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Do Long Lasting Insecticidal Treated Nets Alone and Long Lasting Insecticidal Treated Nets plus Indoor Residual Spraying combination have an effects on *Anopheles arabiensis* Patton (Diptera: Culicidae) population densities, biting cycle, biting places and biting rate; A randomised Control Trial in central and eastern, Sudan.

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ABSTRACT: *A cluster randomized controlled trial, with two interventions (study arms)) Long Lasting Insecticidal Treated Nets (LLIN) alone and a combination of Long Lasting Insecticidal Treated Nets plus Indoor Residual Spraying (LLIN + IRS) was carried out with an objective to identify the species composition, abundance, densities, biting cycle, biting places and human biting rate of Anopheles arabiensis in 30 clusters from four areas Ahoosh, Alhagabdalla, Galabat and New Halfa in central and eastern Sudan. Mosquito samples were collected indoor and outdoor from human dwellings using CDC light trap collection method. Species identifications showed that 94.7 % specimens were Anopheles arabinoses Patton. Mean density was found 1.5 ± 0.5 and 1.1 ± 0.3 for LLIN and LLIN+IRS respectively. The overall density was 1.4 ± 0.3 Anopheles / room / day. 65.7% of Anopheles arabiensis population was fed indoor "Endophagic". The pooled ratios between indoor and outdoor for study arm LLIN alone was 1.7:1 and 1.5: 1 for LLIN+ IRS. No significant difference between biting places of Anopheles arabiensis indoor and outdoor in the study arm LLIN alone and LLIN+ IRS in four study areas. The biting activity of Anopheles arabiensis was found to extend throughout the night with the peak commencing in the early of the night). Indoor Man Biting Rate IMBR was significantly higher compared to outdoor (1.52 and 1.03 bites / person /night) for LLIN study arm, while the Outdoor Man Biting Rate (OMBR) was (0.8 and 0.6 bites / person /night) for LLIN+IRS study arm respectively. The overall mean man biting rate indoor was (1.4 ± 0.34 bites / person /night) , while the mean man biting rate outdoor was (0.7 ± 0.15 bites / person /night). There was a significant difference between indoor and outdoor human biting rate (P < 0.05). The study revealed that Anopheles arabiensis is the dominant malaria vector in Sudan, was fed throughout the night with the peak commencing in the early of the night, bites indoor (Endophagic) and there was no difference in biting places, biting time and man biting rates between combining LLIN with IRS relative to use LLIN alone.*

Keywords: *Anopheles arabiensis, LLIN alone, LLIN+IRS combination, biting habits, Sudan.*

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I. Introduction

Malaria is the most common and devastating disease in the tropics. 247 million among 3.3 billion people at risk in 2006. It has been recently estimated that 200 million people (24.6% of the total population in Africa) live in urban settings where at risk of contracting malaria [1] (Keiser, 2004). *Anopheles gambiae* complex have been known as the most efficient malaria vectors in Afro - tropical region. [2] [3] *Anopheles arbiensis* is the predominant malaria vector reported from all parts of Sudan .[4] [5] [6] [7] [8] [9] Co- existing with *Anopheles gambiae sensu stricto (s. s)*. The siblings of *Anopheles gambiae* are morphologically similar at all levels of their different developmental stages, the adults are different in their biology, biting and resting behaviours. [10Based on many reports analyzed, it seems that at least in some cases, there are advantages of combining LLIN with IRS relative to use either method alone, but that this outcome may be different in certain situations, since there are numerous confounding factors that can affect the results. It is therefore certain that evidence to support or refute this strategy for combinations remains inconclusive and any generalizations for optimal strategies cannot be made. [11] Universal coverage with long lasting insecticide – treated nets (LLINs) or IRS is actively promoted as the main prevention strategy under the WHO endorsed malaria control and elimination plan. [12] The LLINs + IRS combination strategy is mostly recommended for accelerating control in

high transmission areas, where either IRS alone or ITNs alone may not be adequate. [12] [13] Uses of both IRS and LLIN has increased over the last decade as part of the drive towards covering all human populations at risk, saving millions of lives. Combined IRS and LLIN have also been suggested as a means of delaying the emergence of insecticide resistance by using different classes of insecticide for IRS and LLINs. [13] Understanding the behaviour of the malaria vectors and their abundance are essential for malaria control operations.

