically (Gillies and De Meillon 1968; Gillet 1972; Gillies and Coetzee 1987; Kent, 2006). All *Anopheles* specimens that were not dissected were labeled and stored individually in Eppendorf tubes over silica gel for further processing. All samples collected from the field were sent to the centrally located insectary at Nasarawa State University Keffi.

3.4 Determination of Parity

Ovaries from randomly selected female unfed *An. gambiae* s.l. specimens captured by human baited CDC light traps were dissected to determine their physiological age and parity rate as described by Gillies and Wilkes (1963) and WHO (2003). Parity was conducted only in places where the technical expertise was confirmed (Nasarawa and Enugu sentinel sites). Parity was established by observing the degree of coiling of ovarian tracheoles (Detinova 1962, Detinova and Gillies 1964). The parity rate was obtained by determining the number of parous females and dividing by the total number of mosquitoes examined (WHO 2013).

3.5 PCR IDENTIFICATION OF MEMBERS OF THE ANOPHELES GAMBIAE COMPLEX

Anopheles gambiae s.l. mosquitoes collected from the six vector surveillance sites were analyzed for species identification using the Polymerase Chain Reaction (PCR). The samples were collected using pyrethrum spray collection and CDC light trap collection indoors or outdoors. This was a subset of all *An. gambiae* s.l. mosquitoes caught and represented approximately 10% of samples caught across the sentinel sites both indoors and outdoors. All members of the *Anopheles gambiae* complex were analyzed using a standard method. Extracted DNA was amplified using the *Anopheles gambiae* species-specific multiplex PCR (Scott *et al.,*1993). PCR products were separated in agarose gel, stained with ethidium bromide and visualized under UV trans-illuminator. The PCR diagnosis bands for this assay include: a 464 base pair (bp) band for *Anopheles melas*, 390 bp for *An. gambiae* s.s. and 315 bp for *An. arabiensis.* All PCR negative tests were repeated for confirmation.

3.6 *PLASMODIUM* SPOROZOITES ASSAY

To estimate *Plasmodium* infection rate in the mosquito population, Enzyme-Linked Immunosorbent Assay (ELISA) tests for *Plasmodium falciparum*, *Plasmodium ovale* and *Plasmodium malariae* were carried out on a proportion of mosquitoes collected from the field. Head and thorax of each female *Anopheles* mosquito was crushed in Phosphate Buffered Saline (PBS) and tested for the circumsporozoite antigen using an ELISA assay (Burkot *et al.,* 1984). It should be noted that negative samples were re-analyzed prior to final scoring for either PCR or ELISA assay.

4. RESULTS

4.1 ANOPHELINE SPECIES COLLECTED BY THE DIFFERENT COLLECTION METHODS

During the study period Between November 2014 and October 2015, the study teams using baited CDC light traps and PSC sampling methods collected a total of 14,702 *Anopheles* mosquitoes from six sentinel sites. Detailed data are included in Annex A. The species composition of collected mosquitoes follows:

- 13,692 *An. gambiae* s.l.
- 171 *An. funestus*
- 720 *An. coustani/ziemanni*
- 77 *An. nili*
- 21 *An. pharoensis*
- 21 *An. moucheti*

A total of 13,692 (93.13 percent) were *An. gambiae* s.l and 171 (1.16 percent) were *An. funestus*. The difference between the two major vectors, *An. gambiae* s.l and *An. funestus,* was statistically significant ($p<0.0001$). Other species caught were *An. coustani* 720 (4.89 percent), *An. nili* 77 (0.52 percent), *An. pharoensis* 21(0.14) (0.14 percent) and *An. moucheti* 21 (0.14 percent) etc. *An. gambiae* s.l. was common in all the six sites while *An. coustani* was collected in Enugu, Nasarawa and Plateau states, *An. moucheti* was collected from Enugu site only. *An. pharoensis* was only collected from Nasarawa and Sokoto (Annex A-1).

4.2 PYRETHRUM SPRAY CATCH

Between November 2014 and October 2015, the study teams using PSC sampling methods collected a total of 7,882 *Anopheles* mosquitoes. Tables 4- 5 show the PSC results, detailed data is included in Annex A (A-8 to A-15). The mean indoor resting density of female *An. gambiae* s.l. Indoor resting Density (IRD) across the six sentinel sites indicated that higher indoor resting mosquitoes were recorded in Plateau in the months of December, April, and June while Nasarwa Eggon recorded higher numbers in November, January, and February with mean IRD ranging from 3.4-13.9 (Table 4) compared to the other sentinel sites. The highest IRD of 35.8, 38.8, and 16.8 recorded in August, September and October were observed in Sokoto (Figure 2).

The average number of female anophelines found per structure were highest in Sokoto (16.05), followed by Plateau (7.10) and Nasarawa (5.34). The proportion of female anophelines that were fed per structure were also highest in Sokoto state (8.07) and was closely followed by Plateau (6.27) (Table 4).

Figure 2:Indoor Resting Density for all Sentinel Sites, November, 2014 to October 2015

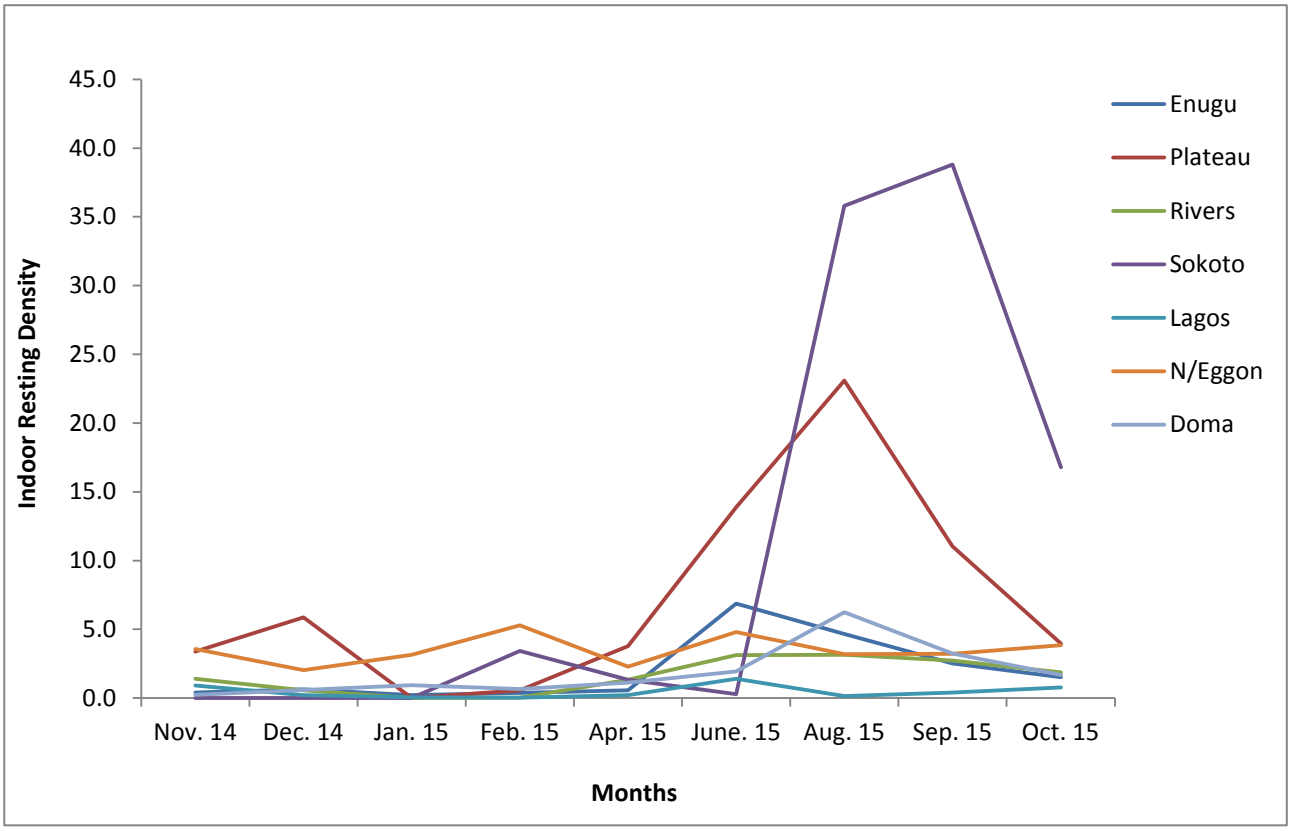


Table 4: Abdominal/Blood digestion stages of mosquitoes collected by PSC, November, 2014 – December, 2015

	# of Structures	# of Occupants	*An. gambiae* s.l. collected	Abdominal/Blood Digestion Stages				Total (HG+G)	Proportion of gravid (HG+G/ UF+HG+G+F)	Female per Structure	Fed per Structure	Fed/human host
				UF^	F^	HG^	G^					
Enugu	288	434	544	15	365	102	62	164	30%	1.89	1.27	0.84
Plateau	288	545	2045	73	1807	56	109	165	8%	7.10	6.27	3.32
Rivers	288	449	455	25	354	38	38	76	17%	1.58	1.23	0.79
Sokoto	192	1259	3082	241	1549	375	917	1292	42%	16.05	8.07	1.23
Nasarawa	288	774	1538	255	691	335	257	592	38%	5.34	2.40	0.89
Lagos	288	849	134	7	119	4	4	8	6%	0.47	0.41	0.14

^ UF – un-fed, F-fed, HG-half-gravid, G - gravid

4.3 HUMAN - BAITED CDC LIGHT TRAP COLLECTIONS

Overall results indicated that higher numbers of *An. gambiae* s.l. were collected indoors than outdoors using CDC light trap method and the difference was statistically significant (χ^2 = 7.220 , df =1; p=0.0072) (Annex A (A-1 to A-7))

In Enugu, a total of 319 (72.67 percent) *Anopheles gambiae* s.l. were collected indoors while 120 (27.33 percent) *An. gambiae* s.l. were collected outdoors. significantly higher number of mosquitoes were collected indoors than outdoors (χ^2 = 40.500, df =1; p<0.0001)

In Lagos State, no significant difference were observed between indoor and outdoor collections (χ^2=0.20; df =1; p= 0.8875) while in Plateau significantly higher of *Anopheles gambiae* s.l were collected indoors than outdoors (χ^2=56.180, df=1, p<0.0001).

In Rivers State, *Anopheles* mosquitoes collected indoors were not significantly different from outdoor collections (χ^2 =3.38; df =1; p=0.0660) .

In Nassarawa state, *An. gambaie* s.l were collected from two LGA of Doma and Nasarawa Eggon . Overall significantly higher numbers of *An. gambaie* s.l were collected outdoors than indoors (χ^2= 29.29, df=1; p<0.0001). The same was observed in *An. coustani* (χ^2= 84.70, df=1; p<0.0001) with *An. coustani* pre

dominantly found in Doma Local Government Area. Unlike in Doma, members of the *An. gambiae* complex were significantly more abundant in Nasarawa Eggon (p<0.0001)
In Sokoto, although higher numbers of *An. gambiae* s.l were collected indoors than outdoors using the human baited CDC light traps, there was no statistical difference

between mosquitoes collected indoors and outdoors (χ^2 =1.32; df =1; p=0.25) (Annex A (A-1 to A-7)(Table 5) .

Table 5: Percentage Total of Mosquitoes Caught in all Sentinel Site using the CDC Light Trap Method

Mosquito Species		*An. gambiae S.l.*	*An. funestus*	*An. nili*	*An. coustani*
Enugu	In	73%	42%	77%	26%
	Out	27%	58%	23%	74%
	P-Value	0.0001S*	0.034S	0.0001S	0.0001S
Lagos	In	51%			
	Out	49%			
	P-Value	0.88NS**			
Nasarawa	In	48%	0%	100%	29%
	Out	52%	100%	0%	71%
	P-Value	0.67NS	0.0001S	0.0001S	0.0001S
Plateau	In	77%	65%		68%
	Out	23%	35%		32%
	P-Value	0.0001S	0.0001S		0.0001S
Rivers	In	57%			
	Out	43%			
	P-Value	0.67NS			
Sokoto	In	53%		20%	
	Out	47%		80%	
	P-Value	0.47NS		0.0001S	

*NB: *S= Significant; **NS= Not significant*

Table 6:Total Number of anophelines Caught in all Sentinel Sites, November, 2014 – October, 2015

Mosquito Species	Enugu			Lagos			Nasarawa			Plateau			Rivers			Sokoto			Total			Overall Total
	CDC		PSC	CDC		PSC	CDC		PSC	CDC		PSC	CDC		PSC	CDC		PSC	CDC		PSC	
	In	Out	In	In	Out	In	In	Out	In	In	Out	In	In	Out	In	In	Out	In	In	Out	In	
An. gambiae S.l.	319	120	544	93	89	134	1116	1212	1538	1331	393	2045	290	215	455	376	340	3082	3525	2369	7798	13692
An. funestus	25	34	22				0	1	0	34	18	31				0	0	6	59	53	59	171
An. coustani	8	23	0				138	339	0	144	68	0							290	430	0	720
An. Nili	49	15	2				1	0	0							2	8	0	52	23	2	77
An. pharoensis							13	7	0							1	0	0	14	7	0	21
An. flavicosta										1	2	0							1	2	0	3
An. malculipalpis	0	1	0							2	3	0							2	4	0	6
An. Moucheti	7	13	1																7	13	1	21
An. pretoriensis				0	11	0				4	4	8							4	15	8	27
An. rufipes										7	6	14							7	6	14	27
An. squamosus	2	0	0				4	1	0	0	3	0							6	4	0	10
An. ziemanni	1	0	0																1	0	0	1
Others										3	4	0							3	4	0	7
Grand Total	411	206	569	93	100	134	1272	1560	1538	1526	501	2098	290	215	455	379	348	3088	3971	2930	7882	14783

4.4 BITING TIME AND LOCATION ACROSS SIX SENTINEL SITES

In Enugu State, higher indoor collections were recorded than outdoors from the months of November 2014 through to October 2015. Indoor peak biting time was recorded from 1-2 am (Figure 3).

