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Screening of *Anopheles culicifacies* Population of Sri Lanka for Sibling Species A

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A total of 1119 *Anopheles culicifacies* mosquitoes collected from various malaria endemic regions in Sri Lanka were examined using two DNA probes Rp217 and Rp234, which enable the differentiation of sibling species A from B and C species of *An. culicifacies*. Sibling species A was found to be absent.

Keywords: *Anopheles culicifacies*, DNA probes, Sibling species

INTRODUCTION

Anopheles culicifacies Giles, *sensu lato* is the major vector of malaria in Sri lanka[1,2]. The taxon *An. culicifacies* exists as an isomorphic species complex comprising four reproductively isolated populations. These have been designated as species A and B[3], species C[4] and D[5]. *An. culicifacies* species A and B have been found in Pakistan[6] and species A has been found in Arabia[7]. The four sibling species have a different distribution pattern in India[8]. In northern and southern India, species A and B are sympatric, whereas, species B and C are sympatric in the western and eastern regions. Species D has been found sympatrically with species A and B in the north-western region and with species A, B and C in central India[9]. Furthermore, the proportion of sibling species

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varied in different geographical regions particularly with seasonal and environmental changes[5]. Recently, another two new populations of species B have been identified in Rameshwaram Island[10]. On the basis of limited cytogenetical studies of *An. culicifacies* populations in Sri Lanka, only species B has been identified so far in the Island[3,11]. The cytogenetic methods of identification are tedious and laborious and are limited to the use of semi-gravid adults or late III or early IV instar larvae. Therefore, a large number of field samples may be lost due to unsuitable preparations. The use of DNA probes offer an attractive alternative to the available technique thus, each and every mosquito can be analysed even in the dry state or 70% ethanol, or isoproponol preserved samples.

A comprehensive study on the sibling species status of *An. culicifacies* presents in Sri Lanka has not yet been carried out[11]. Base line data gathered from such a study would no doubt be important for future vector control strategies. The present study describes the screening of *An. culicifacies* adult mosquitoes collected from various malaria endemic regions in Sri Lanka for sibling species A using two DNA probes Rp217 and Rp234 that have been previously developed in our laboratory[12].

MATERIALS AND METHODS

Mosquitoes

Air-dried samples of laboratory stocks of *An. culicifacies* sibling species A, B and C kindly provided by Dr. S.K. Subbarao, Malaria Research Centre, Delhi India, were used as control mosquito samples.

For the field investigations, mosquitoes were collected from various malaria endemic regions in Sri Lanka during the time period from January 1992 to December 1994 (Table 1). Standard mosquito collection methods[13] such as cattle-baited hut collection (CBHC), cattle-baited trap collection (CBTC), human bait night collection (HBNC), larval collection (LC), pyrethrum spray sheet collection (PSC) and hand collection (HC) were used. Air dried *An. culicifacies* mosquitoes in eppendorf tubes were transported in a desiccator with silica gel to the laboratory and stored at –20°C until further analysis.

Identification of sibling species

Single mosquito DNA extractions were carried out as described by Collins *et al.*[14]. A 1/200 dilution of each DNA sample in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was dot blotted on to nitrocellulose filters and the dot blots were prehybridized for 16 h at 37°C in prehybridization buffer (1M NaCl, 50% formamide, 1% SDS, 10% dextran sulphate, 50 mM Tris-HCl pH 7.6, 100 µg/ml heparin) and further hybridized for 16 h at 37°C with ^{32}P oligolabelled[15] Rp234 (Rep340) and Rp217 (Rep217) DNA probes (specific activity 10^9 cpm/µg DNA). The final wash of the filters were carried out at 30°C in 0.1 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate). The air-dried fil-

Table 1. *An. culicifacies* mosquitoes collected from various regions in Sri Lanka during January 1992 to December 1994 for sibling species status analysis

Malaria endemic area	Collection method/ sites	No. of mosquitoes analysed	Total no. analysed/area
Gomadiyagala	CBHC (I)	217	
	HBNC (I)	22	
	(O)	10	
	LC stream	25	
	Sand pools	12	
	Rock pools	8	
	Paddy fields	13	
	Wells	9	
	HC (I)	44	
	(O)	3	364
Halmillawa	CBHC (I)	137	
	HBNC (I)	17	
	(O)	11	
	LC Stream	32	
	Sand pools	14	
	Rock pools	5	
	Paddy fields	7	
	Wells	12	
	HC (I)	36	
	(O)	2	273
Elivitiya	CBHC (I)	97	
	CBTC (I)	71	
	PSC	14	182
Anuradhapura	CBHC	52	
	CBTC	30	
	PSC	9	91
Matale	CBHC	78	
	PSC	13	91

contd...

Table 1. (*contd.*)

Malaria endemic area	Collection method/ sites	No. of mosquitoes analysed	Total no. analysed/area
Polgahawela	CBHC	69	
	HBNC (I)	12	
	PSC	10	91
Ampara	CBHC	12	
	PSC	8	20
Wellawaya	CBHC	7	7
Total		1119	1119

CBHC — Cattle-baited hut collection; CBTC — Cattle-baited trap collection; HBNC — Human bait night collection; PSC — Pyrethrum spray sheet collection; HC — Hand collection; LC — Larval collection; I — Indoor collection; O — Outdoor collection.

ters were autoradiographed (Kodak XAR-5 film with an intensifying screen) for 16 h at –70°C.

Squash blots with mosquito heads were carried out as described by Hill *et al.*[16]. The squash blots were prehybridized for 16 h at 37°C in a less expensive buffer containing 5 x SSC, 0.5% SDS and 2% skimmed milk and hybridized for 16 h at 37°C with ^{32}P oligolabelled Rp217 (Rep217) DNA probe (specific activity 10^9 cpm/μg DNA). Filters were washed at 60°C in 0.1 x SSC, 0.5% SDS, and the air-dried filters were autoradiographed (Kodak XAR-5 film with an intensifying screen) for 2 h at –70°C.

RESULTS

Table 1 shows the number of mosquitoes collected using six different collection methods during the period of January 1992 to December 1994 and the localities from which they were collected. These data are summarized in Fig. 1. Since different habitats and climatic conditions have been covered by the collection methods during the two year period, and every mosquito in the sample could be examined by the DNA probe method without loss, the possibility of detecting more than one sibling species from a mosquito sample is high particularly with a sample as high as 1000 mosquitoes.

All 1119 DNA samples collected from individual mosquito by the six sampling methods (Table 1), gave a positive hybridization signal with Rp234 and Rp217 when diluted 200 fold and assayed by the dot-blot hybridization technique (Fig. 2). During the latter part of the study, squash blots of mosquitoes were used for the screening of wild caught mosquitoes (Fig. 3). Initial screening was carried out for 25 mosquitoes collected from

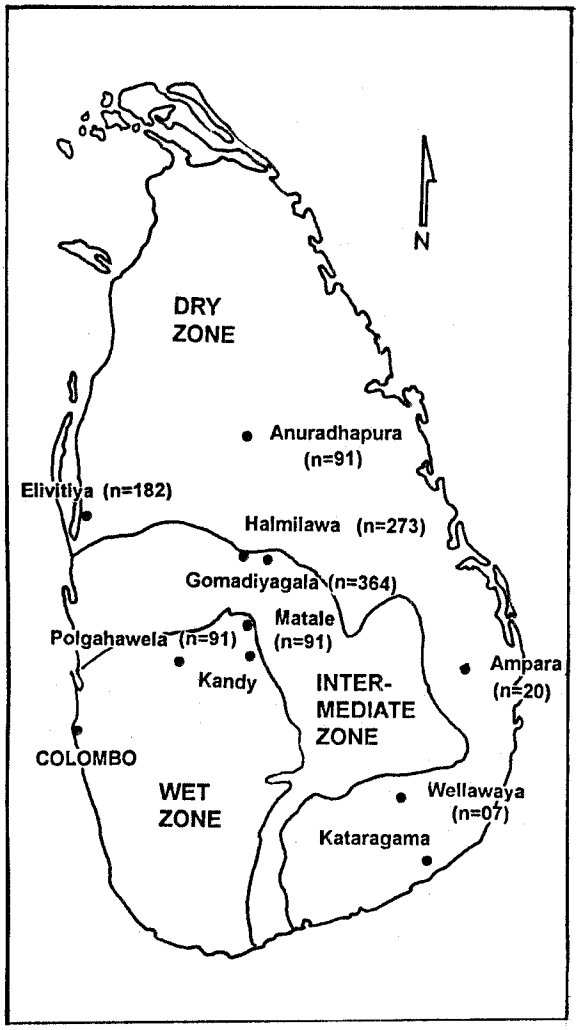

Fig. 1: A Map of Sri Lanka, showing three climatic zones, study villages and the numbers (n) of mosquitoes analysed to date for sibling species status A

Gomadiyagala with the DNA probe Rp217 and all the wild caught mosquito samples tested, gave a very strong positive hybridization signal indicating the absence of sibling species A in the population of wild caught mosquitoes.