II. Material And Methods

Study design

The study was a cluster randomized controlled trial, it was conducted at 30 clusters from four areas Ahoosh, Alhagabdalla, Galabat and New Halfa in central and eastern Sudan with two interventions (study arms) LLINs alone and a combination of LLINs plus IRS.

Study area

The study was carried out at 4 areas in Sudan. 2 areas (Ahoosh and Hag Abdullah) are located in Gezira agriculture scheme this is one of the biggest irrigation schemes in the world (National Pesticide Council, unpublished data). The main occupation of the inhabitants is agriculture cultivating cotton. Climatically the areas have hot dry summer from March to June and cool dry winter from October to February; the average annual rainfall is 225 mm per annum, mainly in July to September. Housing consists of a mixture traditional mud walls with thatched roof construction, and modern brick built houses. Galabat area belongs to Gadarif state, 80 km from Gedarif town and bordering Ethiopia. The area is within the dry savannah region, with a short rainy season during June to September followed by a dry season from October until the end of May. The houses are made of local materials (hay and mud). New Halfa area is located in the semi-arid belt of the Sudan approximately 500 km east of Khartoum within the New Halfa irrigated scheme in Kassala State. The area is classified as dry savannah with rainfall from July to early October ranging between 300 to 411 mm per annum.Temperatures range between 16°C and 45°C. Housing is composed of mixture of hay, mud and brick. Administrative units (Fig1)

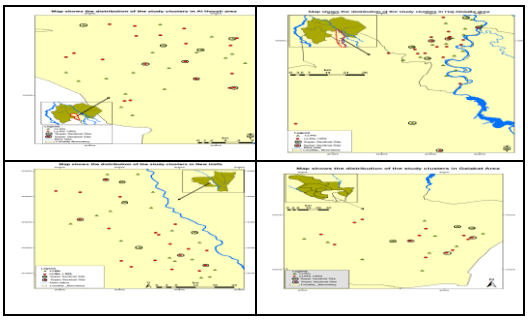

FIgure1: Map showing location of clusters in the four study areas:

Materials

During the study, the following equipment and materials were used : light traps, hand lenses, small Petri dishes, paper cups with net covers , forceps , plastic containers, adhesive tapes, cotton wool, filter papers , a torch and batteries, , mouth aspirators, hammers, nails , silica gel, eppendorf tubes, recording sheets, pens and pencils.

Mosquito collection

Collection by means of Human-baited CDC Light Traps collection methods was used to make monthly collections of indoor and outdoor to catch mosquitoes during the period 2012, 2013 and 2014. Collection by light trap capture was carried out quarterly (3 hours) from 19:00 to 07:00 hours. The collections of mosquitoes were done in 30 clusters; three houses were randomly selected for adult mosquito collections. One CDC light trap is positioned indoors, fitted with incandescent bulbs, and placed close to the human volunteer sleeping under an untreated bed net in his / her usual sleeping place. [14] The light trap was installed at about 1.5 m (5 - 6 ft) above the floor next to the foot of the bed. [15] Trapped mosquitoes removed the next morning. To cover 30 clusters; four teams worked for a total of 8 days per month for three months (September-November) in Alhoosh, Hag Abdllah, and New Halfa and 6 days in Galabat during the transmission season and one month (March) in dry season in each year. Females collected were classified according to their blood meal stages unfed, fed, half-gravid / gravid. Females collected were kept in separate paper cups until identification.