Higher numbers of anopheline bites in Lagos State occurred outdoors from the months of November 2014 through October 2015 with peaks at 11-12pm and 2-3 am. Generally the difference between bites indoors and outdoors was not statistically significant. (χ^2= 0.18, df=1; P=0.67)(Figure 4).

In Plateau State, significantly higher indoor collections were recorded than outdoors from the month of November 2014 through October 2015 (χ^2=518.32; df=1; P<0.0001). Indoor biting peaked in the month of December with the onset on the harmattan before drastically reducing as the harmattan progressed through February 2015. From April through to October peak biting times were 11pm-4a.m indoors and 12-1 a.m outdoors (Figure 5).

In Rivers State, the highest indoor peak biting time was recorded between 3-4 am, while outdoor peaks were recorded between 8-9pm and 1-2 am. (Figure 6).

Peak biting time indoors was recorded between 10-11pm and 12-1am in Doma whereas in Nasarawa Eggon, the peak biting time indoors was 12-1am and 4-5am respectively. Significantly higher indoor biting than outdoors were recorded in Doma through the months of collection (Figure 7), whereas in Nasarawa Eggon, this was not consistently the case.

Slightly higher outdoor biting activities than indoors were observed from the hours of 6-9pm with a peak at 10-11 pm from the months of November through to October. The indoor peak biting time was recorded between 4-5 a.m (Figure 8). In Sokoto, slightly higher outdoor biting activities than indoors were observed from the hours of 6-9pm with a peak at 4-5 a.m from the months of November through to October. The indoor peak biting time was recorded between 5-6 a.m (Figure 9).

Figure 3: Biting Trend of *An. gambiae* s.l., Enugu Sentinel Site, November, 2014 – October, 2015

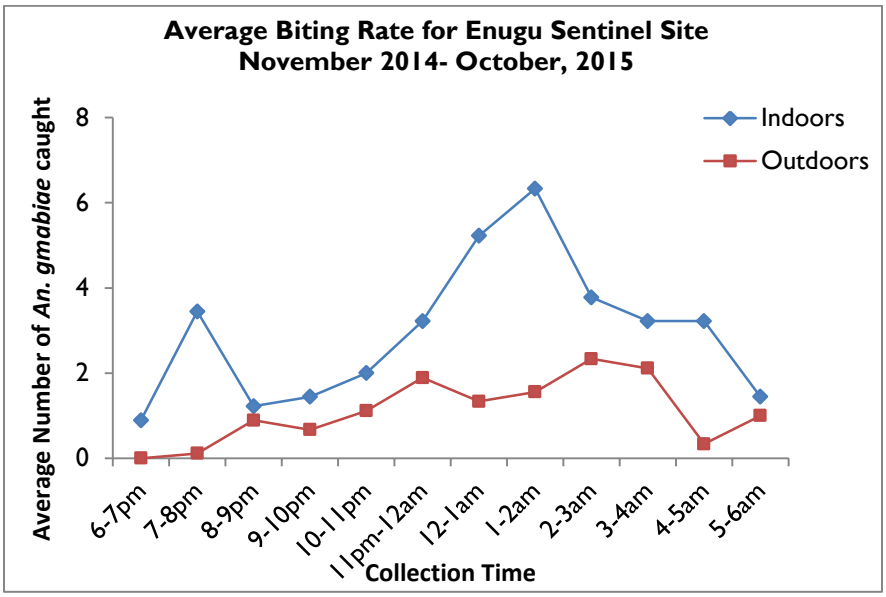


Figure 4: Biting Trend of *An. gambiae* s.l ,Lagos Sentinel Site, November, 2014 – October, 2015

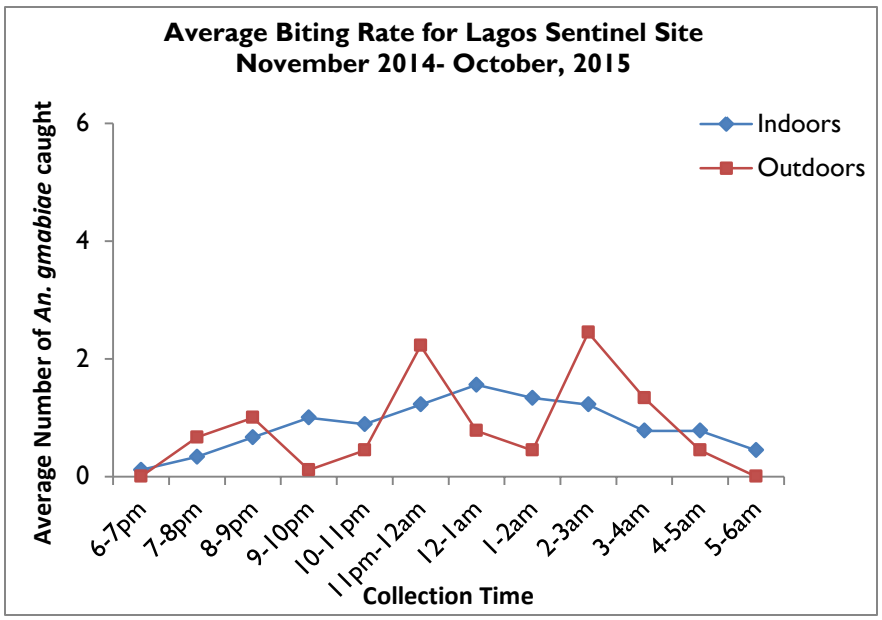


Figure 5: Biting Trend of *An. gambiae* s.l. , Plateau Sentinel Site, November, 2014 – October, 2015

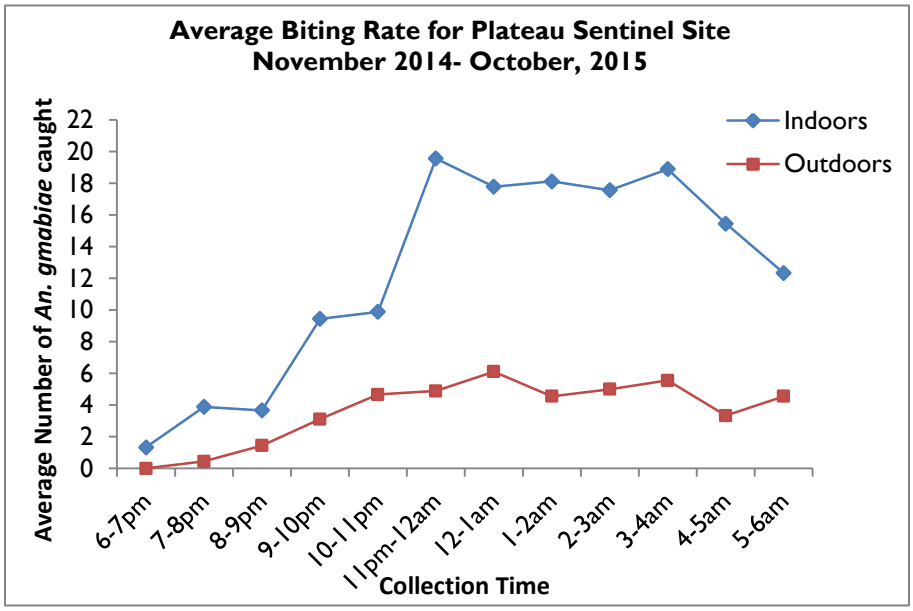


Figure 6: Biting Trend of *An. gambiae* s.l. , Rivers Sentinel Site, November, 2014 – October, 2015

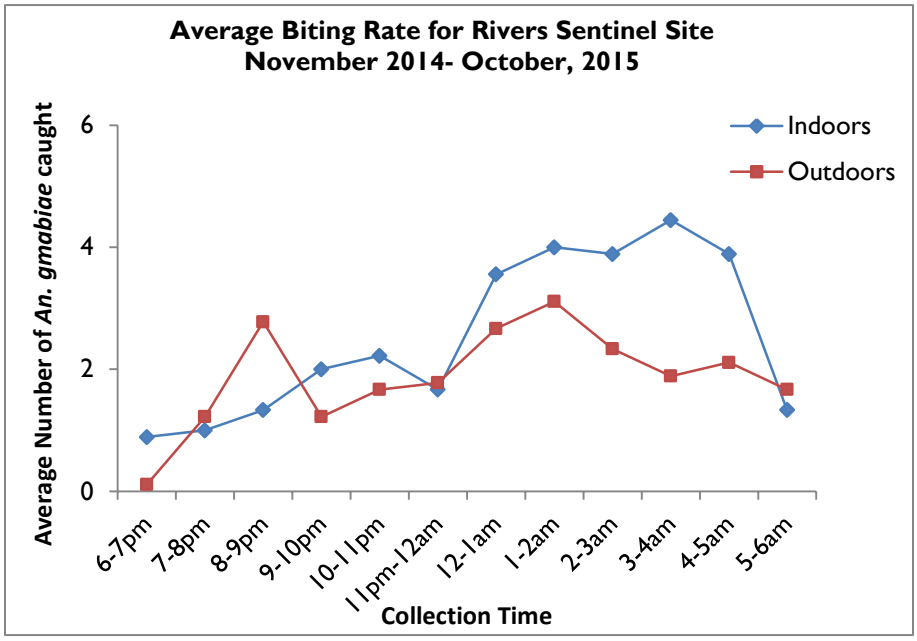


Figure 7: Biting Trend of *An. gambiae* s.l., Doma Sentinel Site, November, 2014 – October, 2015

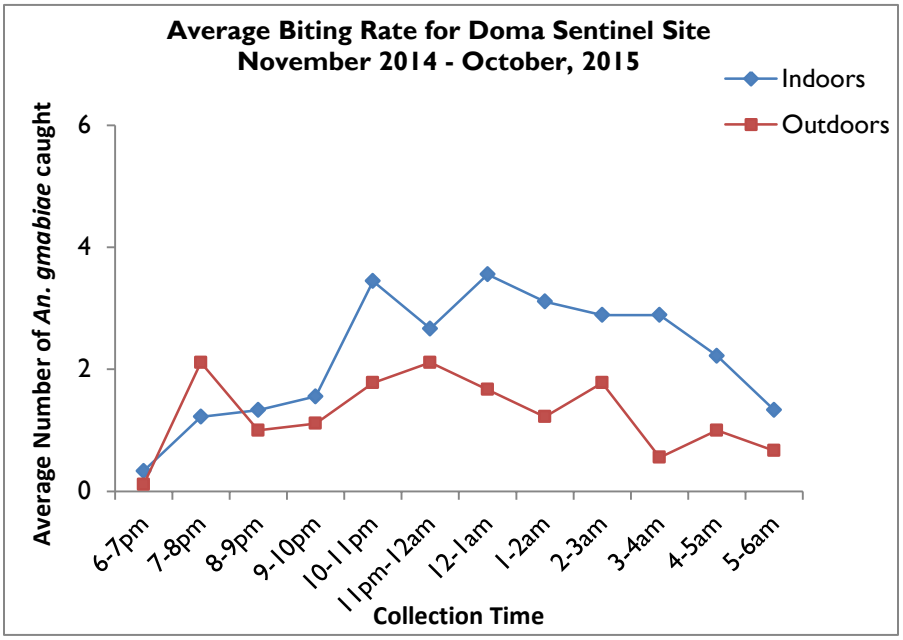


Figure 8: Biting Trend of *An. gambiae* s.l. , Nasarawa Eggon Sentinel Site, November, 2014 – October, 2015

