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Malaria Outbreaks in Villages in North Karnataka, India, and Role of Sibling Species of *Anopheles culicifacies* Complex

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Abstract

Investigations on malaria outbreaks and role of sibling species complex of principal rural malaria vector *Anopheles culicifacies* were carried out in villages in north Karnataka, India from 1997 through 2014. Information regarding densities, resting and breeding habitats of malaria vectors prevalent in the area was also generated so as to formulate an appropriate vector control strategy. The Slide Positivity Rate (SPR), Slide Falciparum Rate (SFR) and Pf proportion was 43.1%, 35.9% and 83.3%, respectively. Three sibling species A, B, and C of *An. culicifacies* were found sympatric with cumulative percent composition of 63.7, 28.2 and 8.1, respectively. Per man hour and per structure densities of *An. culicifacies*, *An. fluviatilis* and *An. stephensi* varied from 0 to 27.5 and 0 to 56.0, 0 to 0.5 and 0 to 7.0 and, 0 to 2.5 and 0 to 7.5, respectively. The proportion of semi-gravid and gravid females was more as compared to fully fed and unfed females which indicated that most of the females rested indoor. Streams/river, wells, seepages and irrigation tanks are the major habitats supporting breeding of *An. culicifacies*. Integrated vector managements by indoor residual spraying of effective insecticide as per national guidelines along with biological control methods especially use of larvivorous fish *Gambusia affinis* and *Poecilia reticulata* are suggested to control malaria in the area.

Keywords

Malaria, *Anopheles culicifacies*, Sibling Species, Biological Control, North Karnataka

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1. Introduction

Malaria is a major communicable disease in Karnataka. The disease was at its peak in 1976 with steady decline till 1984. However, the incidence was on the rise in the mid-1990s and thereafter declined since 2010. During 1996 four districts of north Karnataka, namely Bijapur, Belgaum, Gulbarga and Raichur contributed about 17% of total malaria cases in the state and *P. falciparum* malaria contribution was about 40% [1]. Malaria problem with frequent epidemics was persisting in this area. Malaria outbreaks have been reported from Gabbur and Masarkal PHCs in October-November, 1999 and Masarkal PHC in March-April 2007 [2] [3]. Recently, in April 2014 a sudden increase in malaria cases occurred in Yalgundi village of Annehosur PHC. During the first half of twentieth century lot of work has been carried out on biology of malaria vectors in Karnataka especially at sensu lato level [4] [5]. *An. culicifacies* has been identified as a complex of 5 sibling species A, B, C, D, and E in India with a specific distribution pattern. Further, it is evidenced that species A, C, D, and E are implicated in transmission of malaria while species B has limited role in malaria transmission [6] [7]. Recently, malaria sporozoites have been detected in naturally infected sibling species of the *An. culicifacies* complex in Karnataka [8]. It is therefore, extremely important to generate information up to sibling species level. In the present study, we report malaria outbreaks and sibling species composition of *An. culicifacies* in villages in north Karnataka. In addition, information was also generated pertaining to densities and resting and breeding habits, of malaria vectors prevalent in these villages which would be of immense importance in planning suitable vector control strategy.

2. Methods

Four outbreak investigations were carried out in six villages, Londa, Gulivada of district Belgaum, Hanchinal, Belganoor, Tidagundi of district Bijapur, Narayanpur of district Yadgir during February 1997, ten villages Ramdurg, Kakargal, Masarkal, Guntral, Miapur, Chikka Honakuni, Teggchal, Chintolkunta, Mustoor, and Jagatigal during November 1999, four villages K. Irrebegere, Kampare doddi, G. Irrebegere and Number Doddi during April 2007, and Yalguundi village during August 2014 of district Raichur (**Figure 1**). Blood smears were collected by the local health staff from all fever cases were stained with JSB and examined under microscope for detection of malaria parasite. Malaria positive cases were given treatment as per NVBDCP drug policy. Adult mosquitoes were collected from 4 human dwellings and 4 cattle sheds in the morning hours between 06.00 and 08.00 by oral aspirator and a flash light. Pyrethrum spray collections were also done from one human dwelling and cattle shed in each village. Collected mosquitoes were brought to the laboratory and identified [5]. Malaria vector species *An. culicifacies*, *An. fluviatilis* and *An. stephensi* were separated and their per man hour densities (PMHD) and per structure densities (PSD) were calculated. The gonotrophic condition of *An. culicifacies* was recorded and grouped them as unfed (UF), fully fed (FF), semi-gravid (SG) and gravid (G). In three surveys of February 1997, November 1999 and August 2014, ovaries were pooled from semi gravid females and fixed in modified Carnoy's fixative (glacial acetic acid:methanol: 1:3). Due to low densities, ovarian nurse cells could not be pooled during April 2007 survey. Polytene chromosomal preparations were done and sibling species were identified using karyotype [6] [9]. Hardy Weinberg equilibrium formula was applied to distinguish between the heterozygote and homozygote population of sibling species A of *An. culicifacies* complex. Chi-square (χ^2) values were thus derived and a p value < 0.5 was considered significant. Larval sampling was done from available breeding habitats in all the villages using standard WHO method [10]. Anopheline immature were collected separately from each breeding site, brought to the laboratory and reared till adult emergence for species identification using the key of Nagpal and Sharma [5]. Breeding site-based anopheline species emerged in these surveys were pooled and percentage of individual species (of total anophelines) emerged from each breeding site was calculated. Likewise, percent vector contribution from different breeding habitats was calculated out of total vector species emerged from all breeding habitats.