Morphological identification

All the mosquitoes caught were counted, recorded and identified using morphological features to species with the aid of identification Gillies and Meillon, 1968 manuals. [16]

Species molecular identification

A Subsample of mosquitoes collected resting indoors was identified to species level. Species-specific PCR assay following Scott protocol [16] and genomic DNA extracted following the procedure described by Livak, [18] were used. DNA was re-suspended in 100 µl molecular biology water. Positive controls obtained from known colonies maintained in the insectary at Prof El gaddal National Malaria Research and Training Centre - Sennar. The *Anopheles gambiae* amplification was done in thermal cycle in 94o C for 5 minutes and 30 cycles of denaturation 94o C for 30 seconds, 50o C for 30 seconds of annealing and final extension at 72o C for 30 seconds the end cycle at 72o C for 10 minutes and incubates at 10o C forever. The samples were run on 2% agarose gel which prepared by adding 2 grams agarose gel to 100 ml TBE buffer and 5 µ Ethidium bromide show the result as follow, *Anopheles arabiensis* 315 base pair and *Anopheles gambiae* 390 base pair under Gel documentation system instrument.

Data analysis

Data were analyzed using the Microsoft Excel program and SPSS version16.0. ANOVA was used to evaluate the difference in the density. The Chi-Square test was used to evaluate the difference between biting places and man biting rate of female in the two study arms (LLIN and LLIN+ IRS) in the four study areas during the study period (2012- 2013 and 2014). The P. value less than 0.05 considered significant.

III. Results and Discussion

***Anopheles* Species Identification:**

A total of 720 member of morphologically identified as *Anopheles gambiae* complex subsamples were randomly selected from collected mosquitoes and subjected to species specific polymerase chain reaction PCR test. The majority 94.7 % (682/720) was successfully identified as *Anopheles arabienasis*. (Fig 2 and 3)

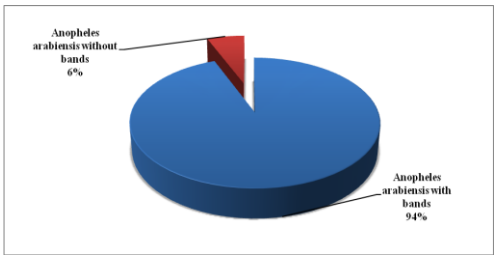

Figure2: PCR analysis results for *Anopheles gambiae* per study areas in 2012- 2013 and 2014

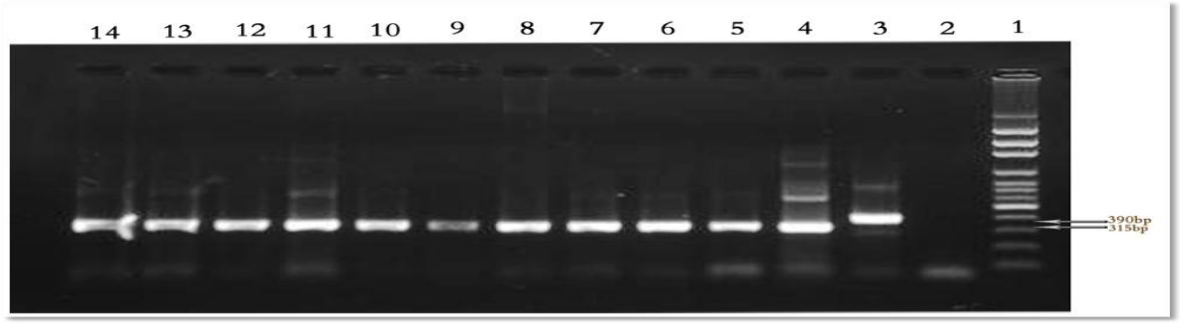

Figure3: Amplified fragments using the species-specific PCR assay for the identification of members of the *Anopheles gambiae* complex, Lanes 1 kb molecular markers from right to left: 2 negative control, 3 *Anopheles gambiae* control, 4 *Anopheles arabiensis* control and then the samples from (5-14) are *Anopheles arabiensis* which are the DNA ladder sizes are 315 bp and 390 bp for *Anopheles arabiensis* and *Anopheles gambiae* respectively.

