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Impact of long-lasting insecticidal nets on host seeking behaviour of *Anopheles fluviatilis* and *Anopheles culicifacies* in hyper endemic *falciparum* area of Odisha state

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Abstract
We observed behavioural changes in the host seeking preferences of two major malaria vectors *Anopheles fluviatilis* and *An. culicifacies* in Koraput and Malkangiri districts of Odisha state after the distribution of Long-lasting insecticidal nets (LLINs). The two vector species collected from the study villages of these districts were subjected to blood meal analysis and sibling species composition. Blood meal analysis results showed that 1.92% and 23.8% of *An. fluviatilis* were found to be fed on human in Koraput and Malkangiri districts, respectively and 5.1% of *An. culicifacies* in Malkangiri fed on human. After the distribution of LLINs, the sibling species S (malaria vector) of *An. fluviatilis* was disappeared from Koraput district, while in Malkangiri district the population of this species was found reduced significantly. There was a rising trend of *An. fluviatilis* sibling species T in both the districts. A change in the feeding behaviour of both the vector species from anthropophagy to zoophagy was observed.

Keywords: *Anopheles fluviatilis, Anopheles culicifacies,* host seeking behavior, Odisha

1. Introduction
Malaria is the most serious public health problem in Odisha state for quite some time [1]. Nearly 1.09 million malaria cases were reported in India during 2016 of which about 43.7% were from Odisha state [2]. With about 3.8% of India's population, 58.2% of *Plasmodium falciparum* burden and 33.7% malaria deaths occurred in Odisha alone during 2016 [2]. The Odisha state has been reporting high proportion (>86.0%) of *P. falciparum* malaria which is known to cause complications and mortality [2]. In India, the primary rural vector of malaria is *Anopheles culicifacies* [3] and *An. fluviatilis* is the important vector in the hills and foothills [4].
Malaria control is mainly based on preventing transmission. Vector control is one of the essential components of any malaria control programme. Long-lasting insecticidal nets (LLINs) are being promoted by WHO and Roll Back Malaria partners as a best vector control tool which is cost effective and sustainable method for protection against malaria [5]. LLINs form a protective barrier around people sleeping under them. The insecticides that are used for treating bed nets either kill or repel the mosquitoes, reducing the number that enter the house and attempt to feed on people inside. In addition, if high community coverage is achieved, the numbers of mosquitoes, as well as their length of life will be reduced and the survived mosquitoes will change the host for blood feeding [5]. Hence, knowledge on host seeking behaviour of anopheline vectors in LLIN used areas is highly essential for further designing appropriate vector control programmes and also in understanding the epidemiology of malaria [6].
Currently, the distribution of LLINs is the major vector control intervention measure being implemented in the districts of Odisha state. Both *An. fluviatilis* and *An. culicifacies* are major malaria vectors in Odisha and have been identified as species complexes [4]. Before distribution of LLINs, *An. culicifacies* was found to be predominantly zoophagic (0.75%) and *An. fluviatilis* was anthropophagic (62.2%) in the state [4]. *An. fluviatilis* in Odisha state was a complex of three sibling species S, T, and U [7]. Among them, species S has been reported to be an efficient malaria vector, T a poor vector, and U a non vector [7, 8]. Species S reportedly was primarily anthropophagic, whereas species T and U were zoophagic [9].

It was reported earlier that the species S was 90.9% and T was 9.1% in the state [10]. In the state, *An. culicifacies* is a complex of three sibling species B, C, and E, with species B and C considered to be poor vectors [4]. The proportion of species E was 43.9%, the overall human blood index of *An. culicifacies* was only 0.75% [4]. Studies on host seeking behaviour would require the information at the sibling species level as the differences in the biological characteristics of members of the complexes have an important bearing on the malaria transmission dynamics. After the mass distribution of LLINs in Odisha state during 2012, no information is available on the host seeking behaviour of *An. fluviatilis* and *An. culicifacies* at their sibling species level. Therefore, a study was undertaken from March 2017 to April 2017 to assess the impact of large scale distribution of LLINs on feeding behaviour of species complexes of *An. fluviatilis* and *An. culicifacies* in Koraput and Malkangiri districts of Odisha state, endemic for *falciparum* malaria since many decades.