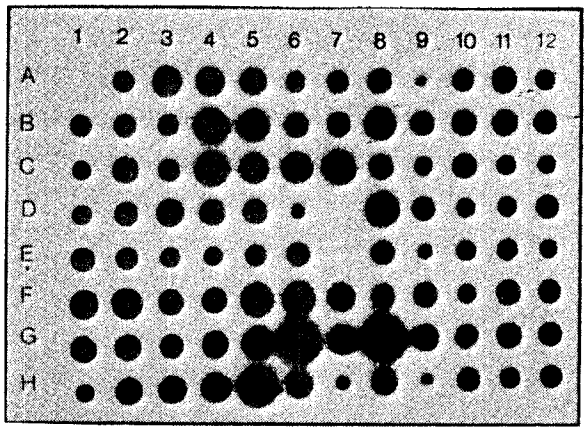

Fig. 2: Dot-blot hybridization of single mosquito DNA extracts (diluted by 200 fold) of wild caught mosquitoes with Rp234 DNA probe (Rp217 gives an identical hybridization pattern)

Control DNA samples: *An. culicifacies* sibling species B (India) H1 and D6; *An. culicifacies* sibling species A (India) A1 and D7; *An. tessellatus* (Sri Lanka) E7; All wild caught mosquitoes gave a positive hybridization signal

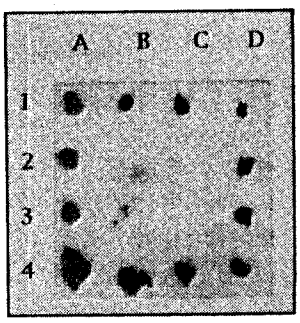

Fig. 3: Squash blot of mosquito heads hybridized with Rp217 DNA probe at 37°C

Control DNA samples: 2B — *An. tessellatus;* 2C — *An. subpictus*; 3B — *Cx. quinquefasciatus*; 3C — *An. culicifacies* species A; All wild caught mosquitoes gave a positive hybridization signal

DISCUSSION

We have previously shown[12,17] that Rp36, Rp217 and Rp234 repetitive sequences could be used as DNA probes to detect *An. culicifacies* form other mosquito species as well as to distinguish species A from B and C using a 200 x dilution of a single mosquito DNA extract in a dot-blot hybridization assay.

Rp217 gave a negative hybridization signal with *An. culicifacies* species A, while it sometimes gave a very faint hybridization signal with other control mosquito samples namely, *An. tessellatus, An. subpictus, Cx. quinquefasciatus* in the squash blot hybridization assay. This indicates that *An. culicifacies* species B contains higher copy number of Rp217 repetitive fragment than that of other control mosquito samples and *An. culicifacies* species A[18]. However, other mosquito species can be morphologically differentiated from *An. culicifacies* using conventional taxonomic criteria prior to screening of *An. culicifacies* wild caught mosquitoes for sibling species A.

Screening of 1119 mosquitoes collected from various malaria endemic regions in Sri Lanka has consistently shown the absence of *An. culicifacies* sibling species A. Twenty-five semi-gravid *An. culicifacies* collected from Gomadiyagala were screened for sibling species status using cytogenetic method, all were identified as sibling species B.

The simplified squash blot hybridization assay using the DNA probe Rp217 would be ideal for the detection of *An. culicifacies* species B and C in the northern region of India where *An. culicifacies* A is predominant, since the detection of a few positive hybridization signal in a largely negative population is an easy task.

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Seasonal Prevalence of *Anopheles stephensi* Larvae and Existence of Two Forms of the Species in an Urban Garden in Calcutta City

SANGITA CHAKRABORTY, SUDIPTA RAY and NEELAM TANDON

A study on the occurrence and seasonal prevalence of *Anopheles stephensi* (principal vector of malaria in Calcutta) larvae in an urban garden in central Calcutta revealed their presence in collections of fresh and/or rain water in various kinds of temporary and permanent breeding habitats. Breeding sites in shady areas were preferred in comparison to those receiving direct sunlight. The density of *An. stephensi* larvae was highest in earthen and lowest in wooden pots throughout the study period, as supported by ANOVA and Wilcoxon's signed rank test. During monsoons the larval density was uniformly high in all four, i.e. earthen, stone, cement and wooden kinds of pots. The variation in the density of *An. stephensi* larvae due to the effect of season and nature of pots was highly significant. Co-existence of *An. stephensi* larvae with *Aedes aegypti, Ae. albopictus* and *Cx. quinquefasciatus* was also observed at times in certain water collections. *An. stephensi* population in the urban garden in central Calcutta was found to comprise of two forms the type and variety *mysorensis*.

Keywords: *Anopheles stephensi*, Breeding sites, Seasonal variation, Type and variety *mysorensis*

INTRODUCTION

Anopheles stephensi has been incriminated as an efficient transmitter of malaria in several metropolitan towns and cities, including Calcutta[1-3]. In view of the resurgence of the disease in the city since the beginning of 1984[4]; a gradual increase in its prevalence

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and incidence during the last one and a half decade, and a sudden spurt in the number of malaria cases during the latter part of 1995, a study on the breeding sites of *An. stephensi*, and seasonal variation in their larval density in such habitats in an urban garden in central Calcutta was conducted. Attempts to confirm Senior-White's[5] observations with respect to the existence of two forms of *An. stephensi*, i.e. type and variety *mysorensis*, in the study area, were also made. The findings are presented in this study.

MATERIALS AND METHODS

Study area

The study was conducted in a 40,000 sq m garden situated in the heart of the city. A large marble palatial building stands on its eastern and a number of one room brickbuilt houses, some with tiled and others with cemented ceilings occupied by a contingent of staff on its western periphery.

A horse stable, with few horses, a cattleshed, with some cows, and a mini zoo consisting of a variety of birds, i.e. ducks, cranes, peacocks, parrots, *mynahs* etc. is also present. A small paddle pool, a *chowbacha* (cemented tanks to store water), and large flower pots made of stone and cement, a few wooden and tin containers used for feeding the animals are distributed randomly in the said area. Abundance of almost all kinds of flowering and perennial plants provide the ecological conditions almost simulating the rural surroundings.

Larval survey

A random survey of various kinds of temporary (tin and earthen pots kept in bird cages, and likely to go dry; crumpled discarded polythene sheets and bags thrown away as waste in the locality; all kinds of containers which hold water temporarily, i.e. those which are liable to be overturned, and flower pots on roadside which contain water only during monsoons), and permanent (containers which hold water throughout the year) water collections in an urban garden in central Calcutta was made to find out the probable (any water collection where anopheline breeding may/or likely to occur), and preferential breeding sites of *An. stephensi* in the study area.

Fortnightly observations on the seasonal variation in the larval density of the species (type form) in wooden, cement, earthen and stone pots placed naturally (in the said garden) were made during February 1995 to January 1996 by recording the number of larvae per dip (average of five dips in all pots of each kind) as per WHO guidelines[6].

An. stephensi population in the urban garden was a composite of two forms (type and the variety *mysorensis*) was a finding perchance. *An. stephensi* larvae collected from various sites were reared to adults in the laboratory and females allowed to oviposit. The length, breadth, number of ridges, and the length of floats of eggs was recorded and the findings compared with the observations made by Senior-White[5]; Subbarao *et al.*[7]; and Sweet and Rao[8].

RESULTS

Survey of aquatic habitats in the urban garden in central Calcutta, revealed the occurrence of *An. stephensi* (type form) larvae in almost all kinds of temporary and permanent accumulations of rain and/or fresh water throughout the study period. The variety *mysorensis* was found to breed only in a few earthen and stone pots, either alone or in association with the type form. Breeding sites (earthen, stone, cement and wooden) in shady areas of the garden and with plenty of algal vegetation (*Rebhularia* sp, *Glaeotrichea* sp, *Calotrix* sp, *P. thophora* and *Cladophora*) were preferred both by the type and variety *mysorensis* as evidenced by a relatively high larval density in these sites.

An. stephensi was found to breed in association with *Aedes aegypti* and *Aedes albopictus* larvae in almost all kinds of (temporary and permanent) breeding sites, provided they contained water in the study area. Co-existence with negligible number of *Culex quinquefasciatus* larvae was also observed occasionally.