Population abundance and density

Density of *Anopheles arabiensis* / room / day

During study period a total of 7444 females were collected of which 84.6 % (6297/7444) collected from indoor of the dwelling by the pyrethrum spray collection method, while 15.4% (1147/7444) were collected by CDC Light Taps from four study areas. The mean density of *Anopheles* was found 682(1.7± SE 0.6), 465 (1.1 ± SE 0.3) female *Anopheles* / room/ day for LLIN and LLIN+IRS while the pool mean density was found 1147(1.4 ± SE 0.3) female *Anopheles* / room/ day. (Tables1 and 2)

Table1: Total number and mean vector densities of *Anopheles* collected indoor by CDC light trap per study arms LLIN and LLIN+IRS per study areas

Study areas	LLIN		LLIN+IRS		Total	
	No. of *Anopheles* collected	Mean ± SE density No./ room / day	No. of *Anopheles* collected	Mean ± SE density No./ room / day	No. of *Anopheles* collected	Mean ± SE density No./ room / day
Alhoosh	246	2.5 ± SE1.16	225	2.2 ± SE 0.83	471	2.4 ± SE 1.157
Alhagabdalla	128	1.2 ± SE 0.36	184	1.6 ± SE 0.29	312	1.42 ± SE .360
Galabat	292	2.8 ± SE 2.01	27	0.3 ± SE 0.15	319	1.55 ± SE 2.01
New Half	16	0.1 ± SE 0.05	29	0.3 ± SE 0.09	45	0.19 ± SE.055
Total	682	1.7 ± SE 0.6	465	1.1 ± SE 0.32	1147	1.4 ± SE0.34

Table2: Total number and mean vector densities of *Anopheles* collected indoor by CDC light trap per study arms LLIN and LLIN+IRS per years

LLIN			LLIN+IRS		Total	
Yeas	No. of *Anopheles* collected	Mean ± SE density No./ room / day	No. of *Anopheles* collected	Mean ± SE density No./ room / day	Total No. of *Anopheles* collected	Mean ± SE density No./ room / day
2012	365	2.33± SE1.54	197	1.10 ± SE0.38	562	1.71± SE0.77
2013	146	0.83± SE0.21	113	0.58 ± SE0.28	259	0.70± SE0.17
2014	171	1.83± SE1.06	155	0.58 ± SE0.28	326	1.73± SE0.63
Total	682	1.7± SE0.60	465	1.1 ± SE0.32	1147	1.4 ± SE00.34

Biting place (indoor / outdoor)

Of the total *Anopheles arabiensis* 1741 was captured indoor and outdoor by CDC light trap collection method, 682(66.9%) and 338(33.1%) was caught indoor and outdoor in LLIN study arm, while 465(64.5%) and 256 (35.5%) was caught indoor and outdoor in LLIN+IRS study arm respectively. (Tables 3 and 4) The percentage of females *Anopheles arabiensis* collected indoor and outdoor by CDC light trap collection method showed variations, ranging from 25% in New Halfa in 2014 to 81.8% in Galabat study area in 2014 for study arm LLIN, while 37.5% in Galabat study area in 2013 to 84.0% in the same study area in 2012 for study arm LLIN+IRS. The mean percentage of *Anopheles arabiensis* captured indoor (61.3% ±SE 2.9) was significantly higher than outdoor (38.7% ± SE 2.9), (P =0.001) (Tables 3 and 4). The pooled ratios between indoor and outdoor for study arm LLIN alone was 1.7:1 and 1.5: 1 for LLIN+ IRS.