Ethical Issue

This study was carried out as per the routine surveillance under the National Vector Borne Disease Control Programme. It does not deal with any individual or personal data.

3. Results

Results of four parasitological surveys are shown in **Table 1**. Out of 818 blood smears examined, 353 were

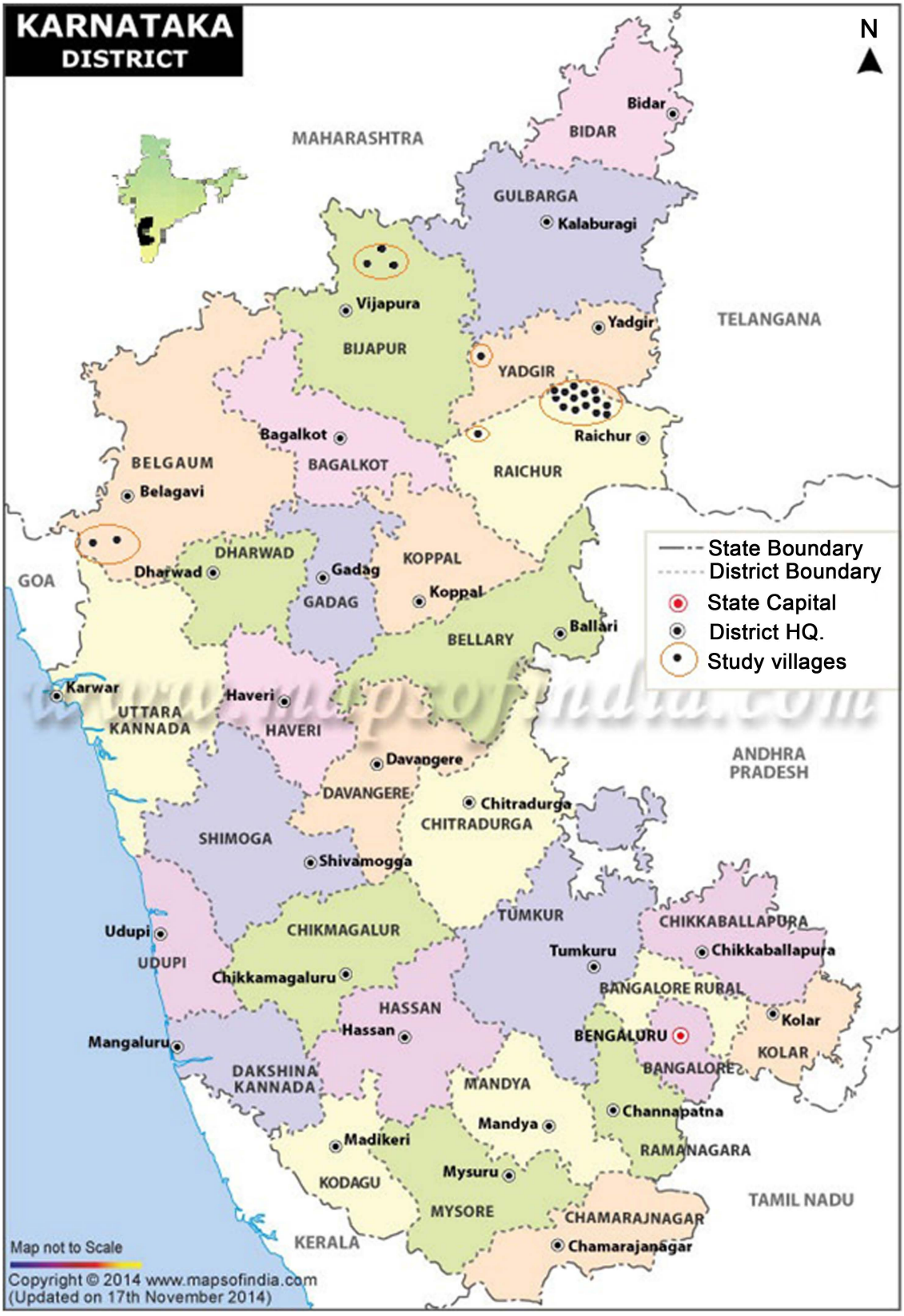

Figure 1. Showing the study villages in north Karnataka, India.

Table 1. Malaria prevalence in the study villages*.

Month/year of survey	BSE	Pv	Pf	Mixed	TPC	SPR	SFR	Pf %
February 1997	24	0	6	0	6	25.0	25.0	**100.0**
November 1999	341	12	225	6	243	71.2	65.9	**95.1**
April 2007	190	27	59	0	86	45.3	31.0	**68.6**
August 2014	263	14	10	0	24	9.12	3.80	**41.7**
Total	818	53	294	6	353	43.1	35.9	**83.3**

BSE—blood slide examined. Pv—*Plasmodium vivax*. Pf—*Plasmodium falciparum.* TPC—total positive cases. SPR—slide positive rate. SFR—slide falciparum rate. Pf%—*Plasmodium falciparum* percentage. *NVBDCP data.

found positive for malaria parasites; 294 were positive for *P. falciparum*, 53 for *P. vivax*, and 6 were positive for mixed infection. Thus, the Slide Positivity Rate (SPR) was 43.1% and the Slide Falciparum Rate (SFR) was 35.9%. Out of positive cases, falciparum proportion was very high (83.3%). The SPR and SFR was higher (>20%) from 1997 to 2007 whereas less (SPR—9.12%, SFR—3.80%) during August 2014. *P. falciparum* percentage was maximum (100%) in 1997 and minimum (41.7%) in 2014.