Observations on the larval density of *An. stephensi* in four kinds (earthen, stone, cement and wooden) of pots throughout the study period revealed that the earthen pots were most thickly populated and wooden pots the least.

The analysis of variance of pots, revealed that the variation in larval density in different kinds of pots is highly significant, the calculated F value 5.47 > table F value 2.60, df = 3, 144 at 5% level. This finding was also supported by Wilcoxon's signed (one sided) rank test from which it was evident that the variation in the larval density between earthen and stone, stone and cement, cement and wooden pots was significant ($T_1 = 0 < t_\alpha = 6$ $\therefore$ earthen pot > stone pot, where N=8, $T_2 = 5.5 < t_\alpha = 11$, $\therefore$ stone > cement pot, where N = 10, $T_3 = 0 < t_\alpha = 11$ $\therefore$ cement pot > wooden pot; N = Sample size, T = Rank test value).

It was also found that the difference in the density of larvae in earthen and stone pots was significant in all the three seasons ($\chi^2 = 6.42$, df = 2, p > table value 5.99 in case of earthen pots and $\chi^2 = 5.99$, df = 2, p = table value 5.99 in case of stone pots) at 5% level.

According to ANOVA (2-way classification, non-interactive mode) the variation in the larval density of *An. stephensi* due to the effect of season, is highly significant (calculated F value 3.39 > table value, 1.79, df = 11, 144) at 5% level.

Studies on the seasonal variation in the larval density of *An. stephensi* revealed the same to be uniformly high in all the four types of pots during monsoons (June to September 1995), and lowest during summer (Feburary to May 1995) (Table 1). The larvae were however, not encountered in the month of March, as most of the breeding sites had dried up. A sudden increase in the larval density in earthen and stone pots was observed during November and December 1995 (Table 1) which is

Table 1. Seasonal variation in the larval density of *An. stephensi* (type form) in four kinds of breeding sites

Seasons	Earthen	Stone	Cement	Wooden
Summer (Feb-May 1995)	3.50	3.25	2.75	2.00
Monsoon (Jun-Sep 1995)	9.25	8.50	7.25	5.75
Winter (Oct 95-Jan 96)	14.25	13.25	6.00	4.25

The data is an average of larval density of four months constituting each season.

attributed to a few smart showers in the beginning of November. Lowest larval density in the cement and wooden pots was due to the emptying of some of the pots of each of the two kinds by the residents.

The variation in the larval density of *An. stephensi* with respect to both the month and nature of pots was highly significant as evidenced by ANOVA (2-way classification; non-interactive mode) where the calculated F value 6.98 > table value 1.44, df = 33, 144 at 5% probability level. The critical density (CD) values of the season signify an equally important role of each of the three seasons on the variation in the larval density of *An. stephensi.*

It was observed that *An. stephensi* larval population in the urban garden comprises of two forms, i.e. the type and variety *mysorensis* (Table 2). The ratio between the two was approximately 2:1 in habitats where they co-existed except in November and December 1995 when the larval population of the latter increased considerably, resulting in the reversal of their proportions.

DISCUSSION

Perennial transmission of malaria has be-

Table 2. Egg measurement of two forms of *An. stephensi*

Species	Total No. of eggs measured	Av. length of eggs	Av. breadth of eggs	Av. length of egg floats	Av. number of ridges on egg floats
An. stephensi (type)	1000	575 μ (535-594 μ)	196 μ (192-216 μ)	299 μ (271-317 μ)	18 ± 1.6 (17-19)
An. stephensi mysorensis (variety)	310	494 μ (452-500 μ)	109 μ (94-118 μ)	220 μ (198-238 μ)	13 ± 1.3 (12-14)

Measurements of eggs of two forms of *An. stephensi* as recorded by Sweet and Rao[8]; Type form : Length of eggs – 555 μ ± 24; Breadth of eggs – 204 μ ± 12; Length of egg floats – 294 μ ± 23; Variety *mysorensis* : Length of eggs – 476 μ ± 24; Breadth of eggs – 106 μ ± 12; Length of egg floats – 218 μ ± 20.

come a common phenomenon in Calcutta city. Effective containment of the disease can be achieved by implementing larval control measures[9]. Knowledge of the breeding habitats and information on the seasonal prevalence of *An. stephensi* in various kinds of breeding habitats is necessary for the containment of the disease.

In the present study, *An. stephensi* larvae were found to occur in all transient and permanent fresh/rain water collections, in the study area indicating that the container positivity rate was cent per cent, throughout the study period (except in March 1995 or in the event of some of the breeding sites being dried up[4,10] (Table 3). It is to be stated that *An. stephensi* larvae, breeding in transient habitats definitely contribute to the adult population. In rainy season, the intensity of breeding in such habitats was almost equal to that in the permanent habitats.

Occurrence of *An. stephensi* larvae in association with *Ae. aegypti, Ae. albopictus* and *Cx. quinquefasciatus* larvae in certain water collections was also observed in the study area and the findings are in corroboration with those of Kumar and Thavaselvam[11].

The findings of the present study also indicate the preference of female *An. stephensi*

Table 3. Average monthly variation in the larval density of *An. stephensi* (type form) in four kinds of breeding sites in relation to rainfall

Year/Months	Type of breeding sites				Av. rainfall (mm)
	Earthen	Stone	Cement	Wooden	
1995					
Feb	2*	2	1	1	0.93
Mar	–	–	–	–	Trace
Apr	4	3	3	2	2.04
May	8	8	7	5	4.74
Jun	10	9	7	6	5.95
Jul	12	11	9	8	12.96
Aug	9	8	7	5	6.93
Sep	8	7	6	4	21.61
Oct	9	6	5	4	7.17
Nov	21	20	9	7	0.80
Dec	19	17	7	5	—
1996					
Jan	8	8	3	1	0.23

*Larval density — The number of larvae per dip (average of five dips in each kind of pot) every fortnight.

to breed in earthen and stone pots (as evidenced by the results of ANOVA and rank tests) in comparison to wooden pots. Choudhury and Sen[4]; Biswas *et al.*[9] and Hati *et al.*[10], also made similar observations.

As mentioned earlier, *An. stephensi* larvae were not found in March 1995 (Table 3) since almost all the breeding sites had dried up. The larvae reappeared in April 1995 which coincided with the first shower, and showed a notable increase in the following three months in all four kinds of pots (Table 3). During the first two months (June and July) of monsoons the larval density was uniformly high in all the four kinds of pots[4,12] (Table 3), a decrease during the latter part of the season (August to September 1995) was due to very heavy rainfall (Table 3) leading to spillage and hence loss of larvae. A peak in November 1995 was again observed in earthen and stone pots due to heavy to moderate rainfall in the preceding two months resulting in optimum water level in the said breeding sites. Earlier workers[9,10] also made similar observations.

As evident from Table 3, monsoons in 1995 were rather inconsistently spaced and erratic which explains the inconsistency in the monthly larval density of *An. stephensi*.

As mentioned, morphometric data (Table 2) of eggs, on comparison with the findings recorded earlier[5,7,8], revealed that *An. stephensi* population in the urban garden in central Calcutta comprises of two forms the type and variety *mysorensis* of which the latter showed a noticeable increase during November and December 1995, in the breeding sites mentioned earlier.

Occurrence of two forms of *An. stephensi* in Calcutta metropolis has added new dimensions to the epidemiology of malaria transmission in the city.

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Efficacy of Neem Oil-Water Emulsion against Mosquito Immatures

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Neem oil-water emulsion was used in mosquito breeding habitats to find out its larvicidal effect on immatures of different mosquito species. Application of 5% neem oil-water emulsion @ 50 ml/sq m in pools and @ 100 ml/sq m in tanks resulted in 100% reduction of III and IV instar larvae of *An. stephensi* after 24 h while, against *Cx. quinquefasciatus* it was 51.6 and 91.2% reduction in the larval density after Day 1 and 14 respectively. Similarly, application of 10% emulsion in desert coolers against *Aedes aegypti* @ 40 to 80 ml per cooler resulted in complete inhibition of pupal production.

Keywords: *Aedes aegypti, An. stephensi, Cx. quinquefasciatus,* Larvicide, Neem oil emulsion

INTRODUCTION

Mosquitoes are the single largest group of insects which transmit many diseases like malaria, filaria, Japanese encephalitis and dengue throughout the world. For the management of vector mosquito population, use of chemical insecticides is now being seriously questioned due to various factors like multiple resistance to insecticides, environmental pollution, and high cost. As an alternative to chemical insecticides effectiveness of neem (*Azadirachta indica*) based natural compounds have been investigated by different workers. Efficacy of neem oil as a repellent has been demonstrated against adult mosquitoes[1-4]. Laboratory trials have also shown the larvicidal action of neem products against different mosquito species[5-9]. Rao *et al.*[10] have shown the control of culicine mosqui-

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toes in rice field using neem cake powder and two other neem formulations. Nagpal *et al.*[11] used wood scrap balls of neem oil to control *An. stephensi* breeding in overhead tanks. This paper presents the results of laboratory and field trials on the efficacy of neem oil-water emulsion against *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*.