Table3: Percentage of *Anopheles arabiensis* biting indoor versus outdoor per study arm LLIN in 2012-2013 and 2014

Study area	2012			2013			2014			Total		Total	Total
	LTIN	LTOUT	%	LTIN	LTOUT	%	LTIN	LTOUT	%	LTIN	LTOUT	%	%
Alhoosh	84	25	77.1	48	42	53.3	114	46	71.3	246	113	68.5	31.5
Alhag Abdalla	34	13	72.3	49	39	55.7	45	14	76.3	128	66	66.0	34.0
Galabat	245	116	67.9	38	20	65.5	9	2	81.8	292	138	67.9	32.1
New Halfa	2	2	50.0	11	10	52.4	3	9	25.0	16	21	43.2	56.8
Total	365	156	70.1	146	111	56.8	171	71	70.7	682	338	66.9	33.1

Table 4: Percentage of *Anopheles arabiensis* biting indoor and outdoor per study arm LLIN+IRS in 2012-2013 and 2014

Study area	2012			2013			2014			Total		Total	Total
	LTIN	LTOUT	%	LTIN	LTOUT	%	LTIN	LTOUT	%	LTIN	LTOUT	%	%
Alhoosh	84	30	73.7	49	47	51.0	92	35	72.4	225	112	66.8	33.2
Alhag Abdalla	80	36	69.0	54	37	59.3	50	36	58.1	184	109	62.8	37.2
Galabat	21	4	84.0	3	5	37.3	3	3	50.0	27	12	69.2	30.8
New Halfa	12	9	57.1	7	5	58.3	10	9	52.6	29	23	55.8	44.2
Total	197	79	71.4	113	94	54.6	155	83	65.1	465	256	64.5	35.5

Biting cycle

Of 1741 *Anopheles arabiensis* collected, 1020 (58.6%) and 721(41.4%) for LLIN and LLIN+IRS study arms. Generally the biting activity initiated at the first quarter (07:00 -10:00) hours. The overall biting times were found 758(43.5 %), 421(24.2 %), 336 (19.4 %) and 226 (13%) in the first, second, third and fourth quarter. The overall percentage of biting activity indoor were found 498 (28.6%), 277 (15.9%), 223 (12.9%) and 149 (8.6%), while it found 260 (14.9%), 144(8.3%), 113 (6.5%) and 77 (4, 4%) of outdoor at the first, second, third and fourth quarter respectively. (Table5) The percentage were found 372(21.4%), 200 (11.5%), 289 (16.6%) and 159 (9.1%) of first, second, third and fourth quarter respectively for the study arm LLIN. The percentages were found 386 (22.2%), 221(12.7%), 47(2.7%) and 67(3.8%) of first, second, third and fourth quarter respectively for LLIN+IRS study arm. (Table 5) The percentages of time biting indoor were found 249(24.4%), 134 (13.1%), 193(18.9%) and

106 (10.4%), while the percentages of time biting outdoor were found 123(12.1%), 66 (6.5%), 96 (9.4%) and 53(5.2%) for LLIN study arm. While the percentages of time biting indoor were found 249 (34.5%), 143(19.8%), 30(4.2%) and 43 (6%), while the percentages of time biting outdoor were found 137 (19%), 78(10.8%), 17(2.4%) and 24(3.3%) for LLIN+IRS study arm. (Table 5) The peak biting activity indoor was 498 (28.6%) at the first quarter (07:00 -10:00) hours and drop gradually to the lowest value 149 (8.6%) at the fourth quarter 04:00 – 07:00 in the next morning, while the peak biting activity outdoor was found 260 (14.9%) and lowest was 77 (4.4%) at the fourth quarter 04:00 – 07:00 in the next morning (Table5 and Fig 4)

Table5 Percentages of biting cycle indoor and outdoor per quarter time study arms LLIN and LLIN+IRS.