2. Materials and Methods

2.1 Study Area

The study was carried out in two of the 10 southern districts of Odisha state, Koraput (latitude 18^0 82' N and longitude 82^0 72' E) and Malkangiri (latitude 17^0 45' N and longitude 81^0 10' E). Koraput district of Odisha covers an area of 8,807 km^2 while Malkangiri district surrounds an area of 5,791 km^2 with a population of 1,379,647 (2011 census) and 641,385 (2014 census conducted by health department), respectively. These districts are hilly with dense forest cover leading to formation of many streams. Dry summer (March to June), wet rainy (July to October), and dry winter (November to February) are the three prevailing seasons. The mean minimum and maximum monthly temperatures in both the districts ranged from 5.0 ^{0}C (December) to 26.8^{0}C (June) and from 27.2^{0}C (January) to 40.0 ^{0}C (May), respectively (Source: District Collectorate Office, Koraput and Malkangiri). The average relative humidity ranged from 30.6% (March) to 85.6% (September). The average total annual rainfall during 2016 was 1447.6mm and 1349.2mm from Koraput and Malkangiri districts, respectively [11]. The major vector control measure currently being carried out is indoor residual spraying (IRS) with DDT or synthetic pyrethroids (deltamethrin and alphacypermethrin) [12]. The last indoor residual spray round was conducted during September 2016 to October 2016. In addition, LLINs were distributed in two districts during 2012 covering all population.

Two Community Health Centres (CHCs) from each district was selected randomly. A total of five villages from each CHC were selected on the basis of random formula in MS-Excel to avoid bias for the study.

2.2 Mosquito Collections

Indoor resting collections in human dwellings (HDs) and cattle sheds (CSs) and outdoor resting collections were performed fortnightly during March 2017 to April 2017. Diurnal resting collections indoors (0600 to 0730h) were made using an oral aspirator and flash light from nine catching stations (six HDs and three CSs) in each village. Each collector spent 10 min in each dwelling collecting resting mosquitoes from the eaves, walls, and roof. Diurnal resting catches also were made outdoors, with collectors spending one man hour (0800–0900 h) in each village from searching natural pit shelters (0.3–0.5 m deep), culverts, and root interstices of trees, and newly dug pit shelters. Artificial pit shelters were pot (round) shaped with a small mouth (15–20 cm) and nearly 0.5 m deep and 0.3–0.5 m wide and were dug on the sides of well shaded mounds of earth. These pit shelters were made at different fixed sites in all the directions around the village. Overall collection effort for day-time resting collections was 80 and 40 man-hours in HDs and CSs, respectively and 80 man-hours outdoors in 20 villages.

2.3 Laboratory processing

All mosquitoes were brought to the Indian Council of Medical Research (ICMR) - Vector Control Research Centre (VCRC), field laboratory located at Koraput, and identified morphologically to genus level. All the culicine mosquitoes were discarded and anopheline mosquitoes were identified to species and the number of females of *An. fluviatilis* and *An. culicifacies* was recorded. Blood meals of the fully fed females of *An. fluviatilis* and *An. culicifacies,* obtained from diurnal resting catches were analyzed to determine the blood meal source using the agar gel diffusion method [13]. The reagents were from MP Biomedicals, (Solon, OH). Before testing the blood meal, the reagents were tested with positive controls to confirm the specificity of the reagents.

The body parts of the individual specimens of *An. An. fluviatilis* and *An. culicifacies* were kept in eppendorf tubes, dried for 4 to 5 h at 90 ^{0}C, and brought to the ICMR-VCRC laboratory, in Puducherry. Two legs were separated from the individual mosquito for DNA extraction and subsequent identification of sibling species using the molecular methods of Manonmani *et al*. [14, 15].

2.4 Comparison with an earlier study

During 2010-2011, as a part of another study (VCRC personal communications), the feeding behaviour and the sibling species composition of *An. fluviatilis* and *An. culicifacies* was studied in these two districts and the results have been discussed elsewhere [4]. The salient features of those results are recapitulated and presented in the results section of the current paper, and compared with the corresponding data obtained during the present study.

2.5 Data analysis

Data were entered into Microsoft Excel spreadsheet and statistical analyses were carried out using SPSS version 16.0. To compare the proportion of human blood fed by malaria vectors and the proportion of sibling species composition of malaria vectors before and after distribution of LLIN, $\chi 2$ tests were used. Probability level of $P< 0.05$ was used for statistical significance.