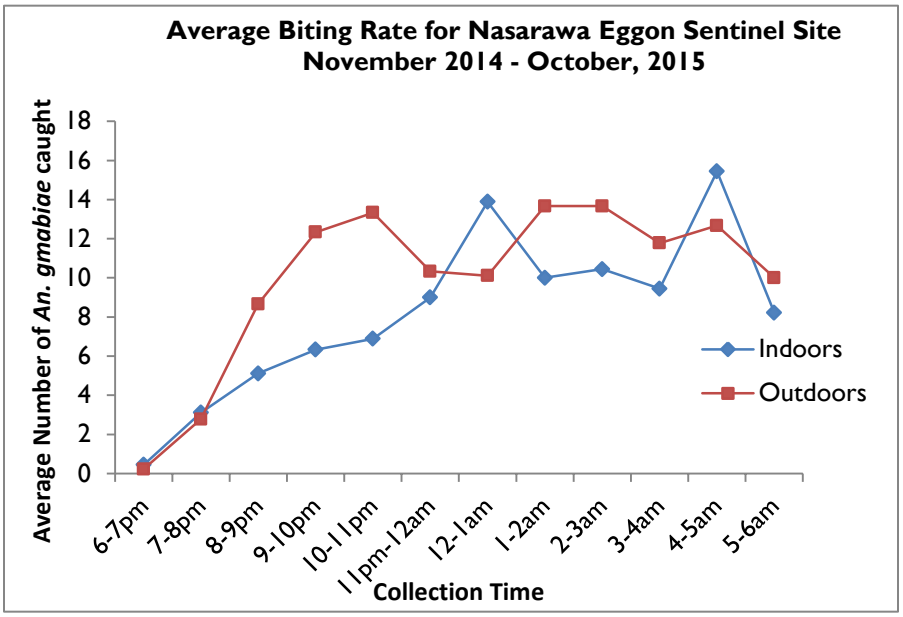
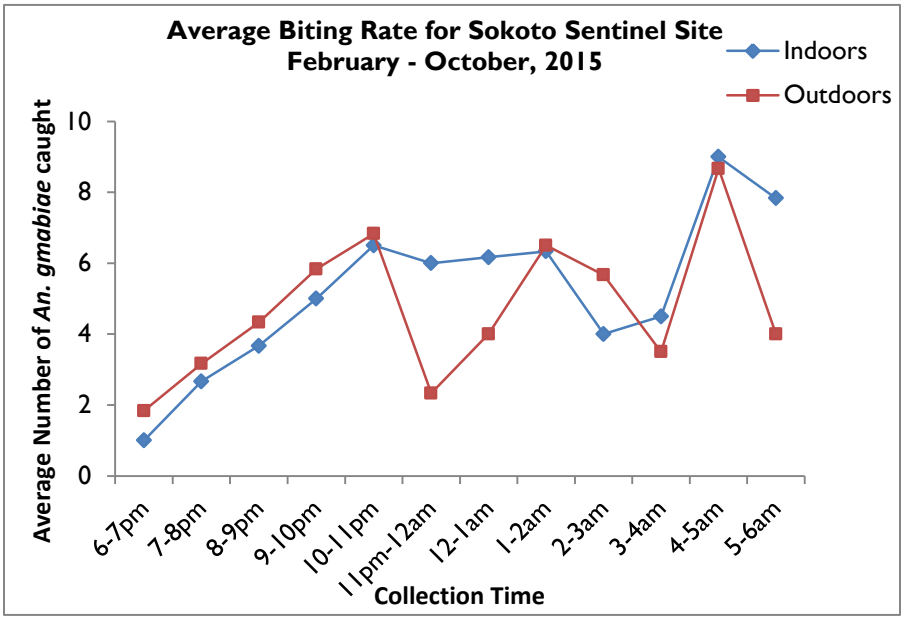


Figure 9: Biting Trend of *An. gambiae* s.l., Sokoto Sentinel Site, November, 2014 – October, 2015



4.5 PARITY

The parity rate was determined at two sites where there was capacity for dissection. *An. gambiae* s.l. caught at Enugu and Nasarawa sentinel sites were dissected for parity. Ovaries from randomly selected female *An. gambiae* s.l. specimens collected using PSC and CDC light trap were dissected to determine the physiological age and parity rate as described by Gillies and Wilkes (1963) and Detinova (1962). Between the months of November 2014 and October 2015, significantly higher rates of parity were observed in both Doma and Nasarawa Eggon in the Guinea savannah which is located in the North Central zone of the guinea savanna ecozone with 6 ½ months(May –September) considered suitable for malaria transmission. A steady trend of parity rates was recorded in Nasarawa Eggon and Doma LGAs both in the Guinea Savannah ecological zone of Nigeria throughout the study period in 2014 (March through October 2014) ranging from 33.3% to 45.8%. A significant variation in the parity pattern was observed in the same study area as higher parity rates ranging from 72.6% to 86.7% were recorded between November 2014 and February 2015. A drastic reduction was recorded as the rains peaked (between April and June, 2015) and ranged from 22.2% to 30.5% while parity peaked again between August and October ranging from 68.2% to 84.2% in the study areas. In Enugu which is mainly a forested area and located in the south east zone, there was generally lower parity rates throughout the study period from March through October 2014, ranging from 9% to 18% whereas between November 2014 and October 2015, higher parity rates were recorded. Parity rate was 30% in November and 25% in January. This peaked by April and remained high throughout the year ranging from 64.8% to 97.2% (Figures 10 and 11).

Figure 10: Monthly Parity of *Anopheles* Mosquitoes in Nasarawa State, November, 2014-October, 2015

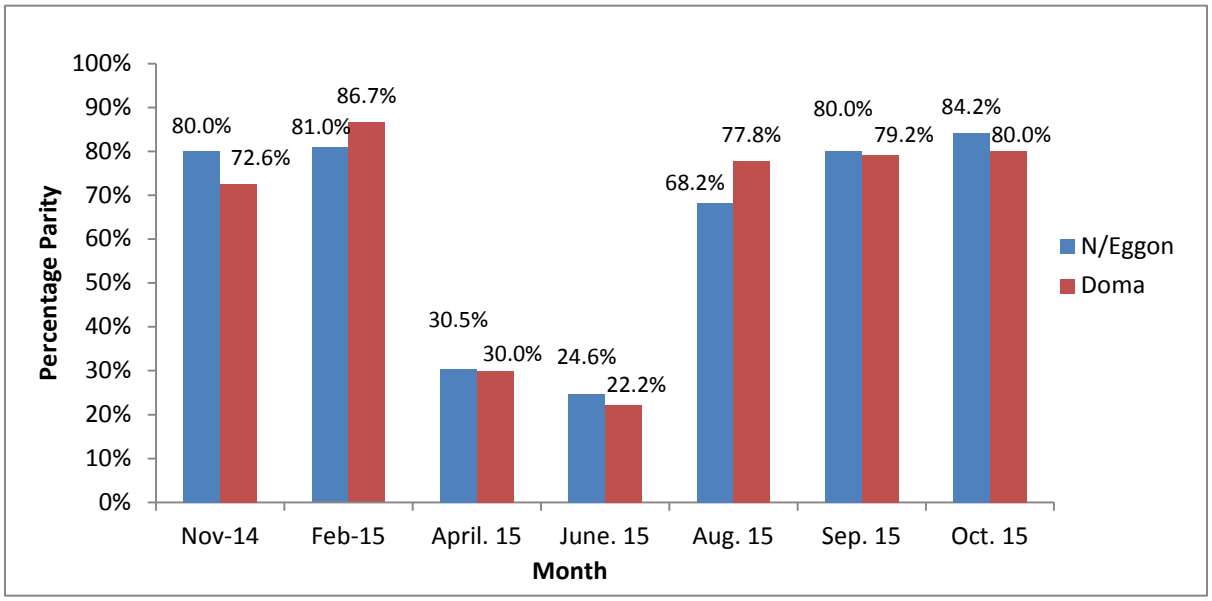
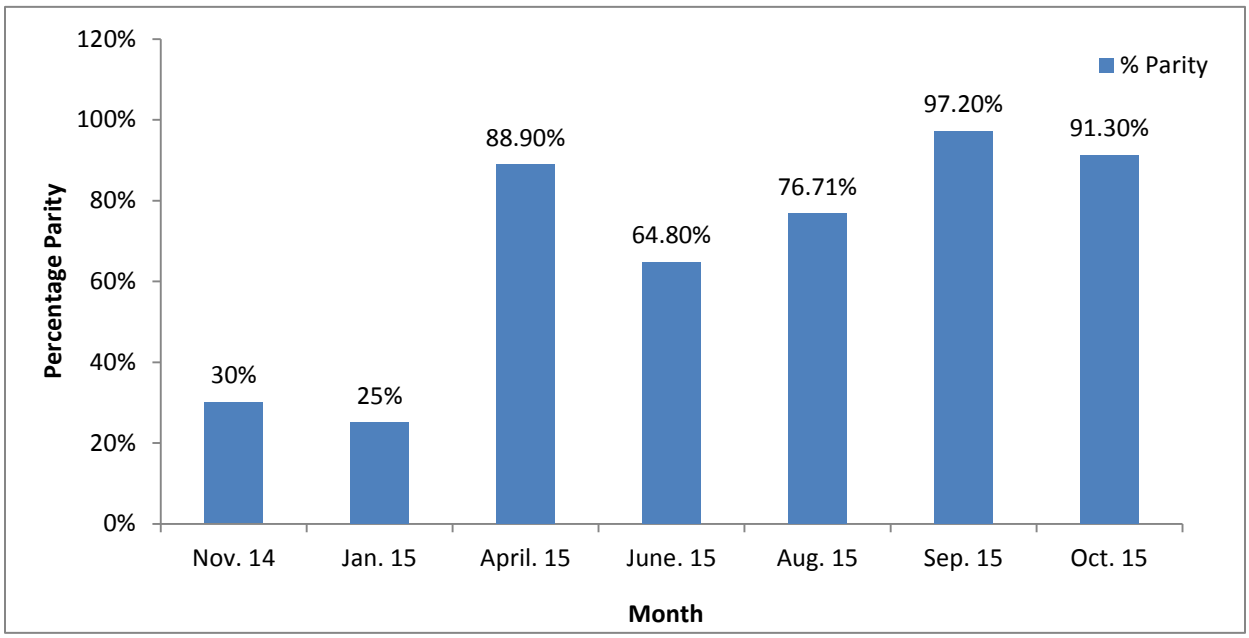


Figure 11: Monthly Parity Rate of *Anopheles* Mosquitoes in Enugu State, November, 2014-October, 2015



4.6 PCR IDENTIFICATION OF MEMBERS OF THE *ANOPHELES GAMBIAE* COMPLEX

Of the 1,250 samples analyzed using Polymerase Chain Reaction (PCR), 745 (60.0%) were amplified and PCR positive for *An.gambiae* s.l. The remaining samples were negative and remained so after repeated tests.

Between 63 and 96% of samples from Lagos, Plateau, Enugu and Nasarawa were PCR positive compared to 47 and 58% of samples from Sokoto and Rivers sentinel sites respectively. *Anopheles gambiae* s.s. was the predominant member of the *gambiae* complex representing 78 to 100% of the *gambiae* population at the different sites. *Anopheles arabiensis* was another member of the group identified by PCR but was found to be absent in Enugu and Rivers sentinel sites (Table 6). None of the other members of the *Anopheles gambiae* complex was found in the specimens analyzed. Overall, a total of 85.7% of mosquitoes that were PCR positive were *An. gambiae* s.s. while *An. arabiensis* represented 14.3% across the six sentinel sites.

Table7: Number of *Anopheles gambiae s.l.* tested and proportion of *An. gambiae* s.s. and *An. arabiensis*

Sites	Total Number Tested	No. tested with proportion positive per collection method							
		PSC		CDC indoor		CDC outdoor		Total	
		An. gambiae s.s.	*An. arabiensis*	*An.*	*An. arabiensis*	*An.*	*An. arabiensis*	*An. gambiae*	*An. arabiensis* (%)
Lagos	**104**	20	2	35	5	27	15	82 (78.8)	22 (21.1)
Plateau	**116**	40	8	32	0	22	14	94 (81.0)	22 (18.9)
Sokoto	**85**	24	6	22	3	22	8	68 (80.0)	17 (20.0)
Enugu	**128**	33	0	75	0	20	0	128 (100)	0
Rivers	**56**	28	0	16	0	12	0	56 (100)	0
Nasarawa	**360**	86	16	132	48	50	28	268 (74.4)	92 (25.5)

4.7 *PLASMODIUM* SPOROZOITES ASSAY

The number of *Anopheles gambiae* positive for *Plasmodium falciparum* infection at each site is presented in table 8 below. Infection rate was highest (7.9%) in Sokoto followed by Enugu (6.6%) and Lagos (5.6%). In spite of the relatively high number of samples from Nasarawa compared to other sites, only 1.8% of the samples were positive for *Plasmodium* infection. Almost all of the positive samples were found in the PSC and CDC collection indoors at all the sites except in Sokoto. Further analysis showed a predominance of *Anopheles gambiae* s.s. infected mosquitoes at all the sites. Aside from Sokoto with 5.9% infected *An. arabiensis,* none of the *Anopheles arabiensis* from the other 5 sites were infected with *P. falciparum* (Table 9).