Quarter	LLIN					LLIN+IRS					LTIN Total	LTOUT Total	LTIN %	LTOUT %
	An. No.	LTIN	LTOUT	LTIN %	LTOUT %	*An.* No.	LTIN	LTOUT	LTIN %	LTOUT %				
07:00-10:00	372	249	123	24.4	12.1	386	249	137	34.5	19.0	498	260	28.6	14.9
10:00-01:00	200	134	66	13.1	6.5	221	143	78	19.8	10.8	277	144	15.9	8.3
01:00-04:00	289	193	96	18.9	9.4	47	30	17	4.2	2.4	223	113	12.9	6.5
04:00-07:00	159	106	53	10.4	5.2	67	43	24	6.0	3.3	149	77	8.6	4.4
Total	1020	682	338	66.9	33.1	721	465	256	64.5	35.5	1147	594	65.9	34.1

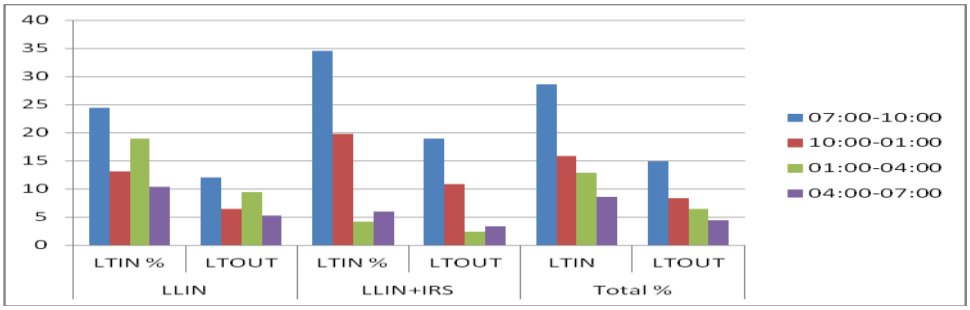

Figure 4: Percentages of biting cycle indoor and outdoor per quarter time per study arms LLIN and LLIN+IRS.

Man biting rate (MBR)

In this study, indirect calculation of the man-biting rate from the spray sheet collection was used, the man-biting rate (per night) is obtained by dividing the total number of fed mosquitoes by the total number of individuals or occupants of the houses used for collection multiplied by the human blood index. (WHO, 2013) Of the *Anopheles arabiensis* females captured 1147(65.9%) and 594 (34.1%) was caught indoor and outdoor. The indoor man biting rate IMBR was (1.52 and 1.03 bites / person /night) for LLIN and LLIN+IRS study arms respectively, while the outdoor man biting rate (OMBR) was (0.8 and 0.6 bites / person / night) for LLIN and LLIN+IRS study arms respectively.(Table6) The overall mean ± SE man biting rate indoor was (1.4 ± 0.34 bites / person /night) , while the mean man biting rate outdoor was (0.7 ± 0.15 bites / person /night) .There was significant difference between indoor and outdoor human biting rate $P < 0.05$ (Table 6) There was no difference between man biting rates for LLIN and LLIN+IRS, study areas and years. The chi-square statistic is 0.0335. The P-value = .854864. This result is not significant at $P < 0.05$.

Table6: Biting rates per study areas per study arms LLIN and LLIN+IRS in 2012 -2013 and 2014

Year	Study area	LLIN				LLIN+IRS			
		LT IN	b/p/n	LTOUT	b/p/n	LT IN	b/p/n	LTOUT	b/p/n
2012	Alhoosh	84	1.75	25	0.5	84	1.8	30	0.63
	Alhag Abdalla	34	0.71	13	0.3	80	1.7	36	0.8
	Galabat	245	6.81	116	3.2	21	0.6	4	0.1
	New Halfa	2	0.04	2	0.04	12	0.3	9	0.2
Total		365	2.03	156	0.9	197	1.09	79	0.43
2013	Alhoosh	48	1	42	0.9	49	1.02	47	1
	Alhag Abdalla	49	1.02	39	0.8	54	1.13	37	0.8
	Galabat	38	1.1	20	0.6	3	0.1	5	0.1
	New Halfa	11	0.23	10	0.2	7	0.15	5	0.1
Total		146	0.81	111	0.62	113	0.63	94	0.52
2014	Alhoosh	114	4.8	46	1.9	92	3.83	35	1.46
	Alhag Abdalla	45	1.9	14	0.6	50	2.08	36	1.5
	Galabat	9	0.5	2	0.1	3	0.2	3	0.17
	New Halfa	3	0.13	9	0.4	10	0.42	9	0.38
Total		171	1.9	71	0.8	155	1.72	83	0.92
Grand total		682	1.52	338	0.8	465	1.03	256	0.6