MATERIALS AND METHODS

Water based neem oil emulsion was prepared by a newly designed water jet mixer. Commercially available neem oil used in the present study had a specific gravity of 0.908 to 0.934 at 30°C and free fatty acid contents of 5 to 7.5% w/w. Laboratory and field trials were carried out using 5 and 10% neem oil-water emulsion as stock solution. Newly emerged larvae of three laboratory reared mosquito species were exposed to different concentrations of neem oil in 250 ml water taken in 500 ml capacity plastic bowls. These concentrations, viz. 0.0125, 0.025, 0.05 and 0.1 ml/l were prepared from the 5% stock solution of neem oil emulsion and used against *Anopheles stephensi* and *Culex quinquefasciatus* while, slightly higher concentration viz. 0.05, 0.1, 0.2 and 0.4 ml/l were used against *Aedes aegypti*. All tests were carried out in three replicates along with parallel untreated controls. Larvae were fed on a mixture of dog biscuit and yeast powder in ratio of 3:2 throughout the experiments. Larvicidal efficacy was ascertained by counting larval mortality at 24 h interval till complete emergence of adult mosquitoes in the control and experimental bowls. Simultaneously, toxicity of neem oil emulsion was evaluated against two non-target predators, mosquitoes fish *Gambusia affinis* and notonectid bugs, *Anisops* spp in the laboratory by exposing ten specimens of each at different concentration. Mortality was recorded after 48 h exposure and LC_{50} values was calculated.

Field trials were carried out in the month of May 1995 against *An. stephensi* in underground basement tanks and pools and against *Cx. quinquefasciatus* in pools and drains. Neem oil-water emulsion (5%) was sprayed @ 50 and 100 ml/sq m with the help of a compression sprayer so as to obtain a concentration of 2.5 and 5 ml/sq m of neem oil respectively. Observation on efficacy of emulsion was recorded after 24 h and subsequently at weekly intervals up to three weeks by recording immature density by taking five dips in pools, tanks and 10 dips in drains. Immature density was estimated by counting I+II and III+IV instar larvae and pupae per dip. Trials against *Ae. aegypti* larvae was carried out in post-monsoon months during September-October in desert coolers, a common breeding site of *Ae. aegypti*. Fifty desert coolers (water tank size 60 x 60 x 20 cm) were taken as experimental and 10 as control in a residential colony of west Delhi. Four doses 20, 40, 60 and 80 ml of 10% neem oil emulsion were used directly with the help of a measuring cylinder of 100 ml capacity so as to obtain a neem oil concentration of 2, 4, 6 and 8 ml per cooler respectively.

Data collected in experimental breeding habitat were compared with control and per cent

reduction (III+IV instar larvae and pupae) was calculated using the Mulla's[12] formula:

$$\% \text{ reduction} = 100 - \frac{C_1}{T_1} \times \frac{T_2}{C_2} \times 100$$

Where, C_1 is number of larvae in untreated control habitat before treatment; C_2, number of larvae in control habitat after treatment; T_1, number of larvae in treated habitat before treatment; and T_2, number of larve in treated habitat after treatment.

RESULTS

Table 1 presents the data on the effect of water based neem oil emulsion on emergence and duration of larval development in the laboratory. The concentrations of neem oil which resulted in the complete inhibition of adult emergence of *An. stephensi*, *Cx. quinquefasciatus* and *Aedes aegypti* were 0.1, 0.1 and 0.4 ml/l respectively. At lower concentrations, 0.0125 to 0.05 ml/l, the duration of larval development was prolonged as compared to control. The LC_{50} value against *Gambusia affinis* and *Anisops* spp were determined as 0.036 and 0.0058 ml/l respectively.

Table 1. Effect of neem oil on growth and development of mosquito immature

Species	Test conc. (ml/l)	% emergence	Av. duration of development from I instar to adult (days)
An. stephensi	0.1	0	–
	0.05	13.3	17.2
	0.025	24.0	17.3
	0.0125	33.3	14.16
	Control	64.0	13.7
Cx. quinque-fasciatus	0.1	0	–
	0.05	9.3	14.6
	0.025	21.3	13.7
	0.0125	29.3	10.7
	Control	89.3	10.6
Aedes aegypti	0.4	0	–
	0.2	6.0	25.6
	0.1	2.0	22.0
	0.05	45.0	10.4
	Control	91.0	11.0

Field trial

Results of field trials against *An. stephensi* are given in Table 2. Application of 5% neem oil-water emulsion @ 50 ml/sq m in pools and @ 100 ml/sq m in underground basement tanks resulted in 100% reduction of III and IV instar larvae within 24 h. The breeding, however, reappeared after Day 7 to Day 10 in pools; and in tanks after Day 14. In one of the experimental pool where notonectid bugs were present the spraying of neem oil emulsion resulted in almost complete mortality of the bugs after 24 h.

Table 3 presents the results of field evaluation of neem oil-water emulsion against *Cx. quinquefasciatus* in pools and drains. Pools treated with 5% emulsion @ 50 ml/sq m showed 51.6% reduction in the density of III and IV instar larvae after 24 h. Subsequently the per cent reduction increased to 65.1 and 91.2% after Day 7 and Day 14. Drains,

Table 2. Field evaluation of neem oil against *An. stephensi* breeding in pools and underground basement tanks

Post-treatment (Day)	Density/dip											
	Control			Pool @ 2.5 ml/sq m					Underground basement tank @ 5 ml/sq m			
	I+II	III+IV	Pupae	I+II	III+IV	%R	Pupae	%R	I+II	III+IV	%R	Pupae
0	1.3	1.0	3.0	38.8	20.0		6.0		0.3	1.25		1.0
1	2.1	2.1	3.0	3.2	0.0	100.0	3.0	50.0	0.4	0.0	100.0	0.0
7	6.0	1.6	4.2	0.0	0.0	100.0	0.0	100.0	0.0	0.0	100.0	0.0
10	3.3	0.5	3.0	1.6	0.8	92.0	0.0	100.0	0.0	0.0	100.0	0.0
14	1.8	0.5	2.0	5.0	7.8	22.0	0.0	100.0	0.0	0.0	100.0	0.0

% R — Per cent reduction.

Table 3. Field evaluation of neem oil against *Culex quinquefasciatus* breeding in pools and drains

Post-treatment (Day)	Control			Treated @ 2.5 ml/sq m				
	I+II	III+IV	Pupae	I+II	III+IV	%R	Pupae	%R
				Density/dip in pools				
0	190	11	0	40	41		16	0
1	266	12.2	1	91	22	51.6	18	0
7	38	10	2	32	13	65.1	7	80.5
10	330	26	8	224	20	79.36	12	91.6
14	189	286	23	70	93	91.2	26	93.7
21	500	8	11	390	218	0	252	0
				Density/dip in drains				
0	21	57	63	57	24		84	0
1	266	223	187	39	9	90.4	8	96.7
7	149	97	43	38	33	19.2	17.8	68.9
10	8	2.9	3.2	0.6	4.6	–	2.4	43.7
14	6.5	12	3	19	3.8	24.79	1.4	65.0
21	59.5	6	1.5	40	4	–	0	100.0

% R — Per cent reduction.

treated with @ 100 ml/sq m showed 90.4% reduction in the density after 24 h, however, breeding reappeared after Day 7 showing only 19.2% reduction.

In each desert cooler application of 10% neem oil-water emulsion @ 20 to 80 ml (equivalent to 2 to 8 ml of neem oil) resulted in sharp reduction in larval density (Fig. 1 and Table 4). Reduction of III and IV instar larvae of *Ae. aegypti* was noticed at all the doses from 20 to 80 ml. At the doses of 40 to 80 ml per cooler 100% control of pupal production was achieved after Day 7 and Day 8, while similar impact was not obtained in the coolers treated with 20 ml emulsion (Fig. 1 and Table 4).