Table8: Number of *Anopheles gambiae* tested and proportion infected with *Plasmodium falciparum*

Sites	Total Number	No. tested (%) positive per collection method						Total No. & % positive
		PSC		CDC indoor		CDC outdoor		
		N	No. (%) +ve	N	No. (%) +ve	N	No. (%) +ve	
Lagos	108	**24**	1 (4.2)	**41**	4 (9.7)	**43**	1 (2.3)	6(5.6)
Plateau	183	**73**	5 (6.8)	**58**	3 (5.2)	**52**	1 (1.9)	9(4.9)
Sokoto	178	**70**	7 (10.0)	**55**	4 (7.2)	**53**	3 (5.7)	14(7.9
Enugu	198	**53**	6 (11.3)	**110**	7 (6.4)	**35**	0	13(6.6)
Rivers	95	**40**	3 (7.5)	**32**	2 (6.2)	**23**	0	5(5.3)
Nasarawa	488	**153**	5 (3.3)	**225**	4 (1.8)	**110**	0	9(1.8)
TOTAL	**1, 250**							

Table7: Number of *Anopheles gambiae s.l* tested and proportion of *An. gambiae s.s.* and *An. arabiensis* infected with *Plasmodium falciparum* across the sentinel sites

Ento. Sentinel Sites	Total Number Tested		Total Number Positive	
	An. gambiae s.s	*An. arabiensis*	*An. gambiae s.s. (%)*	*An. arabiensis (%)*
Lagos	86	22	6 (6.9)	0 (0.0)
Plateau	161	22	9 (5.6)	0 (0.0)
Sokoto	161	17	13 (8.1)	1 (5.9)
Enugu	198	-	13 (6.6)	0 (0.0)
Rivers	95	-	5 (5.3)	0 (0.0)
Nasarawa	396	92	9 (2.3)	0 (0.0)
Total	**1097**	**153**	**55 (5.1)**	**1 (0.7)**

4.8 INSECTICIDE SUSCEPTIBILITY AND MECHANISM OF RESISTANCE

This test was conducted with both the WHO tube and CDC bottle bioassays using non-blood fed adult female *An. gambiae* s.l. reared from larvae and pupae collected at all six sentinel sites. This test was aimed to determine the susceptibility level of the vector population across the different ecological zones. Insecticide susceptibility results indicated that DDT (organochlorine) was strongly resistant across all six sentinel sites. *An. gambiae* s.l. was found to show resistance to the pyrethroids lamdacyhalothrin, deltamethrin and permethrin across all sites but showed susceptibility to alphacypermethrin as observed in Rivers. In the carbamate class susceptibility to bendiocarb was observed across all sentinel sites except Sokoto state while local mosquitoes showed susceptibility to propoxur and primiphos methyl (organophosphate) in Lagos (Tables 10 and 11).

Table8: Test Results (Percent Mortality After 24 Hours) against *An. gambiae* s.l. using WHO Tube Bioassay Method at 95% Confidence Interval (CI).

Class of Insecticides	Insecticides	WHO Tube Bioassay																			
		Plateau			Sokoto			Rivers			Enugu			Nasarawa (Doma)			Nasarawa (N/Eggon)			Lagos		
		Total No. Tested	Total No. Dead	Percentage Mortality	Total No. Tested	Total No. Dead	Percentage Mortality	Total No. Tested	Total No. Dead	Percentage Mortality	Total No. Tested	Total No. Dead	Percentage Mortality	Total No. Tested	Total No. Dead	Percentage Mortality	Total No. Tested	Total No. Dead	Percentage Mortality	Total No. Tested	Total No. Dead	Percentage Mortality
Pyrethroid	**Lambda cyhalothrin**	100	88	88% *R CI 79.98-93.64	100	12	12% R CI 6.36-20.02	100	48	48% R CI 37.9-58.22	100	13	13% R CI 7.1-21.11	100	23	23% R CI 15.17-32.49	100	35	35% R CI 25.73-45.19	100	12	12% R CI 6.36-0.02
Pyrethroid	**Permethrin**	100	97	97% **SR CI 91.48-99.37	100	30	30% R CI 21.24-39.98	100	58	58% R CI 47.712 - 67.801	100	19	19% R CI 11.84-28.07	100	10	10% R CI 4.9-17.62	100	50	50% R CI 39.83-60.17	100	3	3% R CI 0.62-8.52
Pyrethroid	**Deltamethrin**	100	74	74% R CI 64.27-82.26	100	83	83% R CI 74.18-89.77	100	40	40% R CI 30.329 - 50.279	100	84	84% R CI 75.32-90.57	100	59	59% R CI 48.71-68.74	100	85	85% R CI 76.47-91.36	100	7	7% R CI 2.86-13.89
Pyrethroid	**Alphacypermethrin**	100	84	84% R CI 75.32-90.57	100	68	68% R CI 57.92-76.98	100	98	98% ^S CI 92.96-99.75	100	68	68% R CI 57.92-76.98	100	85	85% R CI 76.47-91.36	100	89	89% R CI 81.17-94.38	100	96	96% PR CI 90.07-98.90
Carbamate	**Bendiocarb**	100	100	100% S CI 96.38-100	100	73	73% R CI 63.20-81.39	100	100	100% S CI 96.38-100	100	100	100% S CI 96.38-100	100	100	100% S CI 96.38-1001	100	100	100% S CI 96.38-100	100	100	100% S CI 96.38-1000
Carbamate	**Propoxur**	100	95	95% SR CI 88.72-98.36	-	-	-	100	78	78% R CI 68.61-85.67	100	95	95% SR CI 88.72-98.36	-	-	-	-	-	-	100	98	98% S CI 92.96-99.75
Organo-phosphate	**Pirimiphos-methyl^**	100	74	74% R CI 64.27-82.26	100	63	63% R CI 52.77-72.44	100	59	59% R CI 48.71-68.74	100	52	52% R CI 41.78-62.1	100	100	100% S CI 96.38-1001	100	95	95% SR CI 88.72-98.36	100	100	100 S CI 96.38-100
Organo-chlorine	**DDT**	100	43	43% R CI 33.14-53.29	100	70	70% R CI 60.02-78.76	100	37	37% R CI 27.56-47.24	100	6	6% R CI 2.23-12.60	100	7	7% R CI 2.86-13.89	100	8	8% R CI 3.52-15.16	100	1	1% R CI 0.025-5.45

*NB: *R = Resistance; **SR = Suspected Resistance; ^S = Susceptible*

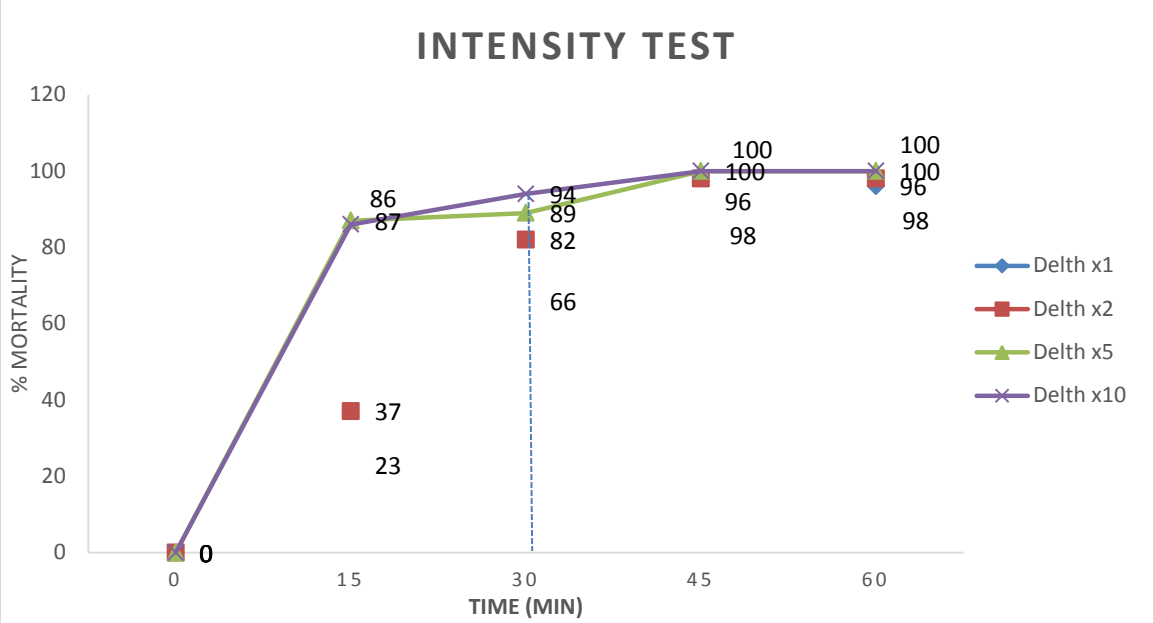
Table 9: Test Results against *An. gambiae s.l.* using CDC Bottle Bioassay Methods at 30 Minutes Diagnostic Time (45 Minutes for DDT) with 95% Confidence Interval (CI)

Class of Insecticides	Insecticides	CDC Bottle Bioassay																		
		Plateau			Sokoto			Rivers			Enugu			Nasarawa (N/Eggon)			Lagos		
		Total No. Tested	Total No. Dead	Percentage Mortality	Total No. Tested	Total No. Dead	Percentage Mortality	Total No. Tested	Total No. Dead	Percentage Mortality	Total No. Tested	Total No. Dead	Percentage Mortality	Total No. Tested	Total No. Dead	Percentage Mortality	Total No. Tested	Total No. Dead	Percentage Mortality
Pyrethroid	**Lambda cyhalothrin**	100	90	90% *R CI 82.38-95.10	100	97	97% **SR CI 91.48-99.38	100	77	77% R CI 67.51-84.83	100	79	79% R CI 69.71-86.51	104	104	100 ^S CI 96.56-100	100	89	89% R CI 81.17-94.38
Pyrethroid	**Permethrin**	100	21	21% R CI 13.49-30.29	100	85	85% R CI 76.47-91.36	100	21	21% R CI 13.49-30.29	100	33	33% R CI 23.92-43.12	100	73	73% R CI 63.20-81.39	100	5	5% R CI 1.64-11.28
Pyrethroid	**Deltamethrin**	100	85	85% R CI 76.47-91.36	100	90	90% R CI 82.38-95.10	100	0	0% R CI 0.00-3.62	100	64	64 R CI 53.79-73.36	100	95	95% SR CI 88.72-98.36	100	15	15% R CI 8.65-23.53
Pyrethroid	**Alphacypermethrin**	100	89	89% R	100	95	95% SR CI 88.72-98.36	100	82	82% R CI 73.05-88.97	100	62	62% R CI 51.75-71.52	100	100	100% S CI 96.38-100	100	60	60% R CI 49.72-69.67
Carbamate	**Bendiocarb**	100	100	100% S CI 96.38-100	-	-	-	100	100	100% S CI 96.38-100	100	100	100% S CI 96.38-100	103	103	100% S CI 96.48-100	100	100	100% S CI 96.38-100
Carbamate	**Propoxur**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Organophosphate	**Pirimiphos-methyl^**	100	7	7% R CI 2.86-13.89	100	86	86% R CI 77.63-92.13%	100	0	0% R CI 0.00-3.62%	100	35	35% R CI 25.73-45.19	100	49	49% R CI 38.86-59.20	100	98	98% S CI 92.96-99.75
Organochlorine	**DDT**	100	12	12% R CI 6.36-20.02	100	83	83% R CI 74.18-89.77	100	0	0% R CI 0.00-3.62	100	18	18% R CI 11.03-26.95	100	68	68% R CI 57.92-76.98	100	57	57% R CI 46.71-66.86