The study showed that *Anopheles arabiensis* was the principal malaria vector in the study areas (94.7 % *Anopheles arabiensis* patton). Our results agreed with the previous studies showed that the predominant vector which has been reported in Northern, Eastern and Central Sudan. . [9] [19] [20] [21]. This study confirmed that *Anopheles arabiensis* is the only member of the *Anopheles gambiae* complex present. The densities of *Anopheles arabiensis* were indifference between study arm LLIN alone and LLIN plus IRS, study areas and between years $P > 0.05$.

The study showed that the biting places of *Anopheles arabiens* were indoor and outdoor. However it was found that no significant difference between biting place in the study arm LLIN alone and LLIN+ IRS in four study areas although this study showed that large feeding of *Anopheles arabiensis* population was occurring indoor "endophagic".This finding supported by the study conducted in Gorgora North – West Ethiopia and Tanzania. [22] [23] Working in northern Uganda showed that 67.3% of biting feeding of *Anopheles gambaie* occurred indoor and 32.7% of biting feeding occurred outdoor. [24]

This study contrasted with previous studies due to the excito- repellent effect of pyrethroids causes the mosquitoes to leave rooms for the outdoors, hence the observed reduction in indoor biting. [4] [14] [6] Three factors appear to determine the biting habits of the *Anopheles arabiensis* 1) rhythmic or periodic activity of the mosquitoes, 2) the microclimate 3) and human habits. However, *Anopheles arabiensis* was higher indoor than outdoor biting (more endophagic than exophagic).

Anopheles arabiensis populations showed a wide range of peak biting times at different sites, with some of this variation being explained by season. One possible explanation for the remaining variation is that peak biting times may reflect the historical use of insecticides. [25] [4] [26] [27]

In this study the biting activity of *Anopheles arabiensis* was found to extend throughout the night with the peak commencing in the early hours of the night (first quarter 1900 – 2200) before the inhabitants go to bed. Similar results were reported in a previous study in Northern Ethiopia which reported that the peak time initiated at early time of the night 1900 hrs after 40 years of DDT IRS [28]

Over 64% of biting activity occurred before 24.00 hours, when people typically retire to bed. This early biting activity may have a negative impact on the efficiency of bed nets to control malaria. However, *Anopheles arabiensis* was higher indoor than outdoor biting (more endophagic than exophagic). Slightly shifts in *Anopheles gambiae s.l.* behaviours were found after spraying campaigns in western Kenya and Tanzania. [14] [29] Where in Burkina Faso- no behavioural changes were observed in vector mosquitoes. [30] Another recent study has also reported early biting cycles in *Anopheles arabiensis, Anopheles pharoensis* and *Anopheles coustani* in Ziway in central Ethiopia. [31] In this study the man biting rate (MBR) recorded for indoors was significantly higher compared to outdoors man biting rate. In this study indirect calculation of the man-biting from the spray sheet collection was used, the man-biting rate (per night) is obtained by dividing the total number of fed mosquitoes by the total number of individual occupant the houses. [32] In this study the man biting rate MBR recorded for indoor was significantly high compared to outdoor man biting rate.

Many previous studies revealed that the combination of LLIN plus IRS had no additional impact on *Anopheles arabiensis* densities a possible explanation for this contradiction is that exposure to treated bed nets. [33] 92.5% coverage of Householder by IRS with bendiocarb and LLINs (PermaNet 2.0) 87% ownership (one LLIN per 2 person) after the LLIN universal coverage campaign in 2012. (National Malaria Control Programme, unpublished data).

IV. Conclusion

arabiensis population was fed indoor more "endophagic than exophagic", the feeding activity extend throughout the night with the peak commencing in the early of the night when people typically retire to bed. This early biting activity may have a negative impact on the efficiency of bed nets to control malaria.

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