The composition of sibling species of *An. culicifacies* is given in **Table 2**. Three sibling species A, B, and C were found sympatric with the cumulative percent composition of 63.7, 28.2 and 8.1, respectively (**Figure 2**). In February 1997 and November 1999 collections, both heterozygote and homozygote inversions were found in sibling species A and the cumulative proportion of sibling species A and C was higher (76%) as compared to August 2014 collection (47%). The proportion of sibling species A and C was maximum (91%) in February 1997 which was less at 24% in November1999 survey. In August 2014, sibling species C was not recorded and the proportions of sibling species A and B were 47% and 53%, respectively. In dry month February, sibling species A was found to be predominant whereas, species B was numerous in wet months of August and November.

Per man hour density (PMHD) and per structure density (PSD) of malaria vectors are given in **Table 3**. PMHD of *An. culicifacies*, *An. fluviatilis* and *An. stephensi* varied from 0 - 27.5, 0 - 0.5 and 0 - 2.5, respectively. Likewise, per structure densities varied from 0 - 56.0, 0 - 7.0 and 0 - 7.5. Fewer densities of *An. culicifacies* was recorded in 1997 to 2007 surveys while more numerous in 2014. *An. fluviatilis* and *An. stephensi* densities did not show any trend.

The trophic status of *An. culicifacies* is presented in **Table 4**. Overall, the proportion of resting stage mosquitoes (87%) was higher than foraging mosquitoes (13%). Out of 670 females examined in four surveys, 3.4% were unfed, 9.6% were full fed and 50.3% were semi-gravid and 36.7% were gravid.