4.9 INSECTICIDE RESISTANCE INTENSITY ASSAY RESULTS FROM THE SIX SENTINEL SITES.

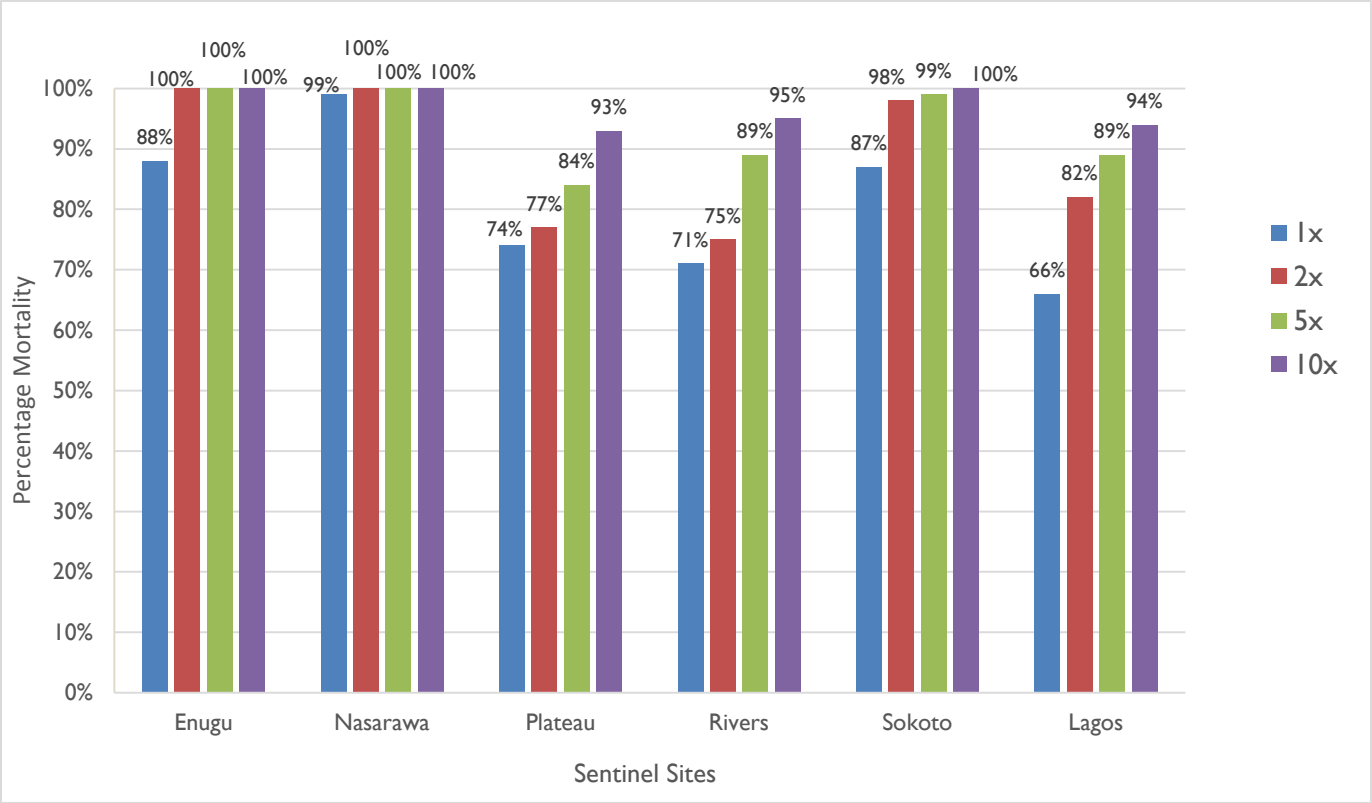
Insecticide Resistance Intensity assays were carried out at all six sentinel sites following the CDC protocol with three to four day old adult *Anopheles* mosquitoes. Four different concentrations of deltamethrin (x1, x2, x5 and x10) were provided by the AIRS project. Each of the stock solution was dissolved in 50ml of absolute (100%) acetone. Approximately 1ml of the mixture was used for coating each CDC bottle following the description in the protocol. Four replicates of each concentration with two controls were tested with a total of 150 mosquitos per concentration. The diagnostic time was 30 minutes . The numbers of mosquitoes dead and alive were recorded at 0, 15, 30, 45 and 60minutes after exposure. In this report the test mortality rates recorded at the diagnostic time of 30 minutes are included. In Lagos, only 66, 82 89% and 94 % of the test population was susceptible in the three concentrations of x1, x2, x5x x 10 respectively.

Figure 12: Determination of maximum resistance intensity level in the mosquito population from Ejirin, Lagos State using four concentrations of deltamethrin.



In Enugu and Sokoto sentinel sites, just like Lagos, local mosquitoes showed resistance at x1 concentration but were susceptible at higher concentrations (Figures 13 and 17). In Plateau and Rivers sentinel sites local mosquitoes showed resistance at x1, x2 and x5 while suspected resistance were recorded at x10 concentrations (Figures 15 and 16). However, susceptibility was recorded at the four different concentrations (x1, x2, x5 and x10) in the Nasarawa sentinel site. (Figure 14).

Figure 13: Pyrethroid (Deltamethrin) Resistance Intensity across all Sentinel Sites



4.10 MOLECULAR IDENTIFICATION AND KDR RESISTANCE ASSAY

4.10.1 M AND S-FORMS AND KDR PCR ASSAYS

A total of 500 *Anopheles gambiae* s.l. mosquito samples that survived the diagnostic concentration of pyrethroids or DDT during susceptibility tests from 2 sites in the Guinea savannah (Nasarawa) and Coastal mangrove (Lagos) were analyszed both for M and S molecular forms and Kdr. These include 250 samples from Lagos and 250 samples from the Nasarawa sentinel sites. The source of the *Anopheles* samples from each site, the proportion positive for *An. gambiae* and *An. arabiensis* by PCR is presented on table 19. Almost all (>99.0%) of the samples DNA analyzed from each site were amplified by PCR. *Anopheles gambiae* s.s and *Anopheles arabiensis* were the only members of the *gambiae* complex found at both sites. In each case *An. gambiae* represented 71.2% and 84.4% of the *An. gambiae* complex population in Nassarawa and Lagos respectively. The remaining were *An. arabiensis* (Table 12). None of the specimens tested was positive for the *Anopheles melas.* At each site, the proportion of *An. arabiensis* was higher in samples from larval collections.

Table 10: Number of *Anopheles gambiae* analyzed and proportion (%) of *Anopheles gambiae* s.s. and *Anopheles arabiensis*

Site	Sample source	Number analysed	PCR identification*	
			***An. gambiae* s.s.**	***An. arabiensis***
Nassarawa	**Indoor collection**	150	114 (76.0)	34 (22.7)
	Resistant mosquito**	100	64 (64.0)	36(36.0)
	Total	**250**	**178(71.2)**	**70(28.0)**
Lagos	**Indoor collection**	80	75 (93.7)	5(6.3)
	Resistant mosquito**	170	136 (80.0)	32 (18.8)
	Total	**250**	**211 (84.4)**	**37 (14.8)**

**Two mosquito samples from each of Nasarawa and Lagos did not amplify after repeated test.*
*** Mosquitoes that survived after exposure to DDT/or Deltamethrin*

4.10.2 PROPORTION OF THE MOLECULAR M AND S-FORM AT EACH SITE

The molecular M and S form of *Anopheles gambiae* occurred in sympatry at both sites. The S form was predominant representing 79.2 and 68. 2% in Nassarawa and Lagos respectively. There was no case of the M form + S-Form bands in a single specimen suggesting the absence of "hybrid State" or contamination from the samples collected.

4.10.3 FREQUENCY OF THE KDR MUTATION IN THE MOSQUITO POPULATION

Analysis of the Kdr PCR assay shows the presence of the West kdr mutation (*kdr-w*) in 3.3% of samples collected indoor at Nassarawa but in 27.0% of samples that survived insecticide exposure. Similar findings were found in samples from the Lagos site with the kdr present in 10.0% of samples collected indoor as against 28.8% in sample that survived the insecticide exposure. At each site the homozygous resistance state (RR) predominated the allelic frequency. The overall frequency of the *kdr-w* was 0.518 and 0.570 in Nasarawa and Lagos respectively (Table 13 and 14). Interestingly none of the molecular M form in Nassarawa or Lagos was positive for the kdr mutation. PCR tests using primers designed for the East African kdr mutation did not show any positive case of the *kdr-e* in all the samples from both sites.

Table 11: Proportion of the molecular M and S-form of *Anopheles gambiae* and the frequency of the Kdr in the mosquito population from Nasarawa

	Indoor collections		Resistant mosquitoes	
	S form	M form	S form	M form
Homozygous (RR)	2	0	21	0
Heterozygous (Rr)	3	0	6	0
Susceptible (rr)	86	23	23	14
Total tested	91	23	50	14
Allelic frequency	**0.038**	**0**	**0.48**	**0**

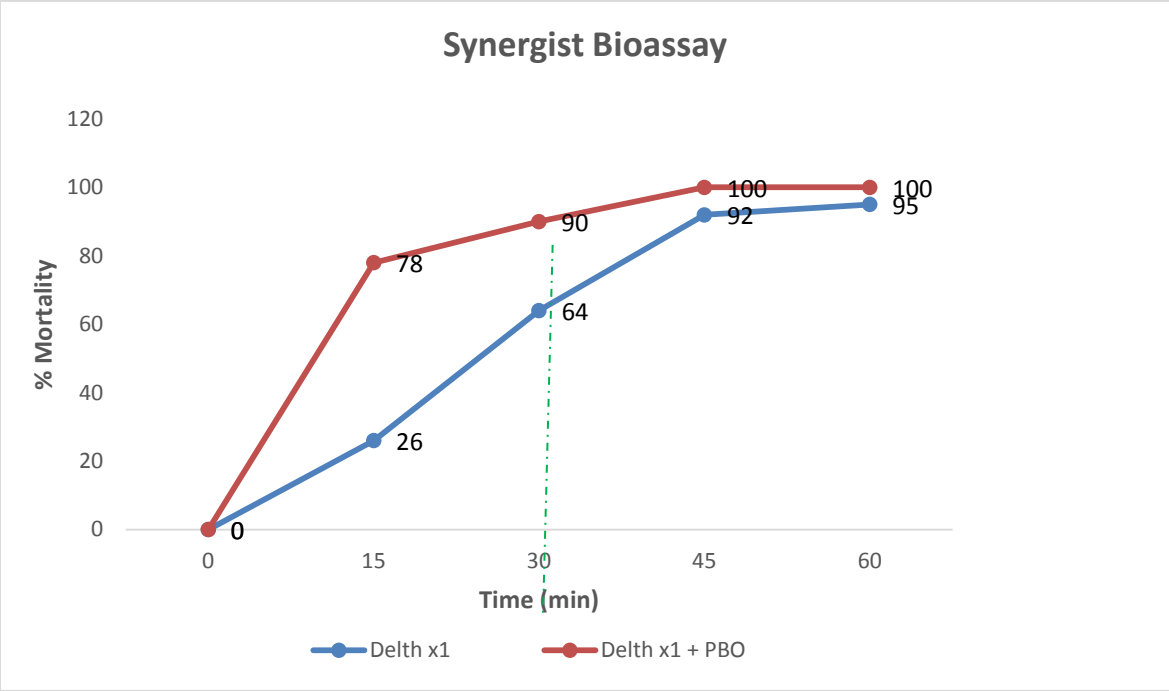
Table 12: Proportion of the molecular M and S-form of *Anopheles gambiae* and the frequency of the Kdr in the mosquito population from Lagos

	Indoor collections		Resistant mosquitoes	
	S form	M form	S form	M form
Homozygous (RR)	2	0	41	0
Heterozygous (Rr)	6	0	8	0
Susceptible (rr)	41	26	46	41
Total tested	49	26	95	41
Allelic frequency	**0.10**	**0**	**0.47**	**0**

4.10.4 SYNERGIST ASSAY WITH THE CDC BOTTLE BIOASSAY IN LAGOS STATE

A synergist test was carried out to investigate the plausible role of metabolic enzymes in insecticide detoxification in the resistant mosquito population from Nassarawa and Lagos sentinel sites. Synergist assays was done using piperonyl butoxide (PBO) an inhibitor of mixed function oxidase on *Anopheles gambiae* from each site (Figure 18; Tables 22 and 23 in Annex c). Susceptibility to deltamethrin was not restored after exposure to deltamethrin at 30 minutes diagnostic time in Lagos. Susceptibility was only restored after 45 minutes of exposure. Mortality post-exposure of synergized samples was 90.0% (n = 100) while unsynergized samples recorded a mortality of 64% (n= 100) but became 92% after 45 minutes of exposure .The partial abolishing of resistance by PBO suggests that the mechanism is related to the synergist is involved in the resistance but it is not the only mechanism involved in this particular case.(Brogdon and Chan 2010;;Annex B: 2015) The differences in mortality post exposure between synergized and unsynergized samples using PBO was statistically significant (χ^2 =8.117, DF = 1, P=0.0044)(Figure 22).

Figure 14: Mortality of synergized and non-synergized population of *Anopheles gambiae* exposed to the diagnostic concentration of deltamethrin in CDC bottle bioassay for Lagos Sentinel Site.



4.10.5 SYNERGIST ASSAY WITH THE CDC BOTTLE BIOASSAY IN NASARAWA STATE

The mortality of un-synergized and synergized mosquitoes from Nasarawa Eggon and Doma exposed to ×1 concentration of Deltamethrin is shown in Figures 19 and 20; tables 24, 25, 26 and 27 in Annex C. The non- synergized Mosquitoes population showed 98.9% and 97.7% mortality at the diagnostic time of 30 minutes compared to the 100% mortality of the synergized population from both sites.

Figure 15: Percentage mortality of Synergized and non-synergized population of *Anopheles gambiae* exposed to the diagnostic concentration of Deltamethrin in CDC bottle bioassay in Nasarawa Eggon.