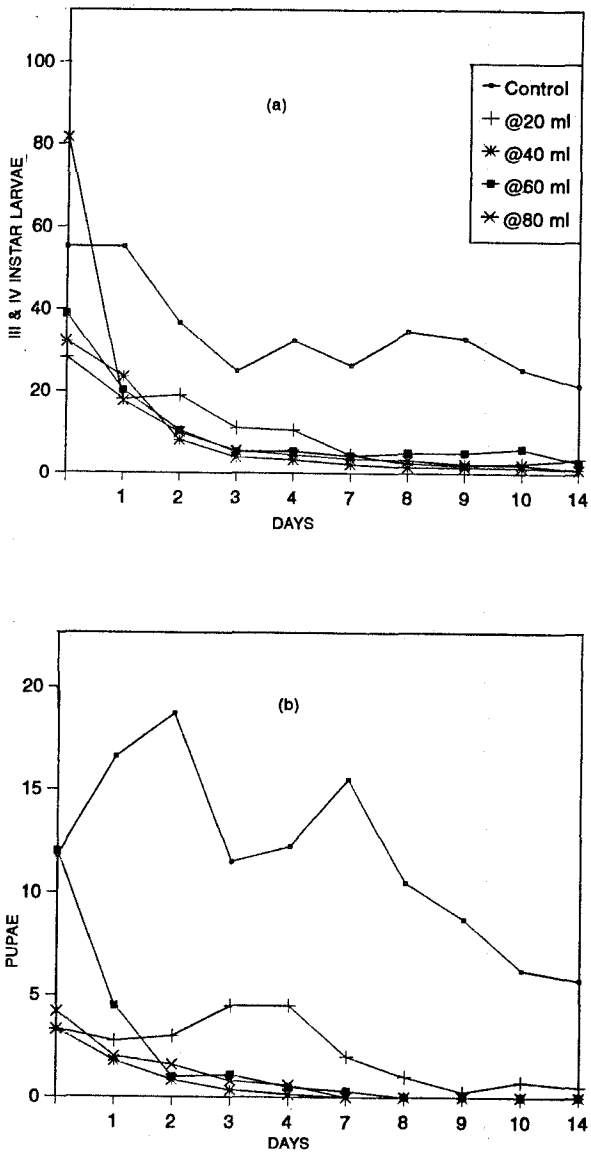

Fig. 1: Effect of 10% neem oil-water emulsion on *Ae. aegypti* (a) larvae, and (b) pupae in desert coolers

DISCUSSION

Results of the present study have revealed that neem oil produced complete inhibition of *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* emergence in the laboratory conditions at a dose of 0.1 to 0.4 ml/l indicating the interference in the hormones controlling metamorphosis and moulting of larvae.

Larvicidal action of various neem products against *Cx. quinquefasciatus*, *An. stephensi* and also against *Ae. aegypti* have been reported by many workers[5,7,8]. Mittal *et al.*[9] in a laboratory study showed that crude neem oil was as effective as any other azadirachtin enriched neem products but the use of crude neem oil in field application faced difficulty due to its low miscibility in water and spreadability. Therefore, a water based emulsion was prepared in a water jet mixer, fitted with a specially designed nozzle which helps in breaking down the oil particles into very small particles to make a stable emulsion.

Table 4. Average density of *Ae. aegypti* immatures in desert coolers treated with 10% neem oil emulsion

Post-treatment (Day)	Control		@ 20 ml		@ 40 ml		@ 60 ml		@ 80 ml	
	III+IV	Pupae	III+IV	Pupae	III+IV	Pupae	III+IV	Pupae	III+IV	Pupae
0	55.2	11.8	28.33	3.33	32.2	3.33	38.9	12.0	81.6	4.2
1	55.2	16.6	18.0	2.75	23.5	1.78	20.25	4.5	17.6	2.0
2	36.7	18.7	19.0	3.0	8.07	0.85	10.37	1.0	9.9	1.6
3	25.0	11.5	11.0	4.5	3.92	0.35	5.25	1.1	5.5	0.8
4	32.5	12.25	10.5	4.5	3.28	0.14	5.37	0.5	4.5	0.6
7	26.5	15.5	4.5	2.0	2.21	0.0	4.25	0.3	3.55	0.0
8	34.75	10.5	2.5	1.0	1.54	0.0	5.0	0.0	3.25	0.0
9	33.0	8.75	2.0	0.25	1.54	0.0	5.0	0.0	2.25	0.0
10	25.5	6.25	2.5	0.75	1.4	0.0	6.0	0.0	2.12	0.0
14	21.5	5.75	3.5	0.5	1.0	0.0	2.8	0.0	0.87	0.0

This water based emulsion is readily miscible in water and disperse uniformly and remains stable for atleast six months.

Field trials with neem oil emulsion have shown up to 100% reduction in late instar larvae and pupae in small stagnant water habitats, however, in drains reduction was not so sharp, probably due to natural flow of water in drains. In a desert cooler 40 ml of 10% neem oil emulsion was found to be sufficient for controlling *Ae. aegypti* breeding up to 14 days. Present study has clearly demonstrate that neem oil emulsion can be used as a effective larvicide, however, its adverse killing effect on the beneficial predators like *Gambusia affinis* and *Anisops* restricts its use randomly in natural habitat. It is, however, important to mention here that Rao *et al.*[10] did not observe any adverse impact of neem cake against non-target organism in rice fields. In the context of present study neem oil emulsion can still be used as a larvicide to control mosquito breeding in coolers, overhead tanks, curring tanks and containers where beneficial fauna is not present.

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Species Diversity and Interspecific Associations among Mosquitoes in Rice Agro-ecosystem of Kheda District, Gujarat

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Rice agro-ecosystem of Kheda district in Central Gujarat was inhabited by 14 species of anophelines and 15 species of culicines. *Anopheles subpictus* and *Culex vishnui* gr dominated the respective groups. Species diversity in rice fields as well as in associated habitats remained high during non-monsoon (*rabi*) period. There existed several positive and negative interspecific associations. Malaria vector *An. culicifacies* showed positive association with *An. subpictus, An. annularis, An. pallidus* and *Cx. quinquefasciatus* but was found negatively associated with *An. nigerrimus, Cx. vishnui* and *Cx. tritaeniorhynchus.*

Keywords: Interspecific associations, Mosquitoes, Rice agro-ecosystem

INTRODUCTION

In immature stages different species of mosquitoes occupy the same habitat and form part of a single guild. The co-existence of more than one species in a habitat at a given time indicates that species of the same nature and preference interact with each other, breed simultaneously and invade the habitat at the same time for feeding and development. This interaction is governed by interspecific association. The degree of association varies from habitat-to-habitat and within the habitat depending upon prevailing physico-ecological conditions. Earlier studies on species association in different breeding habitats were made by Lounibas[1], Reisen *et al.*[2] and Bhatt *et al.*[3]

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Rice agro-ecosystem is conceptualized as a complex of interactions between various factors which determine the breeding and prevalence of different mosquito species at various stages of the growth of the crop. A number of mosquito species including the vectors of some well-known human diseases have been reported to breed in rice fields and associated habitats[4-6]. Present study was undertaken to find out the species diversity and interspecific associations in different habitats in the rice agro-ecosystem of Kheda district, Gujarat for better understanding of the mosquito bionomics and for planning effective control measures.

MATERIALS AND METHODS

Kheda district in central Gujarat is one of the leading and prosperous districts among the 19 districts of the state due to well developed agriculture and ample irrigation facilities. The topography, climate, rainfall and other features of the district have been described earlier[7,8]. Rice is one of the most important food crops of Kheda. Its cultivation pattern and agronomic practices have already been described elsewhere[5,6]. Twenty seven rice fields in nine villages of three talukas, viz. Anand, Matar and Petlad which have canal irrigation and are best suited for rice cultivation were selected. In each village three rice fields were monitored on weekly basis during two *rabi* (January to June) and two *kharif* (July to December) seasons commencing from June 1988. Random sampling was also done from the rice fields of adjoining talukas to ensure bigger sample size for studying the mosquito fauna of rice agro-ecosystem.

Sampling for mosquito immatures was done from all the habitats of rice agro-ecosystem, viz. nurseries, rice field channels, rice fields and post-harvest rice fields every week using a standard larval dipper (9.5 cm dia and 300 ml capacity). Larvae and pupae were recorded and transferred to the plastic containers and brought to the laboratory for adult emergence. Larval food containing the mixture of powdered dog biscuits and yeast tablets was provided. Adults emerged were anaesthetized with ether and identified using the keys of Christophers[9] and Barraud[10].

The mean diversity (H') per individual was calculated for each habitat during both the seasons to evaluate the apportionment of individuals among species by applying the uncertainity function as described by Shanon and Weaver[11]. However, to quantify the association of different species occurring together, C_8 index of association of Hurlbert[12] which ranges from +1 (complete association) to –1 (complete dissociation) was used. Statistical significance was assessed with the corrected χ^2 formula as described by Pielou[13] for approximating a discrete distribution. Fisher's exact test was applied when a cell value was less than or equal to five.