Species specific breeding sources of anopheline mosquitoes in the study villages are presented in **Table 5**. Wells and seepage supported the breeding of maximum numbers of anopheline species *An. culicifacies* (23.4%), *An. stephensi* (10.4%), *An. annularis* (1.3%), *An. subpictus* (28.6%), *An. vagus* (1.3%), *An. pseudojamesi* (1.3%), *An. barbirostris* (28.6%) and *An. nigerrimus* (5.2%) and viz. *An. culicifacies* (7.5%), *An. stephensi* (2.5%), *An. subpictus* (77.5%), *An. pseudojemesi* (0.6%) *An. barbirostris* (3.1%), *An. nigerrimus* (3.1%), *An. theobaldi* (6.2%), *An. vagus* (0.6%), respectively followed by streams *An. culicifacies*(66.3%), *An. fluviatilis* (2.2%), *An. subpictus* (3.4%), *An. pseudojemesi* (20.2%), *An. barbirostris* (3.4%), *An. jeyporiensis* (4.5%), Irrigation tank *An. culicifacies* (28.6%), *An. annularis* (19.0%), *An. subpictus* (35.7%), *An. nigerrimus* (7.1%), *An. vagus* (9.5%), Borrow pits *An. culicifacies* (6.4%), *An. annularis* (3.2%), *An. subpictus* (80.6%), *An. pallidus* (9.7%), Irrigation canals *An. culicifacies* (90.0%), *An. stephensi* (10.0%) and Cemented tank *An. culicifacies* (5.0%), *An. subpictus* (95.0%). Paddy field *An. subpictus* (71.4%), *An. barbirostris* (14.3%), *An. nigerrimus* (14.3%) and tap pits *An. subpictus* (100.0%).

Overall, contribution of malaria vector *An. culicifacies* was 55.4% from streams/river, 14.9% from wells, 9.9% each from seepages and irrigation tanks, 7.4% from canals, 1.7% from borrow pits and 0.8% from cemented tanks in the villages studied. *An. stephensi* contribution was mainly from wells (61.5%) and seepages (30.8%) followed by irrigation canals (7.7%). *An. fluviatilis* contribution (100.0%) was only from streams/river (**Table 6**).

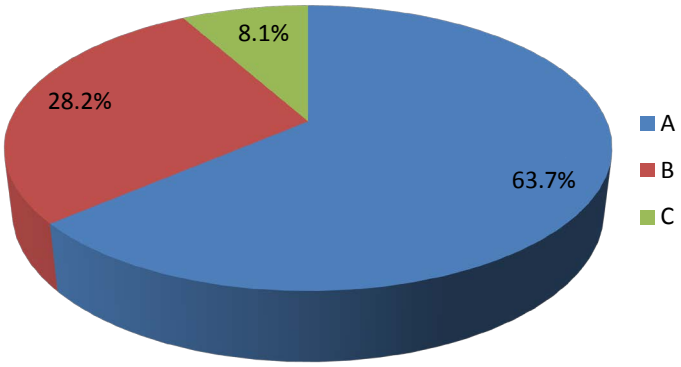

Figure 2. Proportion of *Anopheles culicifacies* sibling species A, B and C in the study villages.

Table 2. Sibling species composition of *Anopheles culicifacies* in the study villages.

Month/year of collection	Total No. of specimens estimated	Sibling species composition					
		Species A				Species B	Species C
		A	A'	A/A'	Total		
February 1997*	82	10	33	26	69 (84.1)	7 (8.6)	6 (7.3)
November 1999**	25	1	1	0	2 (8.0)	19 (76.0)	4 (16.0)
August 2014	17	8	0	0	8 (47.1)	9 (52.9)	0
Total	124	19 (15.3)	34 (27.4)	26 (21.0)	79 (63.7)	35 (28.2)	10 (8.1)

Figures in parentheses are percentage composition, Note: sufficient ovary samples were not available in April 2007 survey. *Subjected to Chi-square test: χ^2 value—1.587 (n.s.). **Subjected to Chi-square test: χ^2 value—2.000 (n.s.)

Table 3. Malaria vector(s) densities in the study villages.

Month/year of survey	*An. culicifacies*		*An. fluviatilis*		*An. stephensi*	
	PMHD	PSD	PMHD	PSD	PMHD	PSD
February 1997	8.75	17.0	0.17	0.12	0	0.21
November 1999	4.79	ND	0	ND	0	ND
April 2007	0.75	4.5	0	0	1.25	7.5
August 2014	18	56	0	7	0	0
Average	8.07 (0 - 27.5)	25.8 (0 - 56.0)	0.04 (0 - 0.5)	2.37 (0 - 7.0)	0.31 (0 - 2.5)	2.57 (0 - 7.5)

Figures in parentheses indicate range. PMHD—per man hour density. PSD—per structure density. ND—not done.

Table 4. Trophic status of malaria vector *Anopheles culicifacies* in the study villages.