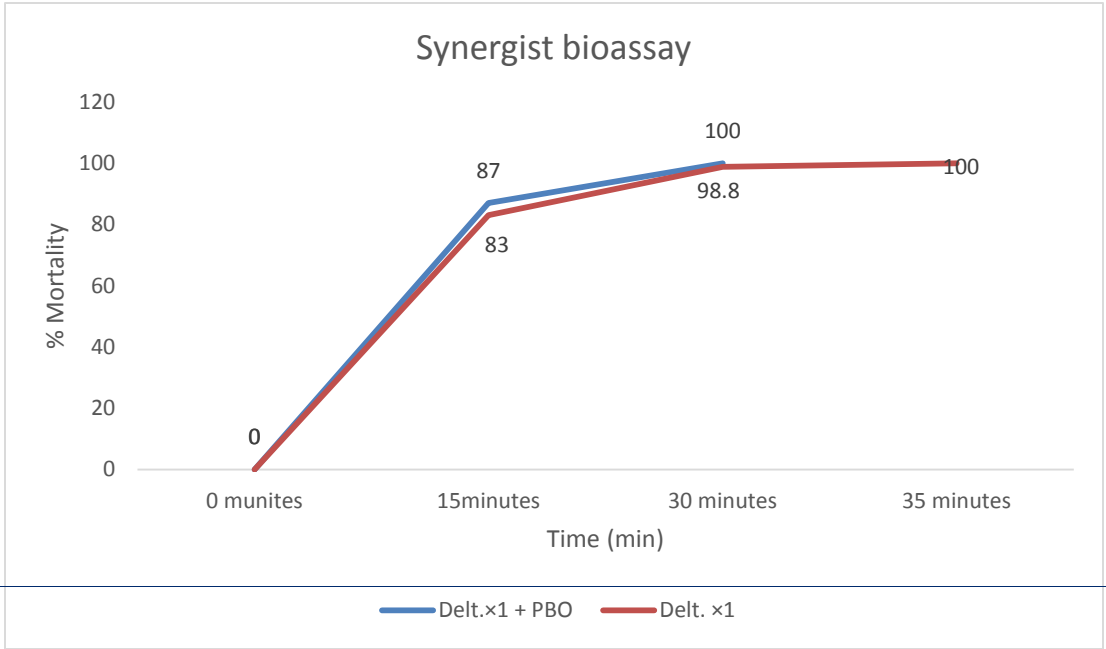
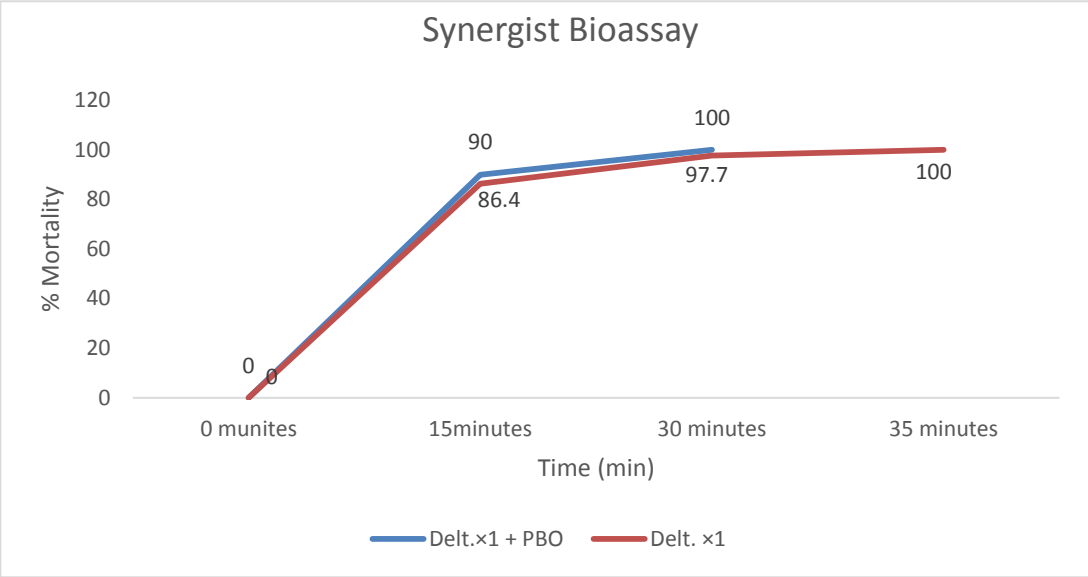


Figure 16: Percentage mortality of Synergized and non-synergized population of *Anopheles gambiae* exposed to the diagnostic concentration of deltamethrin in CDC bottle bioassay in Doma.



5. Discussion, Lessons Learned, and Challenges

1. This study found *An. gambiae* s.s. to be predominant among the *An. gambiae* complex in most of the study sites followed by *An. arabiensis*. Both *An. gambiae* *An. arabiensis*, the second most predominant species of the *An. gambiae* complex found in this study, is very flexible over its ecological range and behavior. Incidentally unlike last year, they were not found in Enugu and Rivers sentinel sites. This could be due largely to seasonal variations across the various ecological zones. It is both exophilic and endophilic, as well as anthropophilic and zoophilic (Coetzee *et al.,* 2000; Mafuyai, 2010).

2. The presence of *An. funestus* was observed markedly in two sentinel sites of Plateau and Enugu states with swampy areas. Peak collections of *An. funestus* in Guinea Savannah area of Plateau was in December with significant numbers collected indoors earlier in November as the rains gradually recede. In the swampy cum forested areas of Enugu, *An. funestus* peak was observed in April almost at the onset of rains similar to the previous year's findings which had its peak in the month of March. This is as observed by Coetzee *et al.* (2006) and WHO (2006). *An. funestus* observed in most of the study areas mainly rest indoors and are highly anthropophagic (human biters) (Gillies and De Meillon 1968; Service 1961; WHO 2007). It was observed predominantly in Plateau (rice farm and irrigated farms) and humid, forested Enugu State.

3. Significant numbers of *An. coustani* were observed in collections from the Guinea Savannah area of Nasarawa and Plateau States while a smaller number was found in the forested area of Enugu. It has been suggested that since many secondary vectors are exophilic and exophagic, they could potentially sustain malaria transmission after the main endophilic and endophagic vectors have been reduced by indoor vector control measures such as use of IRS or insecticide-treated bed nets (ITNs) (Gillies 1964, Wilkes *et al.*1996, Antonio-Nkondjio *et al.* 2006, Fornadel *et al.* 2011). Recent studies have indicated that *An. coustani* is playing a major role in outdoor transmission (Mwangangi *et al.* 2013). Fornadel *et al. (*2011) earlier observed an increased anthropophily in *An. coustani*. Effective malaria control programs should therefore include tools that target both indoor and outdoor transmission.

4. Findings in this study revealed that between 63 and 96% of samples from Lagos, Plateau, Enugu and Nasarawa were PCR positive compared to 47 and 58% of samples from Sokoto and Rivers sentinel sites respectively. *Anopheles gambiae* s.s. was the predominant member of the *gambiae* complex representing 78 to 100% of the *gambiae* population at the different sites. *Anopheles arabiensis* was the other member of group identified by PCR but absent in Enugu and Rivers sentinel. Poor storage of mosquitoes may have led to DNA degeneration and may have accounted for the low DNA amplification recorded in Sokoto and Rivers. The program is working closely with Principal Investigators on sample storage. Overall, 85.7% of mosquitoes

that were PCR positive were *An. gambiae* s.s. while *An. arabiensis* represented 14.3%. *An. arabiensis* was found at four sites (Lagos, Nasarawa, Sokoto and Plateau). Although previous findings have shown the presence of *An. arabiensis* in Rivers sentinel site (AIRS Report 2014 unpublished), it was absent in this present study while same species has remained absent in Enugu. Although the mangrove and rainforest areas are dominated by *An. gambaie* s.s., it is not uncommon to find *An. arabiensis,* (as in the case of Lagos) which has been described as a savannah vector.

5. The highest *Plasmodium falciparum* sporozoite infection rate was recorded in Sokoto (7.8%) followed by Enugu (6.6) and Lagos (5.5). Previous findings have shown that prevalence of malaria infection among pregnant women in sokoto ranged from 41.6%- 59.5 percent. This is considerably higher than other sahelian regions such as Maiduguri where a prevalence of 22.1% was reported among pregnant women (Fana *et al.,* 2015). Transmission is seasonal with peak period from May through December in Sokoto and a peak prevalence of 59.5% (Fana *et al*.,2015; MPP 2015). This high malaria prevalence could explain why many of the indicators were found to be highest in Sokoto during this study compared to other sentinel sites.

6. Biting times of An. gambiae s.l. are epidemiologically important. In this study, conducted at the peak of the dry season in Nigeria, significant indoor biting activity in An. gambiae s.l. was observed to have peaked at different times and varied by location and time in the rainforests and guinea savannah states of Enugu, Plateau and Nasarawa States. Early morning indoor biting was high in Plateau, Nasarawa and Sokoto sentinel sites. On the contrary, significant outdoor biting was observed in the mangrove coastal states of Lagos and Rivers States as well as Doma LGA of Nasarawa, a site where a significant number of An. coustani were collected in a previous study. Generally across all the sentinel sites, the peak biting period was about the time most inhabitants were in bed, the use of LLINs could help reduce human vector contact and the risk of infective bites reduced.

7. The steady trend of parity rates recorded in Nasarawa Eggon and Doma LGAs both in the Guinea Savannah ecological zone of Nigeria throughout the study period in 2014 (March through October 2014) ranging from 33.3% to 45.8%. The significant variation in the parity pattern observed in Nasarwa Eggon and Doma in this study as compared to earlier findings could be as a result of seasonal variiations. In Enugu, a progressive increase was recorded during the study period compared to the previous year where parity rate remained generally low.

8. The increase in parity indicates an increase of older mosquito vectors and the risk of the vectors being infected and re-infected during subsequent feeding and increase in transmission as recorded by WHO (2013).

9. Findings from this study revealed that An. gambiae s.l. showed resistance to the pyrethroids - (lamdacyhalothrin, deltamethrin, and permethrin) across all sites, although susceptibility to alphacypermethrin was observed in Rivers. In the carbamate class susceptibility to bendiocarb was observed across all sentinel sites except Sokoto state while propoxur and pirimiphos methyl (organophosphate) was susceptible in Lagos but suspected resistance was recorded in Plateau and Enugu sentinel sites. This study shows that although pyrethroid resistance is widespread, the intensity of the resistance in Nigeria is not high. However, the low mortality for the tests on

primiphos methyl across the different sites could be due to problems with the stability of insecticides used for the tests.

10. Findings from this study indicated that the molecular M and S form of Anopheles gambiae occurred in sympatry at both sites. The S form was predominant representing 79.2 and 68. 2% in Nasarawa and Lagos respectively (Table 2 and 3). There was no case of the M form + S-Form bands in a single specimen suggesting the absence of "hybrid state" or contamination from the samples collected.

11. Analysis of the Kdr PCR assay shows the presence of the West kdr mutation (kdr-w) in 3.3% of sample collected indoor at Nasarawa but in 27.0 % of samples that survived insecticide exposure. Similar findings were found in samples from the Lagos site with the kdr present in 10.0 % of sample collected indoor as against 28.8% in sample that survived the insecticide exposure. At each site the homozygote resistant state (RR) predominated the allelic frequency.The overall frequency of the kdr- was 0.518 and 0.570 in Nasarawa and Lagos respectively. Results of the insecticide susceptibility test and the kdr assay indicate that the kdr gene is present at a lower frequency compared with reports from other West African countries. In Benin, Cote D'Ivoire and Mali for instance kdr frequencies ranging from 80 to 90% has been reported in areas where the mosquito populations have shown resistance to pyrethroid insecticides (Akogbeto and Yakoubou, 1999; Chandre *et al*., 1999).

While the mosquitoes were susceptible in Nasarawa N/Eggon with mortality of 98.8% (n = 100), in Lagos synergist assays performed using PBO, an inhibitor of monooxygenase showed that susceptibility to deltamethrin was partially restored in the deltamethrin after exposure to PBO in Lagos. This indicates that in Lagos monooxygenases might not be the only resistant mechanism involved.

6. CHALLENGES

- Occasional security concerns in some of the surveillance states (Rivers, Nasarawa, and Plateau). Measures taken to mitigate this challenge was an assessment of security situation prior to commencement of surveillance activities across all six sentinel sites.

- National Malaria Elimination Programme (NMEP's) inability to participate in site supervision visits due to financial constraints. Steps taken to mitigate this challenge is that NMEP is now scheduled to participate in supervision visits in the 2016 AIRS Nigeria work plan.

7. RECOMMENDATIONS

- Entomological surveillance should be scaled up by establishing malaria vector sentinel sites in all PMI – supported states in Nigeria.
- The number of sentinel sites for insecticide resistance monitoring per state should be increased from 1 to 4 to generate more representative data to guide insecticide resistance management in line with PMI guidelines.
- Entomological impact assessment of LLIN distribution in PMI supported States should be carried out to link epidemiological data with entomological data.
- More efforts should be made to Assist the NMEP to develop IRM plan for Nigeria
- The PMI-funded insectary in Keffi could serve as a training center of sentinel site Technicians across the country in basic surveillance techniques.
- Pirimiphos-methyl was found to be show resistance across the sentinel sites except Lagos. It sis suggested that premeasured dosages prepared using actellic cs be used in further tests as recommended by Bill Brogdon
- Synergist tests show that resistance is not due to monooxygenases and that PBO does not restore susceptibility – one recommendation is that PBO nets would therefore not be effective in Nigeria and we should continue with regular LLINs.
- Pyrethroid resistance does not seem to be high in intensity in most states -- This is an indication that resistance will probably not cause operational failure, LLINs are likely to still be efficacious to use.
- Sokoto has particularly poor indicators, may be worth additional study (bed net coverage and usage?) to see why this is a problem.
- Problems with species PCR not working in some sites: recommend checking with sites on following sample storage protocols properly, checking a subset of morphological IDs to make sure they are not misidentifying other species as *An. gambiae*.