RESULTS AND DISCUSSION

Mosquito composition in rice agro-ecosys-

tem is shown in Table 1 and species diversity is depicted in Figs. 1 and 2. C_8 indices of association are given in Tables 2 and 3.

Nurseries

Mosquito species breeding in rice nurseries comprised 13 species of anophelines and five species of culicines. *An. subpictus* was the most dominant among the former, whereas *Cx. vishnui* gr dominated among the culicines. Species diversity was found maximum during non-monsoon (*rabi*) crop. Forty-six pairings of the non-monsoon (*rabi*) crop revealed one positive and two negative associations, whereas 39 pairings of the mon-

Table 1. Per cent composition of different mosquito species in rice agro-ecosystem

Species	N	RFC	RF	HRF	Mean	Season of prevalence
Anophelines						
An. annularis	12.06	3.77	5.43	24.12	11.34	NM
An. barbirostris	0.73	11.18	1.86	3.70	4.36	+
An. culicifacies	0.91	20.83	5.32	2.51	7.39	NM
An. nigerrimus	19.92	16.36	7.68	20.55	16.12	+
An. subpictus	56.67	42.09	73.53	32.65	51.23	M
An. tessellatus	5.48	2.93	4.83	1.05	3.57	NM
Other anophelines	4.23	2.84	1.35	15.42	5.96	+
Total adults emerged	547	715	18,776	1513	21,551	
Total species emerged	13	9	14	12	14	
Culicines						
Ae. taeniorhynchoides	0	0.18	4.89	0	1.26	NM
Cx. bitaeniorhynchus	0.29	2.23	1.27	0.59	1.09	+
Cx. fuscanus	0.87	2.41	3.91	1.23	2.10	NM
Cx. quinquefasciatus	8.21	7.99	7.19	0.09	5.87	NM
Cx. vishnui gr	87.09	86.05	81.43	93.55	87.03	M
Other culicines	3.51	1.14	1.31	4.54	2.62	+
Total adults emerged	341	538	12,858	2020	15,757	
Total species emerged	5	9	15	9	15	

Composition is based on the adult emergence from the immatures collected from fixed as well as random rice fields; N — Nurseries; RFC — Rice field channels; RF — Rice fields; HRF — Harvested rice fields; NM — Non-monsoon; M — Monsoon; (+) denotes more or less equal distribution during both the seasons.

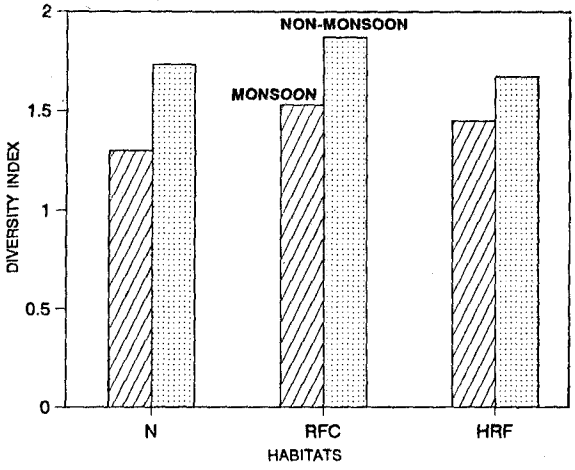

Fig. 1: Species diversity in habitats associated with rice fields

soon (*kharif*) crop showed five positive and three negative associations. *An. subpictus* showed positive association with *Cx. quinquefasciatus* ($p < 0.05$) during non-monsoon (*rabi*) and negative association with *An. nigerrimus* ($p < 0.05$) in the monsoon (*kharif*) season.

Rice field channels

A total of nine anopheline and the equal number of culicine species were encountered in rice field channels. *An. subpictus* and *Cx. vishnui* gr were found to be the predominating species in respective groups. Malaria vector *An. culicifacies* was found breeding profusely in field channels. Diversity index remained high during non-monsoon (*rabi*) period. Forty-seven pairings of the monsoon (*kharif*) crop revealed 10 positive and three negative associations. *An. subpictus* was found negatively associated with *An. barbirostris* ($p < 0.05$). *An. annularis* showed positive association with *An. pallidus*, *An. tessellatus* and *Cx. vishnui* whereas *An. nigerrimus* exhibited positive association with *An. aconitus* ($p < 0.05$).

Forty-one pairings of the non-monsoon (*rabi*) crop revealed seven positive and three negative associations. *An. subpictus* was found positively associated with *An. annularis* ($p < 0.01$) and *An. culicifacies* ($p < 0.05$) and the latter showed positive association with *An. annularis* ($p < 0.05$) too.

Rice fields

Immatures collected from rice fields revealed the presence of 14 anopheline and 15 culicine species. *An. subpictus, An. culicifacies, An. nigerrimus, An. annularis, An. tessellatus* and *Cx. vishnui* gr were found to be the predominant mosquito species inhabiting rice fields. Low species diver-

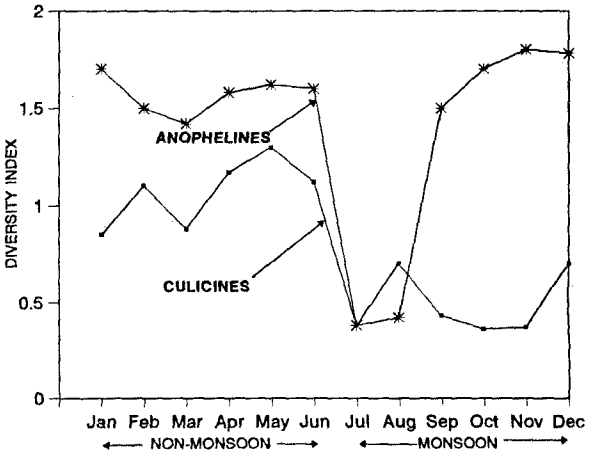

Fig. 2: Monthwise mosquito diversity in rice fields

sity in respect of anophelines was found in the months of July and August due to dominance of *An. subpictus* and there was an increase in diversity index with the decrease in the density of *An. subpictus* in later months. No definite pattern was however, shown by the culicines. Ninety pairings of the monsoon (*kharif*) period of rice cultivation yielded 23 positive and 14 negative associations. *An. culicifacies* showed positive association with *An. subpictus* ($p < 0.01$), *An. pallidus* and *Cx. quinquefasciatus* ($p < 0.05$) but showed strong negative association with *An. nigerrimus* ($p < 0.001$) and *Cx. vishnui* ($p < 0.05$). *An. subpictus* exhibited positive association with *An. vagus* ($p < 0.05$) but was found negatively associated with *An. barbirostris, An. nigerrimus* ($p < 0.001$) and *Cx. vishnui* ($p < 0.01$). *An. annularis* was positively associated with *An. pallidus* ($p < 0.01$) and *An. stephensi* ($p < 0.05$), and showed negative association with *Cx. vishnui* ($p < 0.05$). *An. barbirostris* exhibited positive association with *An. nigerrimus* and *Cx. seniori* ($p < 0.01$), whereas *Cx. vishnui* showed positive association with *Cx. fuscanus* ($p < 0.01$) and negative with *Aedes aegypti* ($p < 0.05$). Associations between other species were statistically non-significant.

One hundred and thirty-four pairings of the non-monsoon (*rabi*) crop produced 39 positive and 27 negative associations. *An. culicifacies* showed strong positive associa-

Table 2. Co-efficient of association (C_8) among different mosquito species in rice fields

Monsoon crop

Species	*A.su.*	*A.ba.*	*A.ni.*	*A.pa.*	*A.st.*	*A.va.*	*C.vi.*	*C.q.*	*C.fu.*	*C.se.*	*Ae.a.*
A.cu.	0.060** (30)	–	–0.702+ (5)	0.240* (4)	–	–	–0.322* (17)	0.353* (3)	–	–	–
A.su.	–	–0.641+ (12)	–0.533+ (30)	–	–	1.0* (4)	–0.173** (79)	–	–	–	–
A.an.	–	–	–	0.406** (6)	0.406* (3)	–	–0.221* (27)	–	–	–	–
A.ba.	–	–	0.102** (40)	–	–	–	–	–	–	1.0** (3)	–
C.vi.	–	–	–	–	–	–	–	–	0.818** (11)	–	–0.623* (2)
Total (+)ve	179	77	148	12	6	4	220	7	12	3	10
Total (–)ve	233	335	264	400	406	408	192	405	400	409	402

contd...