Month/year of collection	Total No. of specimens estimated	Un fed (UF)	Full fed (FF)	Semi gravid (SG)	Gravid (G)
February 1997	496	10 (2.0)	42 (8.5)	227 (45.8)	217 (43.7)
November 1999	104	9 (8.7)	11 (10.6)	64 (61.5)	20 (19.2)
April 2007	14	1 (7.1)	1 (7.1)	9 (64.3)	3 (21.5)
August 2014	56	3 (5.3)	10 (17.9)	37 (66.1)	6 (10.7)
Total	670	23 (3.4)	64 (9.6)	337 (50.3)	246 (36.7)

Figures in parentheses indicate percentage.

Table 5. Species specific anopheline breeding sources in the study villages.

Breeding site	An. cul.	An. flu.	An. ste.	An. ann.	An. sub.	An. pse.	An. bar.	An. jey.	An. nig.	An. the.	An. pal.	An. vag.	Total
Stream/river	59 (66.3)	2 (2.2)	0 (0.0)	0 (0.0)	3 (3.4)	18 (20.2)	3 (3.4)	4 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	89
Well	18 (23.4)	0 (0.0)	8 (10.4)	1 (1.3)	22 (28.6)	1 (1.3)	22 (28.6)	0 (0.0)	4 (5.2)	0 (0.0)	0 (0,0)	1 (1.3)	77
Irrigation canal	9 (90.0)	0 (0.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	10
Irrigation tank	12 (28.6)	0 (0.0)	0 (0.0)	8 (19.0)	15 (35.7)	0 (0.0)	0 (0.0)	0 (0.0)	3 (7.1)	0 (0.0)	0 (0.0)	4 (9.5)	42
Seepages	12(7.5)	0 (0.0)	4 (2.5)	0 (0.0)	124 (77.5)	1 (0.6)	5 (3.1)	0 (0.0)	5 (3.1)	8 (6.2)	0 (0.0)	1 (0.6)	160
Borrow pit	2 (6.4)	0 (0.0)	0 (0.0)	1 (3.2)	25 (80.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (9.7)	0 (0.0)	31
Paddy field	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	10 (71.4)	0 (0.0)	2 (14.3)	0 (0.0)	2 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	14
Tap pit	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	17 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	17
Cemented tank	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)	19 (95.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	20
Total	113 (24.6)	2 (0.4)	13 (2.8)	10 (2.2)	235 (51.1)	20 (4.3)	32 (6.9)	4 (0.9)	14 (3.0)	8 (1.7)	3 (0.7)	6 (1.4)	460

An. cul.—*Anopheles culicifacies*. An. flu.—*Anopheles fluviatilis*. An. ste.—*Anopheles stephensi*. An. ann.—*Anopheles annularis*. An. sub.—*Anopheles subpictus*. An. pse.—*Anopheles pseudojemesi*. An. bar.—*Anopheles barbirostris*. An. jey.—*Anopheles jeyporiensis*. An. nig.—*Anopheles nigerrimus*. An. the.—*Anopheles theobaldi*. An. pal.—*Anopheles pallidus*. An. vag.—*Anopheles vagus*.

Table 6. Percent vector contribution by different anopheline breeding habitats in the study villages*.

Breeding site	No. of vectors emerged		
	An. culicifacies	*An. fluviatilis*	*An. stephensi*
Stream/river	67 (55.4)	2 (100.0)	0 (0.0)
Well	18 (14.9)	0 (0.0)	8 (61.5)
Irrigation canal	9 (7.4)	0 (0.0)	1 (7.7)
Irrigation tank	12 (9.9)	0 (0.0)	0 (0.0)
Seepage	12 (9.9)	0 (0.0)	4 (30.8)
Borrow pit	2 (1.7)	0 (0.0)	0 (0.0)
Paddy field	0 (0.0)	0 (0.0)	0 (0.0)
Tap pit	0 (0.0)	0 (0.0)	0 (0.0)
Cemented tank	1 (0.8)	0 (0.0)	0 (0.0)
Total	121	2	13

Figures in parentheses indicate percentage. *Adult mosquitoes were collected from emergence of the field collected samples of immature stages.

4. Discussion

The high SPR, SFR and Pf% observed in the present investigation was due to inadequate surveillance, and radical treatment and resistance to chloroquine of *P. falciparum* cases. Similar trend has been reported in the past resulting in to outbreak of malaria [11]. A high degree of resistance to chloroquine (87%) and to sulphadoxine pyrimethamine (75.8%) by *in vivo* was recorded from the same area [12]. After the introduction of Artemisinin-based Combination Therapy (ACT) as per the revised 2005 national drug policy [13] the Pf percentage declined drastically in the area.