3. Results

3.1 Collection of *An. fluviatilis* and *An. culicifacies* in the study villages

In Koraput district, a total of 104 and 150 adult female *An. fluviatilis* and *An. culicifacies* were collected during the study period, respectively. Among them, 6.7 % (n= 7) and 93.3 % (n= 97) *An. fluviatilis* were collected form cattle sheds and outdoors, respectively. Human dwellings were not productive for the collections of *An. fluviatilis.* Among the *An. culicifacies* collection, 22 % (n= 33) were from human dwellings and 78.0 % (n= 117) were from cattle sheds. No *An. culicifacies* was collected from the outdoors.

In Malkangiri district, a total of only 21 *An. fluviatilis* were

collected from all the study villages. Among them, 71.4 % (n= 15) and 28.6 % (n= 6) were collected from human dwellings and cattle sheds, respectively. A total of 156 *An. culicifacies* was collected from the district, 29.5% (n=46) of them were from human dwellings and 70.5 % (n= 110) were from the cattle sheds. Outdoors of Malkangiri was not productive for both the vector species.

3.2 Sibling species composition of *An. fluviatilis* and *An. culicifacies*

Analysis of 20 specimens of *An. fluviatilis* collected from Koraput district showed that all were species T (reported to be non vector). In the case of *An. culicifacies,* all the 24 (100 %) belonged to B/C/E sibling complexes (Table 1).

In Malkangiri district, out of 21 *An. fluviatilis* tested, 11 (52.4%) were sibling species S and the remaining 10 (47.6%) were T. Among 28 *An. culicifacies* tested, all were comprised of B/C/E sibling complexes (Table 1). Due to logistics issues, sibling species complexes of *An. culicifacies* tested from both the districts could not be separated individually.

3.3 Blood meal identification of *An. fluviatilis* and *An. culicifacies*

Of the 104 blood meals of *An. fluviatilis* from Koraput district tested, 1.92% had fed on human and 98.08% on bovine, indicating the propensity towards cattle feeding. In the case of *An. culicifacies*, of the 150 blood meals tested from the same district; none had fed on human and all (100.0%) fed on bovine, signifying a highly zoophagic behaviour of this species (Table 2).

In Malkangiri district, a total of 21 blood meal samples of *An. fluviatilis* were tested of which 23.8% fed on human and 76.2% on bovine. Out of 156 blood meals of *An. culicifacies* tested, 5.1% was found to feed on human and 94.9% on bovine, suggesting very low anthropophagic nature of this species (Table 2).

3.4 Sibling species wise blood meal identification

Analysis of blood meal identification of *An. fluviatilis* with respect to sibling species complex in Koraput district showed that, all population belongs to the species complex T and among them 10.0% were anthropophagic, which is a significant finding in this study and the remaining 90.0% were zoophagic. The population of *An. culicifacies* in this district comprised of sibling species complexes of B/C/E and they all were zoophagic (100.0%) (Table 1).

In Malkangiri district, the population of *An. fluviatilis* sibling S was 52.4% and among them, 45.5% were anthropophagic and the remaining 54.5% were zoophagic. However, sibling species T was found to be 100.0% zoophagic. In case of *An. culicifacies*, 28.6% of sibling species B/C/E were found to be anthropophagic and remaining 71.4% were zoophagic (Table 1).

3.5 Comparison with an earlier study

The data on feeding preference of *An. fluviatilis* s. l. and *An. culicifacies* s. l. and the sibling species composition of these two vectors obtained from the survey conducted during 2010-2011 in Koraput and Malkangiri districts were compared with the data collected during the current survey. In Koraput district, the proportion of *An. fluviatilis* fed on human was 70% before LLIN distribution and after five years of distribution of LLINs, the corresponding value reduced to 1.92%. The difference in the percentage reduction was significant ($\chi2$ = 82.83, p = 0.000001). In case of *An. culicifacies,* the proportion fed on human was 3.0% and the corresponding value become 0 after the LLINs distribution. In Malkangiri district, while 91.0% of *An. fluviatilis* fed on humans before the LLINs distribution, the corresponding value was reduced significantly ($\chi2$ = 30.71, p = 0.000001) to 23.8% after five years of distribution of LLINs. In case of *An. culicifacies,* the proportion was reduced from 7.0% to 5.1%, which was not significant ($\chi2$ = 0.36, p = 0.55).