Table 2. (*contd.*)

Non-monsoon crop

Species	*A.su.*	*A.an.*	*A.ba.*	*A.fl.*	*A.te.*	*A.ni.*	*A.pa.*	*C.q.*	*C.tr.*	*C.bi.*	*C.fu.*	*Ae.t.*	*Ma.u.*
A.cu.	0.134^{+} (78)	0.293^{+} (43)	–	–	–	–0.650^{+} (9)	–	0.211^{+} (60)	–0.893^{+} (2)	–	–	–	–
A.su.	–	0.237** (59)	–0.526** (5)	–	–0.429^{+} (20)	–0.466^{+} (33)	–	0.425^{+} (114)	–0.513^{+} (22)	–	–	–0.729^{+} (5)	–
A.an.	–	–	–	–	–	–	0.644** (5)	0.129^{+} (47)	–0.598** (7)	0.339* (6)	–	–	–
A.ba.	–	–	–	1.0^{+} (2)	–	–	–	–	–	–	–	–	–
A.te.	–	–	–	–	–	0.084** (28)	–	–0.373* (14)	–	–	–	0.392^{+} (19)	1.0** (2)
A.ni.	–	–	–	–	–	–	–	–0.416^{+} (23)	0.155** (35)	–	–0.468* (10)	–	1.0* (2)
C.vi.	–	–	–	–	–	–	–	0.552^{+} (143)	–	–	–	–0.252* (21)	–
C.q.	–	–	–	–	–	–	–	–	–0.548^{+} (13)	–	0.228** (36)	–0.572** (5)	–
Total (+)ve	259	100	23	2	7	134	7	165	98	13	79	40	2
Total (–)ve	302	461	538	559	485	427	554	396	463	548	482	521	559

Figures in parentheses are the joint occurrences; *A.cu. — An. culicifacies; A.su. — An. subpictus; A.ba. — An. barbirostris; A.ni. — An. nigerrimus; A.pa. — An. pallidus; A.st. — An. stephensi; A.va. — An. vagus; A.an. — An. annularis; A.fl. — An. fluviatilis; A.te. — An. tessellatus; C.q. — Cx. quinquefasciatus; C.vi. — Cx. vishnui; C.fu. — Cx. fuscanus; C.se.— Cx. seniori; C.bi. — Cx. bitaeniorhynchus; C.tr. — Cx. tritaeniorhynchus; Ae.t. — Ae. taeniorhynchoides; Ae.a. — Ae. aegypti; Ma.u. —Ma. uniformis*; Due to large numbers, statistically insignificant associations have been excluded from the table; $^{*}p<0.05$; $^{**}p<0.01$; $^{+}p<0.001$.

tion with *An. subpictus, An. annularis* and *Cx. quinquefasciatus* ($p < 0.001$) but was negatively associated with *An. nigerrimus* and *Cx. tritaeniorhynchus* ($p < 0.001$). *An. subpictus* was found positively associated with *An. annularis* ($p < 0.01$) and *Cx. quinquefasciatus* ($p < 0.001$) but showed negative association with *An. tessellatus, An. nigerrimus, Cx. tritaeniorhynchus, Ae. taeniorhynchoides* ($p < 0.001$) and *An. barbirostris* ($p < 0.01$). *An. annularis* showed positive association with *An. pallidus* ($p < 0.01$), *Cx. quinquefasciatus* ($p < 0.001$) and *Cx. bitaeniorhynchus* ($p <$

0.05) but was negatively associated with *Cx. tritaeniorhynchus* ($p < 0.01$). *An. barbirostris* and *An. fluviatilis* produced maximum C_8 value and also showed strong positive association ($p < 0.001$). *An. tessellatus* was found positively associated with *An. nigerrimus, Mansonia uniformis* ($p < 0.01$) and *Ae. taeniorhynchoides* ($p < 0.001$) and negatively with *Cx. quinquefasciatus* ($p < 0.05$). *An. nigerrimus* showed positive association with *Cx. tritaeniorhynchus* ($p < 0.01$) and *Masonia uniformis* ($p < 0.05$) but was recorded negatively associated with *Cx. quinquefasciatus* ($p < 0.001$) and *Cx. fuscanus* ($p < 0.05$). *Cx. vishnui* showed strong positive association with *Cx. quinquefasciatus* ($p < 0.001$) and negative association with *Ae. taeniorhynchoides* ($p < 0.05$). *Cx. quinquefasciatus* was found positively associated with *Cx. fuscanus* ($p < 0.01$) but showed negative association with *Cx. tritaeniorhynchus* ($p < 0.001$) and *Ae. taeniorhynchoides* ($p < 0.01$).

Post-harvest rice fields

Post-harvest rice fields were also found to support prolific mosquito breeding. A

Table 3. Co-efficient of association (C_8) among different mosquito species in habitats other than rice fields

Non-monsoon						Monsoon				
Species	*A.su.*	*A.cu.*	*A.an*	*A.ni.*	*A.ba.*	Species	*A.su.*	*A.an.*	*A.ni.*	*A.st.*
				Nurseries						
C.q.	0.547*	–	–	–	–	*A.ni.*	–0.522*	–	–	–
				Rice field channels						
A.an.	1.0**	0.210*	–	–	–	*A.ba.*	–0.543*	–	–	–
A.cu.	0.528*	–	–	–	–	*A.pa.*	–	0.582*	–	–
						A.te.	–	1.0*	–	–
						A.ac.	–	–	1.0*	–
						C.vi.	–	0.078*	–	–
				Harvested rice fields						
A.pa.	0.381*	–	0.540**	–	–	*A.pa.*	–	0.463**	–	1.0**
A.cu.	0.648**	–	–	–	–					
A.ba.	–	–	–	0.851+	–					
A.ac.	–	–	–	0.642**	0.599+					
C.vi.	0.232*	–	0.241**	–	–					
C.tr.	–	–	–	–	1.0*					

Due to large numbers, statistically insignificant associations have been excluded from the table; *$p < 0.05$; **$p < 0.01$; +$p < 0.001$. Abbreviations used for the species have already been elaborated in Table 2, except *A.ac.* which denotes *An. aconitus*.

total of 12 anopheline and nine culicine species were encountered breeding in post-harvest rice fields. Species diversity maximized during non-monsoon (*rabi*) period. Seventy pairings of the non-monsoon (*rabi*) crop revealed 25 positive and three negative associations. *An. subpictus* was positively associated with *An. culicifacies* ($p < 0.01$), *An. pallidus* and *Cx. vishnui* ($p < 0.05$). *An. annularis* showed positive association with *An. pallidus* and *Cx. vishnui* ($p < 0.01$). *An. nigerrimus* was found strongly associated with *An. barbirostris* ($p < 0.001$) and *An. aconitus* ($p < 0.01$). Positive associations of *An. barbirostris* were also found with *An. aconitus* ($p < 0.001$) and *Cx. tritaeniorhynchus* ($p < 0.05$).

One hundred and two pairings of the monsoon (*kharif*) crop yielded 17 positive and one negative association. *An. pallidus* showed positive association with *An. annularis* and *An. stephensi* ($p < 0.01$). Associations between other species were not found statistically significant.

There was no common association between any species in rice nurseries and field channels during both the cropping seasons due to variation in species dominance, whereas a common positive association of *An. annularis* and *An. pallidus* in post-harvest rice fields was presumably due to presence of extensive growth of aquatic vegetation which provided favourable breeding conditions for both the species. In rice fields some species showed common positive and negative associations during both the crops, viz. *An. culicifacies* with *An. subpictus* and *Cx. quinquefasciatus* (+ve), *An. culicifacies* and *An. nigerrimus* (–ve) and *An. subpictus* with *An. barbirostris* and *An. nigerrimus* (–ve) and *An. annularis* with *An. pallidus* (+ve), which may be due to prevalence of these species in the said habitat and presence of similar micro-climatic conditions required for the proliferation of these species. However, negative associations between species might have been due to change in breeding time and habitat preference of two species.

Positive associations of *An. annularis* with *An. culicifacies, An. stephensi* and *An. subpictus* in paddy fields have been reported in previous observations as well[3]. Positive associations between species may occur due to interspecific attraction and/or common preference for a particular habitat, however, negative associations may result due to interspecific repulsion and/or variation in preference for a particular habitat. Diversity function characteristically decreased when fauna was dominated by a large number of few species and increased when there were equitable number of specimen among species. Diversity index, thus, provides and indication of high species equitability and is usually maximized in heterogenous habitat with a large number of species and is minimized in a homogenous habitat with a small number of species.