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ANNEX A:

A - 1 Total anophelines caught using Human Baited CDC Light Trap at Enugu Sentinel Site, November 2014 – October, 2015

Mosquito Species			*An. gambiae S.l.*	*An. funestus*	*An. coustani*	*An. Moucheti*	*An. nili*	*An. squamosus*	*An. malculipalpis*	*An. ziemanni*	**Total**
2014	**November**	In	14	3	1	4	4				26
		Out	3	0	4	4	0				11
	December	In	1	0	4		3	2			10
		Out	0	1	6		0	0			7
2015	**January**	In	3	1	0		0				4
		Out	5	0	2		3				10
	February	In	1	4		3	1				9
		Out	0	0		3	0				3
	April	In	5	14	0	0	10				29
		Out	5	27	5	5	5				47
	June	In	175	3	1		20				199
		Out	57	2	4		2				65
	August	In	58	0			5				63
		Out	29	1			2				32
	September	In	37	0	1		1		0	1	40
		Out	9	2	0		1		1	0	13
	October	In	25	0	1	0	5				31
		Out	12	1	2	1	2				18
Anopheline Total		In	319	25	8	7	49	2	0	1	411
		Out	120	34	23	13	15	0	1	0	206
Overall			439	59	31	20	64	2	1	1	617

A – 2: Total anophelines caught using Human Baited CDC Light Trap at Lagos Sentinel Site, November 2014 – October, 2015

Mosquito Species			*An. gambiae S.l.*	*An. pretoriensis*	**Total**
2014	**November**	In	62		62
		Out	69		69
	December	In	5		5
		Out	7		7
2015	**January**	In	2		2
		Out	1		1
	February	In	0		0
		Out	0		0
	April	In	0	0	0
		Out	1	4	5
	June	In	17	0	17
		Out	9	4	13
	August	In	2	0	2
		Out	0	1	1
	September	In	2	0	2
		Out	0	2	2
	October	In	3		3
		Out	2		2
Anopheline Total		In	93	0	93
		Out	89	11	100
Overall			182	11	193

A – 3: Total anophelines caught using Human Baited CDC Light Trap at Plateau Sentinel Site, November 2014 – October, 2015

Mosquito Species			*An. gambiae S.l.*	*An. funestus*	*An. coustani*	*An. flavicosta*	*An. pretoriensis*	*An. rufipes*	*An. squamosus*	*An. maculipalpis*	*Others*	**Total**
2014	**November**	In	23	5	1	1	2	5			2	39
		Out	10	8	8	1	0	1			3	31
	December	In	94	22	120	0	1	0			1	238
		Out	23	8	43	1	0	4			1	80
2015	**January**	In	2	1						1		4
		Out	1	0						1		2
	February	In	4	2			0	0		1		7
		Out	2	0			2	0		1		5
	April	In	8	0			0	2				10
		Out	7	1			0	1				9
	June	In	193	1	0							194
		Out	28	0	1							29
	August	In	355	1	2				0			358
		Out	46	0	0				1			47
	September	In	621		13		1		0	0		635
		Out	260		14		2		2	1		279
	October	In	31	2	8		0					41
		Out	16	1	2		0					19
Anopheline Total		In	1331	34	144	1	4	7	0	2	3	1526
		Out	393	18	68	2	4	6	3	3	4	501
Overall			1724	52	212	3	8	13	3	5	7	2027

A – 4: Total anophelines caught using Human Baited CDC Light Trap at Rivers Sentinel Site, November 2014 – October, 2015

Mosquito Species			*An. gambiae S.l.*	**Total**
2014	**November**	In	20	20
		Out	30	30
	December	In	19	19
		Out	37	37
2015	**January**	In	18	18
		Out	12	12
	February	In	0	0
		Out	8	8
	April	In	23	23
		Out	8	8
	June	In	52	52
		Out	38	38
	August	In	102	102
		Out	39	39
	September	In	37	37
		Out	27	27
	October	In	19	19
		Out	16	16
Anopheline Total		In	290	290
		Out	215	215
Overall			505	505

A – 5: Total anophelines caught using Human Baited CDC Light Trap at Doma Sentinel Site, November 2014 – October, 2015

Mosquito Species			*An. gambiae S.l.*	*An. funestus*	*An. coustani*	*An. pharoensis*	*An. squamosus*	**Total**
2014	**November**	In	10		0			10
		Out	6		3			9
	December	In	5		1			6
		Out	18		3			21
2015	**January**	In	7					7
		Out	10					10
	February	In	4		2			6
		Out	8		0			8
	April	In	27	0	3		2	32
		Out	5	1	2		0	8
	June	In	102		10	4		116
		Out	38		27	4		69
	August	In	33		26	3		62
		Out	29		95	1		125
	September	In	30		52			82
		Out	12		34			46
	October	In	13		43		2	58
		Out	10		173		1	184
Anopheline Total		In	231	0	137	7	4	379
		Out	136	1	337	5	1	480
Overall			367	1	474	12	5	859

A – 6: Total anophelines caught using Human Baited CDC Light Trap at Nasarawa Eggon Sentinel Site, November 2014 – October, 2015

Mosquito Species			*An. gambiae S.l.*	*An. coustani*	*An. nili*	*An. pharoensis*	**Total**
2014	**November**	**In**	105				105
		Out	86				86
	December	**In**	123				123
		Out	137				137
2015	**January**	**In**	43				43
		Out	46				46
	February	**In**	29	1			30
		Out	64	0			64
	April	**In**	17	0			17
		Out	39	1			40
	June	**In**	304			3	307
		Out	346			2	348
	August	**In**	176			3	179
		Out	270			0	270
	September	**In**	59				59
		Out	54				54
	October	**In**	29	0	1		30
		Out	34	1	0		35
Anopheline Total		**In**	885	1	1	6	893
		Out	1076	2	0	2	1080
Overall Total			1961	3	1	8	1973

A – 7: Total anophelines caught using Human Baited CDC Light Trap at Sokoto Sentinel Site, November 2014 – October, 2015

Mosquito Species			*An. funestus*	*An. gambiae S.l.*	*An. nili*	*An. pharoensis*	Total
2015	**February**	**In**	0	35	2		37
		Out	0	11	8		19
	April	**In**		9			9
		Out		11			11
	June	**In**		2		1	3
		Out		2		0	2
	August	**In**		172			172
		Out		105			105
	September	**In**		98			98
		Out		168			168
	October	**In**		60			60
		Out		43			43
Anopheline Total		**In**	0	376	2	1	379
		Out	0	340	8	0	348
Overall Total			0	716	10	1	727

A – 8: Total anophelines caught using PSC at Enugu Sentinel Site, November 2014 – October, 2015.

Mosquito Species		*An. gambiae* S.I.	*An. funestus*	*An. coustani*	*An. Moucheti*	*An. nili*	An. squamosus	*An. malculipalpis*	*An. ziemanni*	**Grand Total**
2014	November	9	4	0	0	0				13
	December	16	4	0		0	0			20
2015	January	7	0	0		0				7
	February	6	5		0	1				12
	April	12	5	0	1	0				18
	June	217	3	0		0				220
	August	148	0			1				149
	September	81	0	0		0		0	0	81
	October	48	1	0	0	0				49
Anopheline Total		544	22	0	1	2	0	0	0	569

A – 9: Total anophelines caught using PSC at Lagos Sentinel Site, November 2014 – October, 2015

Mosquito Species		*An. gambiae S.I.*	*An. pretoriensis*	**Grand Total**
2014	November	29		29
	December	7		7
2015	January	2		2
	February	1		1
	April	7	0	7
	June	45	0	45
	August	5	0	5
	September	13	0	13
	October	25		25
Anopheline Total		134	0	134

A – 10: Total anophelines caught using PSC at Plateau Sentinel Site, November 2014 – October, 2015

Mosquito Species		*An. gambiae S.l.*	*An. funestus*	*An. coustani*	*An. flavicosta*	*An. pretoriensis*	*An. rufipes*	*An. squamosus*	*An. maculipalpis*	*Others*	**Grand Total**
2014	November	99	7	0	0	1	1			0	108
	December	173	12	0	0	0	3			0	188
2015	January	0	0						0		0
	February	8	4			0	6		0		18
	April	109	6			2	4				121
	June	443	1	0							444
	August	739	0	0				0			739
	September	353		0		0		0	0		353
	October	121	1	0		5					127
Anopheline Total		2045	31	0	0	8	14	0	0	0	2098

A – 11: Total anophelines caught using PSC at Rivers Sentinel Site, November 2014 – October, 2015

Mosquito Species		*An. gambiae S.l.*	**Grand Total**
2014	November	45	45
	December	18	18
2015	January	0	0
	February	1	1
	April	43	43
	June	100	100
	August	101	101
	September	87	87
	October	60	60
Anopheline Total		455	455

A – 12: Total anophelines caught using PSC at Doma Sentinel Site, November 2014 – October, 2015

Mosquito Species		*An. gambiae S.I.*	*An. funestus*	*An. coustani*	*An. pharoensis*	*An. squamosus*	**Grand Total**
2014	November	8		0			8
	December	19					19
2015	January	30					30
	February	21		0			21
	April	36	0	0		0	36
	June	62		0	0		62
	August	200		0	0		200
	September	104		0			104
	October	54		0		0	54
Anopheline Total		534	0	0	0	0	534

A – 13: Total anophelines caught using PSC at Nasarawa Eggon Sentinel Site, November 2014 – October, 2015

Mosquito Species		*An. gambiae S.I.*	*An. coustani*	*An. nili*	*An. pharoensis*	**Grand Total**
2014	November	114				114
	December	65				65
2015	January	101				101
	February	169	0			169
	April	73	0			73
	June	154			0	154
	August	102			0	102
	September	103				103
	October	123	0	0		123
Anopheline Total		1004	0	0	0	1004

A – 14: Total anophelines caught using PSC at Sokoto Sentinel Site, November 2014 – October, 2015

Mosquito Species		*An. gambiae S.l.*	*An. funestus*	*An. nili*	*An. pharoensis*	Grand Total
2015	February	104	6	0		110
	April	44				44
	June	9			0	9
	August	1146				1146
	September	1242				1242
	October	537				537
Anopheline Total		3082	6	0	0	3088

A – 15: Trends of Indoor Resting Density of *Anopheles* Mosquitoes across all Sentinel Sites, November 2014 – October, 2015

Month	Sentinel Sites	# of Rooms	Total # of Anopheles Caught	Indoor Resting Density
Nov-14	Enugu	32	13	0.4
	Plateau	32	108	3.4
	Rivers	32	45	1.4
	Sokoto	32	0	0.0
	Lagos	32	29	0.9
	N/Eggon	32	114	3.6
	Doma	32	8	0.3
Dec-14	Enugu	32	20	0.6
	Plateau	32	188	5.9
	Rivers	32	18	0.6
	Sokoto	32	0	0.0
	Lagos	32	7	0.2
	N/Eggon	32	65	2.0
	Doma	32	19	0.6
Jan-15	Enugu	32	7	0.2
	Plateau	32	0	0.0
	Rivers	32	0	0.0
	Sokoto	32	0	0.0
	Lagos	32	2	0.1
	N/Eggon	32	101	3.2
	Doma	32	30	0.9
Feb-15	Enugu	32	12	0.4
	Plateau	32	18	0.6
	Rivers	32	1	0.0

Month	Sentinel Sites	# of Rooms	Total # of Anopheles Caught	Indoor Resting Density
	Sokoto	32	110	3.4
	Lagos	32	1	0.0
	N/Eggon	32	169	5.3
	Doma	32	21	0.7
Apr-15	Enugu	32	18	0.6
	Plateau	32	121	3.8
	Rivers	32	43	1.3
	Sokoto	32	43	1.3
	Lagos	32	7	0.2
	N/Eggon	32	73	2.3
	Doma	32	36	1.1
Jun-15	Enugu	32	220	6.9
	Plateau	32	444	13.9
	Rivers	32	100	3.1
	Sokoto	32	9	0.3
	Lagos	32	45	1.4
	Nasarawa (N/Eggon)	32	154	4.8
	Nasarawa (Doma)	32	62	1.9
Aug-15	Enugu	32	149	4.7
	Plateau	32	739	23.1
	Rivers	32	101	3.2
	Sokoto	32	1146	35.8
	Lagos	32	5	0.2
	Nasarawa (N/Eggon)	32	102	3.2
	Nasarawa (Doma)	32	200	6.3
Sep-15	Enugu	32	81	2.5
	Plateau	32	353	11.0
	Rivers	32	87	2.7
	Sokoto	32	1242	38.8
	Lagos	32	13	0.4
	Nasarawa (N/Eggon)	32	103	3.2
	Nasarawa (Doma)	32	104	3.3
Oct-15	Enugu	32	49	1.5
	Plateau	32	127	4.0
	Rivers	32	60	1.9
	Sokoto	32	537	16.8
	Lagos	32	25	0.8
	Nasarawa (N/Eggon)	32	123	3.8
	Nasarawa (Doma)	32	54	1.7

ANNEX B: 2015 VECTOR SUSCEPTIBILITY AND SYNERGIST ASSAYS

INSECTICIDE SUSCEPTIBILITY TESTS

Insecticide susceptibility tests were carried out using the standard WHO protocol (WHO, 2013) and CDC bottle bioassay.