In Koraput district, before distribution of LLINs, the composition of *An. fluviatilis* S was 70.3% and species T was 29.7%. During the current study, it was observed that the sibling species S was not found in the study villages while the sibling species T was significantly increased from 29.7% to 100.0% ($\chi2$ = 42.11, p = 0.000001). Before distribution of LLINs, *An. culicifacies* population in the district comprised of only two sibling species, species B (69.6%) and species C (30.4%); but after distribution of LLINs, the composition of sibling species complexes was changed to B,C and E.

In Malkangiri district, before distribution of LLINs, the sibling species composition of *An. fluviatilis* showed 97.1% of species S which was significantly ($\chi2$ = 103.13, p = 0.000001) reduced to 52.38% after distribution of LLINs. In the case of *An. culicifacies*, species B was the predominant one constituting 76.1% followed by species C (23.7%) and A (0.3%) before distribution of LLINs. However, the composition of sibling species complexes changed to B, C and E after distribution of LLINs.

Table 1: Sibling species wise blood meal identification

District	Anopheline species	Number of samples tested	Sibling species/complex (%)	% Fed on	
				Human	Bovine
Koraput	*An. fluviatilis*	20	T (100.0%)	10.0	90.0
	An. culicifacies	24	B/C/E (100.0%)	0.0	100.0
Malkangiri	*An. fluviatilis*	21	S (52.38%)	45.45	54.55
			T (47.62%)	0.0	100.0
	An. culicifacies	28	B/C/E (100.0%)	28.6	71.4

Table 2: Results of blood meal identification

District	Anopheline species	Number of samples tested	% Fed on	
			Human	Bovine
Koraput	*An. fluviatilis*	104	1.92	98.08
	An. culicifacies	150	0	100
Malkangiri	*An. fluviatilis*	21	23.80	76.19
	An. culicifacies	156	5.13	94.87

4. Discussion

In India, the vector species complexes responsible for the majority of malaria transmission (*An. culicifacies*, *An. fluviatilis*, and *An. stephensi*) are primarily zoophilic [8, 16, 17] feeding much more on cattle than humans [18]. However, the degree of zoophily and exophily varies by species type (these species occur as species complexes) and the development of optimal control tactics depends on understanding the specific feeding and resting behaviour of the species complexes within particular locations [17]. Our study took place in Odisha state in India, where nearly half of all *P. falciparum* cases in India and over a third of all deaths due to malaria occur, even though only 3.8% of the total population of the country resides in this state [2]. Two districts in Odisha state, Koraput and Malkangiri, where the current study was carried out has a long history of high malaria incidence [19]. From 2010 to 2016, the annual parasite incidence (API, which is the number of clinical cases per 1000 people per year) in these districts ranged from 17.7 to 60.3 (*Source*: Data from the Office of the Chief District Medical Officer, Koraput and Malkangiri). Earlier studies of malaria vector mosquitoes in these two districts indicate there are two key species complexes responsible for transmission. *An. culicifacies* (a mix of B, C, and E types, which are largely zoophagic) maintains low level malaria transmission year round and drives a peak in transmission during the monsoon season when mosquito numbers increase [15, 20]. Classically, there was then a second peak in transmission driven by a short-term seasonal upsurge in anthropophilic *An. fluviatilis* (a mix of S which is anthopophagic and T which is zoophagic) during the retreating monsoon period [20]. Whether present-day species complexes and feeding behaviour match these historical patterns was unknown. In the current study, therefore, we examined the sibling species composition and feeding behaviour of *An. fluviatilis* and *An. culicifacies* by a cross sectional study conducted across 20 study villages in Koraput and Malkangiri districts after the mass distribution of LLINs during 2012.

Nanda *et al*. in 2000 reported that *An. fluviatilis* in India has been established as a complex of 4 sibling species (S, T, U and V), of which species S is highly anthropophagic and an efficient vector of malaria which contributes to ~15 per cent of annual cases [21]. Sibling species T is widely distributed but is largely zoophagic. During 2010 to 2011, an in-depth entomological study was conducted in southern districts of Odisha state, which showed that *An. fluviatilis* and *An. culicifacies* played the role in malaria transmission [4]. *An. fluviatilis* was found to be anthropophagic and sibling species S was predominant over sibling species T. In the current study, we found a shift in *An. fluviatilis* from the anthropophagic S to zoophagic T in both the districts. In Koraput district, a total of 10% of sibling species T was found to feed on human, which is an important observation in the current study and ought to have a significant bearing in the epidemiology of malaria in the district. After distribution of LLINs, species S was disappeared in Koraput district, while in Malkangiri district this species had reduced significantly. In case of species T, there was a significant increase in its population in both the districts after distribution of LLINs. It was also revealed that there was a shift in host feeding preference of both the sibling species of *An. fluviatilis* from anthropophagy to zoophagy. This could be due to the fact that after distribution of mosquito nets the mosquitoes were unsuccessful in finding a blood meal during their normal active host-searching period and the mosquitoes might be killed or repelled, thereby caused a drastic reduction in the density of sibling species S.