An. culicifacies, *An. fluviatilis* and *An. stephensi* are the malaria vector species collected from the study villages. PMHD and PSD revealed that *An. culicifacies* is the predominant vector species followed by *An. stephen-*

si and *An. fluviatilis.* Streams/river, wells, seepages and irrigation tanks are the major contributory (>90%) larval habitats for *An. culicifacies. An. stephensi* contribution was from wells, seepages and irrigation canals and *An. fluviatilis* from streams/river. Three sibling species A, B and C of *An. culicifacies* were found sympatric in the study villages. In February 1997 and November 1999 collections, both the inversion heterozygotes and homozygotes were found in sibling species A. Since the "I" inversion which is polymorphic in species A is also diagnostic for species D [14]. The data for these surveys were analysed and the populations were found to be in Hardy Weinberg equilibrium with non significant Chi-square (χ^2) values (1.587—February 1997 survey, 2.000—November 1999 survey; $p > 0.5$). This suggests random mating between the alternative forms, and "I" inversion is polymorphic in species A population from this area. The proportion of species A was found to be predominant in the month of February which is a dry month whereas, species B in wet months August and November. These results are, by and large, in conformity with earlier observations [15]. Overall, there appears to be direct relationships, besides other factors, between high incidence of malaria and the presence of higher proportion of vector sibling species A and C of *An. culicifacies* in this area like previous findings in stone quarry area of Allahabad district [16]. The cumulative proportion of potent malaria vector sibling species A and C of *An. culicifacies* was higher in February and November 1999 collections when malaria incidence was more as compared to August 2014 collection when malaria incidence was also comparatively lesser. However, a low proportion of sibling species A with high malaria in November 1999 collection might be due to malathion fogging because the study villages were continuously under DDT spray for the last several years and at the time of survey in addition four rounds of malathion fogging were also covered and *An. culicifacies* was found to be resistant to DDT and susceptible to malathion [17]. It has also been reported that Species A remains more susceptible to DDT than species B in areas where both A and B are sympatric and DDT has been withdrawn for long periods [18]. In areas with species B and C sympatric association, species C develops resistance to malathion at a faster rate than did species B [19] and species A at a slower rate than species B [20].

The SG and G appearance of female abdomen demonstrate as resting stage of mosquitoes and the females with UF and FF are the indicative of the seeking stages (for blood meal or resting places). The ratio of resting stages to seeking stages (G, SG/UF, FF) indicate a tendency to rest inside/outside. The higher proportion of resting stage mosquitoes (87%) than foraging mosquitoes (13%) in the present investigation suggested indoor resting habit (endophilic) of this species.

The current strategy for vector control is indoor residual insecticide spraying. Presently, DDT, Malathion and Synthetic pyrethroids are being used for vector control in this area. Recently, malaria vector *An. culicifacies* has been reported to be resistant to DDT, malathion and Synthetic Pyrethroid Lambdacyhalothrin in this area [21]. Since most of the vector mosquitoes rest inside, indoor spraying of an effective insecticide as per the national guideline may be adopted for controlling malaria, especially the epidemics in this area. However, the high cost and poor coverage of insecticide spray, warrants integrated vector control strategy. Biological control methods such as use of larvivorous fish *Poecilia* and *Gambusia* and biolarvicide *Bacillus thuringiensis* have been found effective to control malaria in Karnataka [22]. In the present study, streams/river, wells, seepages and irrigation tanks are the main sources of malaria vector *An. culicifacies* breeding. *Gambusia* may be released in big water bodies like irrigation tanks and streams/river and *Poecilia* in confined water bodies like wells. Biocide may be applied in the seepages. Release of larvivorous fish has been initiated in this area and need strengthening. Recently, insecticide treated bed nets especially Long Lasting Insecticide treated Nets (LLINs) have also shown promising results in controlling malaria [23]. Hence, feasibility of LLINs may also be tried to control malaria in this area especially in the villages situated at the banks of river.

5. Conclusion

The present study on malaria outbreak investigations indicated the lack of proper surveillance and timely action. The high densities of malaria vector *An. culicifacies* and its sibling species A might be responsible for high malaria prevalence and need to adopt proper vector control strategy in the area especially the integration of biological control methods. In the mid-1990s, most of the malaria cases were reported from the districts of south Karnataka, and use of larvivorous fish controlled the vector populations. From 2009 onwards, this programme was extended in the entire Karnataka and resulted in reduction of malaria cases in Karnataka in the subsequent years [24] [25]. However, limited focal malaria cases are reported in some areas for which routine vector surveillance is needed.

Conflicts of Interest Statements

Declared none.

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