WHO SUSCEPTIBILITY TEST PROCEDURE

WHO insecticide susceptibility test kits and impregnated papers were used for this test (Figure 2). Two to three- day old, non-blood-fed adult female *An. gambiae* s.l. mosquitoes collected around the sentinel sites were tested. Batches of 20-25 mosquitoes were exposed for 60 minutes to test papers impregnated with permethrin (0.75 percent), deltamethrin (0.05 percent), alphacypermethrin (0.75 percent), lambdacyhalothrin (0.05 percent), propoxur (0.05 percent), bendiocarb (0.13 percent), DDT (4.0 percent) and primiphos-methyl (0.25 percent). The tests were carried out in four replicates across the sentinel sites while, control experiments with a batch of 20 - 25 mosquitoes collected from the same sites were also set up. In this case, the mosquitoes were exposed to untreated papers impregnated with mineral oils. The controls were in two replicates and where control mortality was observed to be between 5 percent and 20 percent, corrected mortality was determined using Abbott's formula:

$$\frac{(\%\ \text{observed mortality} - \%\ \text{control mortality}) \times 100}{100 - \%\ \text{control mortality}}$$

Test results were discarded where control mortality was above 20 percent (Abbott, 1925).

The mosquitoes used for the tests were preserved individually in Eppendorf tubes and labeled appropriately for identification and further analysis (Figure 2).

CDC Bottle Bioassay

Preparation of CDC Test Bottles

The CDC test bottles were washed with detergents, rinsed with tap water and air - dried for 2 hours to achieve complete dryness.

Five bottles were used for the test: four were coated with appropriate insecticide while one served as control. The bottles were labeled with the name and concentration (µg/bottle) of the insecticide to be coated. The date of the experiment was also labeled on the lid and the bottle.

Serial dilutions of each of the insecticide concentrations (permethrin (0.75%), lambdacyhalothrin (0.05%), alpha-cypermethrin (0.75%), deltamethrin (0.05%), bendiocarb (0.1%), primiphos-methyl (0.25%) and DDT (4%)) were prepared by adding 1ml of the stock solution (49ml acetone + 1ml insecticide) to the treatment bottles, per the CDC bottle bioassay protocol. The control bottle was treated with 1ml of acetone only.

To achieve proper coating of the bottles with the insecticides, the bottles were rotated in all directions making sure that the inside of the caps was well coated during the process. After the interior of the bottle was completely coated with insecticide, caps were removed and bottles were rolled on table mat (Figure 3) until all acetone has evaporated. The bottles were left open without the lids on an undisturbed clean mat overnight to ensure that all acetone had dissipated. While drying, they were covered with a cloth to protect them from the light.

Bioassay Procedures

The bottles were lined up with their lids open. A group of 20-25 female *Anopheles* mosquitoes (2-3 days old) were collected with an aspirator from the rearing cages and introduced into each of the five bottles, including the control (Figure 3). The mosquitoes were prevented from escaping by covering the mouth of the bottle and replacing the lid. The number of mortality in each test bottle was recorded at start (Time 0) and after every 15 minutes until all are dead, or up to 2 hours (Brogdon and Chan 2010).

The resistance status of mosquito samples tested with the WHO tube test was determined according to the latest WHO criteria (2013) as follows:

- Mortality rates between 98 percent and 100 percent indicate full susceptibility
- Mortality rates between 90 percent and 97 percent require further investigation
- Mortality rates < 90 percent, the population is considered resistant to the tested insecticides.

The resistance status of mosquito samples tested using the CDC bottle assay was determined according to the CDC criteria (Brogdon and McAllister, 1998; CDC, 2010). The susceptibility thresholds were at the diagnostic time of 30 minutes for pyrethroids, carbamates and organophosphates and 45 minutes for DDT.

Resistance Mechanism Analysis

The need to identify underlying resistance mechanism(s) and to estimate the frequency of the knock down resistance (kdr) gene in the mosquito population informed the decision to carry out knock down resistance (Kdr) analysis of *Anopheles* mosquitoes from two of the sentinel sites: Nasarawa Eggon in Nasarawa State and Epe in Lagos State which were selected for these tests. AIRS project contracted Nigeria Institute for Medical Research (NIMR) based in Lagos to conduct this analysis.

SYNERGIST ASSAY WITH THE CDC BOTTLE BIOASSAY

Synergist test was carried out to investigate the plausible role of metabolic enzymes in insecticide detoxification in the resistant mosquito population from Nassarawa and Lagos sentinel sites. Synergist assays was done using piperonyl butoxide (PBO) an inhibitor of mixed function oxidase on *Anopheles gambiae* from each site. The synergist assay was carried out using 4% piperonyl butoxide (an inhibitor of mixed function oxidases) in CDC bottle assay as detailed in the study protocol described by Brogdon, and Chan (2010).

MOLECULAR IDENTIFICATION AND *KDR* RESISTANCE ASSAY

All *Anopheles* mosquitoes tested were analyzed using PCR for species-specific identification. DNA extracted from each specimen using the standard method was amplified using the *Anopheles gambiae* species specific multiplex PCR.

A subset of the *Anopheles* that survived the insecticide exposure were knocked down in a refrigerator, preserved individually on desiccated silica gel together with the dead mosquitoes and analyzed for the presence of the knockdown resistance (*kdr)* mutation using allele-specific PCR diagnostic tests designed for the West African *kdr* mutation (Martinez-Torres *et al.,* 1998)

M AND S-FORMS AND KDR PCR ASSAYS

The aim of this analysis is to determine the proportion of the molecular M and S form of *Anopheles gambiae* from samples collected at Nasarawa and Lagos sentinel sites The specimens analysed were selected from two sources: (i) mosquito collected from human dwellings indoor and (ii) mosquito that survived insecticide exposure during routine insecticide susceptibility tests at both sites. The M and S-Forms and Kdr PCR assays were preceded by a priori polymerase Chain Reaction (PCR) assay for identification of members of the *Anopheles gambiae* complex (Scott *et al*.,1993). This includes DNA extraction using essential extraction kits followed by PCR analysis. Based on the outcome of the species specific-PCR assay, aliquot of DNA from each sample was processed for subsequent test to identify the molecular M and S using established protocols (Favia *et al.,* 1994; Della Torre *et al.,* 2001). The presence of the knock down resistance alleles was tested as earlier described. The presence of both the west (*kdr-w*) and East (*Kdr-e*) African kdr mutations were determined using specific primers and protocols designed for these assays (Martinez-Torres, 1998). Specifically, the West African kdr genotype is characterized by three different PCR bands: 293bp common to both susceptible and resistant specimens; 137bp susceptible band and 195bp kdr band. The presence of the three bands in a single specimen indicates heterozygous.

The frequency of the kdr gene was calculated using established genotype formula:

f (R) = (2RR + Rr) / 2n. Where f = frequency, n = number of sample analyzed, RR = number of homozygote resistant, Rr = number of heterozygous resistance.

DATA ANALYSIS

Data were analyzed using SAS software (Statistics SAS Institute Inc., Cary, NC 27513, USA) and statistics GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA. Ento guidance statitistical analysis tool and T-Test and Chi-square test with Yates formula were used to determine differences in the abundance of *Anopheles* mosquitoes (indoors and outdoors) at P = 0.05 level of significance. Fisher's exact test and Analysis of Variance (ANOVA) were used for further analysis. All graphs were plotted using Microsoft Excel.

SYNERGIST ASSAY WITH THE CDC BOTTLE BIOASSAY IN LAGOS STATE

B - 1: Mortality of non-synergized mosquito exposed to deltamethrin in Lagos

Time (min)	Bottle 1		Bottle 2		Bottle 3		Bottle 4		TOTAL DEAD		Control	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	TOTAL	%	Alive	Dead
0	25	0	25	0	25	0	25	0	0	0	50	0
15	20	5	20	5	17	8	17	8	26	26	50	0
30	7	18	14	11	5	20	10	15	64	64	50	0
45	1	24	1	24	2	23	4	21	92	92	49	1
60	0	25	1	24	0	25	4	21	95	95	49	1

B - 2: Mortality of PBO-synergized mosquito exposed to deltamethrin in Lagos

Time (min)	Bottle 1		Bottle 2		Bottle 3		Bottle 4		TOTAL DEAD		Control	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	TOTAL	%	Alive	Dead
0	25	0	25	0	25	0	25	0	0	0	100	0
15	7	18	4	21	6	19	5	20	78	78	100	0
30	2	23	1	24	2	23	5	20	90	90	100	0
45	0	25	0	25	0	25	0	25	100	100	100	0
60	0	25	0	25	0	25	0	25	100	100	100	0

SYNERGIST ASSAY WITH THE CDC BOTTLE BIOASSAY IN NASSARAWA STATE

B - 3: Mortality of non-synergized mosquito exposed to deltamethrin in Nasarawa Eggon.

Time (min)	Bottle 1		Bottle 2		Bottle 3		Bottle 4		TOTAL DEAD		Control	
	Alive	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**
0	21	0	20	0	25	0	27	0	0	0	25	0
15	6	15	2	18	5	20	3	24	77	83	25	0
30	0	21	0	20	0	25	1	26	92	98.8	25	0
35	0	21	0	20	0	25	0	27	93	100	25	

B - 4: Mortality of PBO-synergized mosquito exposed to deltamethrin in Nasarawa Eggon

Time (min)	Bottle 1		Bottle 2		Bottle 3		Bottle 4		TOTAL DEAD		Control	
	Alive	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**
0	25	0	25	0	25	0	25	0	0	0	25	0
15	6	19	2	23	4	21	1	24	87	87	25	0
30	0	25	0	25	0	25	0	25	100	100	25	0

B - 5: Mortality of non-synergized mosquito exposed to deltamethrin in Doma.

Time (min)	Bottle 1		Bottle 2		Bottle 3		Bottle 4		TOTAL DEAD		Control	
	Alive	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**
0	22	0	21	0	20	0	25	0	0	0	20	0
15	6	16	2	19	1	19	3	22	76	86.4	20	0
30	1	21	0	21	1	19	0	25	86	97.7	20	0
35	0	22	0	21	0	20	0	25	88	100	20	0

B - 6: Mortality of PBO-synergized mosquito exposed to deltamethrin in Doma.

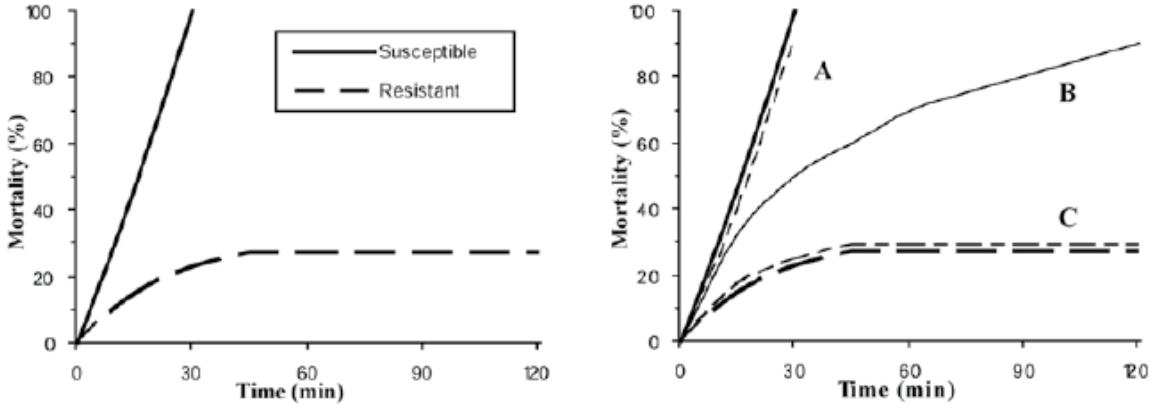
Time (min)	Bottle 1		Bottle 2		Bottle 3		Bottle 4		TOTAL DEAD		Control	
	Alive	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**	**Alive**	**Dead**	**Alive**	**Dead**	**Alive**
0	26	0	25	0	24	0	25	0	0	0	25	0
15	6	20	3	22	1	23	0	25	90	90	25	0
30	0	26	0	25	0	24	0	25	100	100	25	0

Evaluation of Insecticide Resistance mechanisms (synergist assays). (Brogdon and Chan, 2010).

Once a synergist is used on the resistant population, one of three things might happen (Figure 10b):

a. Resistance to the insecticide is abolished (time-mortality line A), which suggests that the mechanism related to that synergist is playing a role in the insecticide resistance observed;
b. Resistance to the insecticide is partially abolished (time-mortality line B). This suggests that the mechanism related to that synergist is involved in the resistance, but it is not the only mechanism involved in this particular case;
c. Resistance to the insecticide is unaffected (time-mortality line C). This indicates that the mechanism related to that synergist is not involved in the resistance.

It is also possible to determine if a target site mechanism, such as the presence of the kdr gene (sodium channel mutation) or insensitive acetylcholinesterase, is involved. This is done by using the synergists in combination. Their combined use will not abolish the resistance in the bioassays when a target site mechanism is present. It is crucial in areas where pyrethroids and/or DDT are used to evaluate the relative role of detoxification and target site mechanisms involved in a particular incidence of resistance. A target site mechanism confers DDT–pyrethroid cross-resistance, while a detoxification mechanism may or may not.



Effects of synergists on resistant vector populations. Figure 10a shows data for a population of resistant vectors compared to a susceptible population. Figure 10b shows the three possible outcomes of synergist exposure (Line A: Resistance to the insecticide is abolished; Line B: Resistance to the insecticide is partially abolished; and Line C: Resistance to the insecticide is unaffected).