The species *An. culicifacies* is widely distributed in India and comprised of five sibling species provisionally designated as species A, B, C, D and E [3]. It was earlier reported that Species B and C were found both in forest and plain areas of Sundargarh, Koraput and Malkangiri districts of Odisha [4, 21]. In addition, species A was also reported earlier in Malkangiri district [4]. The interesting result obtained from the study is a variation in composition of *An. culicifacies* siblings before and after distribution of LLINs. We found *An. culicifacies* to be a mix of zoophilic B, C, and E types. After distribution of LLINs, sibling E appeared in Koraput and Malkangiri districts and sibling A was disappeared from Malkangiri. *An. culicifacies* showed a high preference to feed on human blood (3.0%) in Koraput district before distribution of LLINs and surprisingly, no *An. culicifacies* was found fed on human blood after distribution of LLINs in the district. Similar decreasing trend was also observed in Malkangiri district. This clearly indicates that there was a shift in host seeking behaviour of *An. culicifacies* after mass distribution of LLINs.

Behavioural changes in *Anopheles* are a functional adjustment to external environment. Use of insecticide impregnated mosquito nets is one of the possible causes that make the mosquito exophilic and exophagic. This is one of the avoidance mechanisms where mosquitoes avoid coming into contact with insecticide. Many studies conducted elsewhere showed that LLIN effectiveness is threatened by shifts in biting behaviour of vector mosquitoes, from nocturnal towards crepuscular, with mosquitoes actively host searching during the early morning and/or evening, when many LLIN users are not under their net [22, 23, 24]. It was also observed increased outdoor feeding after exposure to insecticide treated nets (ITNs) in Tanzania [25]. Such a shift could occur simply if mosquitoes are unsuccessful in finding a blood meal during their normal active host-searching period [26, 27]. Changes in the feeding habits of *An. gambiae* was also noticed in Burkina Faso [28] where it was highly anthropophilic before the use of mosquito nets and after the wide use of which the chance of the vector encountering a human host lessened and it showed an alternative preference for cattle. Similar observation was noticed in the current study. The tropic deviation to cattle for *An. funestus* was also reported in the Kilombero Valley of Tanzania [29]. From the same study, a shift in the feeding time upon exposure to insecticides for *An. gambiae* has also been reported. Another study showed the biting activity of *An. funestus* shifted from HD to outside dwellings after the intervention of LLINs and the exophagy rate rose from 45 to >70%.

The limitations of this study are many. The sample size of *An. fluviatilis* processed for blood meal source was too low to draw further conclusion and this was one of the limitations of this study. The second limitation is that, the study does not covered three seasons of the year. Due to logistics B, C and E sibling species of *An. culicifacies* could not be separated which adds a limitation. However, the strength of the current study is that the study was conducted in a highly malarious endemic area for several decades where both *An. fluviatilis* and *An. culicifacies*, play the major role in malaria transmission. The status of their sibling species composition and host seeking behaviour was known before distribution of

LLINs. Therefore, the information regarding the changes in the sibling species composition and host seeking preference of both the vector species after mass distribution of LLINs in the study area would help the policy makers to design further an appropriate optimal malaria control strategy in hilly eco-epidemiological settings.

5. Conclusion

The study indicated a rising trend of *An. fluviatilis* sibling species T in both the districts after the distribution of LLINs. There was a change in host seeking behaviour of both the vector species from anthropophagy to zoophagy. The study also suggests that the current information of the bionomics of sibling species of *An. fluviatilis* and *An. culicifacies* would help in understanding the dynamics of malaria transmission in the study area and would facilitate the planning of appropriate and effective control measures. In the current study area, where the zoophagic vectors dominate malaria transmission, the conventional approaches of IRS and LLINs may not be sufficient to achieve the malaria elimination, even if implemented at maximal achievable coverage. However, current policy restricts IRS to domestic dwellings. Hence, a more comprehensive approach that includes the treatment of cattle sheds with non-repellent chemicals could facilitate malaria control, where the majority of malaria mosquitoes are zoophagic.

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