With the increase in human population and new irrigation schemes, the area under rice cultivation has also increased significantly in last few years. The prolonged water-logging with fast changing ecological condi-

tions and extensive surface area of this habitat offer favourable breeding conditions to a number of mosquito species including disease vectors of malaria, filariasis and Japanese encephalitis. Hence a better understanding of the breeding pattern of mosquitoes, their inter-relationship and prevalence in different stages of the growth of the crop and associated habitats with this ecosystem will be helpful to design better and appropriate control measures to check the disease transmission.

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SHORT NOTES

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Mosquito Breeding Associated with Urban Sewage System in Anand City (Gujarat)

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Keywords: Mosquito potential, Sewage system

Rapid growth of population and industrial installations in unplanned and unbalanced manner in urban areas result into alarming increase in disposal of waste water affecting thus adversely smooth process of ecosystem[1]. In urban, semi-urban and townships unmanaged sewage disposal from sewage plants creates a lot of mosquitogenic potential due to lack of proper infrastructure facilities, maintenance and basic understanding of the disease. Which beside causing a noxious mosquito bites to the local inhabitants also add extra burden on disease prevalence. Hence the present study was attempted to find out the mosquitogenic potential of the sewage disposal plants for feasibility of its management to check the mosquito proliferation.

Study was conducted from July 1994 to June 1995 at sewage disposal plant site of Anand city (population 1,10,226) which is an upcoming town in central Gujarat. The capacity of the plant is about 13.02 million litre water per day which along with eight sedimentation ponds covers an area of six ha. Whenever the

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quantity of water exceeds the capacity particularly during monsoon most of the water is released untreated which flows and get accumulated in low-lying areas of about 35 ha. The same get infested by water hyacinth and other aquatic weeds and become perennial source for mosquito breeding.

Fortnightly larval and pupal collections were made from the pond, pools and other water collections around the sewage treatment plant using standard larval dipper (9.5 cm dia; 300 ml capacity) and brought to the laboratory for rearing and adult emergence. Emerging adults were identified using the keys of Christophers[2] and Barraud[3]. Presence of aquatic plants in and around the catchment area of sewage treatment plant were also noted. Water samples from the same site were also collected and analysed for some chemical parameters as per Trivedi and Goel[4]. Data obtained were later processed and analysed statistically.

Studies on mosquitogenic potential of sewage water disposal site of Anand City revealed that both anopheline and culicine breed in more or less equal proportions throughout the year. The larval density during summer and winter remained below 10 per dip, however, it maximized during monsoon and reached up to 40 per dip. Among the mosquitoes found associated with this type of ecosystem were anophelines (67%), culicines (32%) and *Aedes* sp (0.11%). The composition of different mosquito species found breeding at sewage disposal site is given in Table 1. *An. subpictus* (60%) was pre-

Table 1. Per cent composition of different mosquito species based on larval emergence

Species	Summer		Monsoon		Winter		Total	
	No.	%	No.	%	No.	%	No.	%
An. subpictus	63	56.76	374	64.15	99	51.03	536	60.36
An. barbirostris	1	0.90	0	0.00	27	13.91	28	3.15
An. annularis	0	0.00	0	0.00	12	6.18	12	1.35
An. nigerrimus	16	14.41	0	0.00	3	1.15	19	2.14
An. stephensi	0	0.00	0	0.00	1	0.52	1	0.11
*Cx. vishnui**	13	11.71	93	15.95	34	17.52	140	15.77
Cx. gelidus	14	12.61	108	18.52	12	6.19	134	15.09
Cx. tritaeniorhynchus	2	1.80	6	1.03	3	1.55	11	1.23
Cx. fuscanus	2	1.80	1	0.17	3	1.55	6	0.67
Aedes sp	0	0.00	1	0.17	0	0.00	1	0.11
Total	111	12.50	583	65.65	194	21.85	888	100.0

*Cx. *vishnui* sub group also includes *Cx. pseudovishnui.*

dominant anopheline species followed by *An. barbirostris* (3.15%), *An. nigerrimus* (2.14%), *An. annularis* (1.35%) and *An. stephensi* (0.11%), the later was present only in post-monsoon months due to dilution of organic contents of the water. *An. annularis* also showed predilection for winter months and was absent during summer and monsoon months. It has been reported to be a dominant species in winter months in previous observations also[5]. The presence of water hyacinth and other aquatic weeds probably supports its breeding[6]. *An. subpictus* is an ubiquitous species and its profuse breeding mainly occur in monsoon months[7]. Occurrence of *An. stephensi*, which is primarily a container breeder mosquito may be due to certain ecological compulsion.

Among culicines, *Cx. vishnui* (15.7%), *Cx. gelidus* (15.09%) were found to be dominant species followed by *Cx. tritaeniorhynchus* (1.23%), *Cx. fuscanus* (0.67%) and *Aedes* sp (0.11%). The predominance of *Cx. vishnui* gr of mosquitoes in such water collections is attributed mainly to the presence of aquatic vegetation such as algae, water-hyacinth, *Ipomea* and *Hydrilla*.

Chemical analysis of water samples collected from the catchment site of sewage treatment plant showed high organic contents in the water. pH of the water was found to be 8.0 to 8.5 and high contents of chloride, carbonates, alkalinity and hardness supported the prolific breeding of *Culex* gr of mosquitoes and some tolerant anopheline species such as *An. subpictus*. Other anophelines were found only when there was some dilution of these chemical contents (Table 2).

Due to numerous ecological changes in urban environment and its direct impact on vector behaviour, the problem of urban malaria and other vector-borne diseases is increasing day-by-day. Present study indicates that beside providing support to pest mosquitoes the sewage disposal ecosystem also provides shelter to disease vectors such as *An. stephensi* and *Cx. vishnui* gr. The management of such water bodies along with better understanding between human-environment inter-relation, interaction between several biotic and abiotic factors and socio-cultural practices is hence of utmost importance to combat the re-emergence of vector-borne diseases.

Table 2. Chemical characteristics of sewage water samples

Chemical contents	Concentration (mg/l)	
	Mean ± SD	Range
pH	8.2±0.3	8.0–8.5
DO	1.9±1.4	0.6–3.5
PA	493.3±115.0	380–610
TA	635.3±71.4	558–699
Ca^{++}	77.2±6.5	69.7–81.7
Mg^{++}	20.1±5.5	15.6–26.3
CO_3^{-}	278.0±103.9	160–356
Chloride	168.9±5.1	163.3–173.2
Hardness	274.0±9.1	264–282

DO — Dissolved oxygen; PA — Phenolphthalein alkalinity; TA — Total alkalinity.

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Antimalarial Herbs against Chloroquine-resistant *P. yoelii nigeriensis* in Mice

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Keywords: Chemosuppressive, Medicinal plants, *P. yoelii nigeriensis*, Prophylactic activity

It is noteworthy that modern facilities are absent in rural areas where the majority of West African people live. As such, most people living in such areas depend on plant extracts for the treatment of many diseases including malaria. In the traditional set-up, the feverish patient usually consumes either the aqueous or alcoholic decoction of the traditional plants[1]. The decoctions usually include barks, root and leaves of different plants. Sometimes, they are taken from one type of herb[2].

According to Sofowora[3], the development of resistant strains is commoner with that of the synthetic than with that of the natural antimalarials. Furthermore, natural products are generally safer to mammals including man. These products are degradable unlike some synthetic compounds that are highly persistent in environment. In view of this, there is an increasing need and interest for research into natural products in an attempt to discover effective, safer and cheap antimalarial drugs.

The leaf and bark extracts of *Azadirachta indica* contain a prostaglandin synthetase inhibitory action[4] and are also anti-inflammatory and mildly anti-pyretic[5]. *Enantia chlorantha* (bark and leaf) which is used for sore treatment, fevers, cough, vulnerary ulcer, haemostatic and febrifuge by traditional healers contains alkaloids, lignin, saponins

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and tannins[6]. Also, *Morinda lucida* was reported to contain anthraquinones which showed *in-vitro* activity against *Plasmodium falciparum* and also possess antifungal properties. *M. lucida* is used locally in the treatment of yellow fever and jaundice[7]. The leaves of *Cymbopogon giganteus* contains volatile oil, hespiridin bitter principles, and is used as flavouring agent, stimulant and antipyretic[8]. *Vernonia amygdalina* is used locally for the treatment of fever, as an anthelminthic, aphrodisiac and contains highly oxygenated germacranolides such as glaucolides and hirsutinolides. These plants grow commonly in Nigeria are most frequently used locally for the treatment of malaria and other infections.

This study, therefore, reports on the chemosuppressive and prophylactic activities of some traditional antimalarial herbs against chloroquine-resistant *Plasmodium yoelii nigeriensis* in mice. The test plants were: *Cymbopogon giganteus* (lemon grass) leaves; *Enantia chlorantha* (*awopa-Yoruba*) bark; *Morinda lucida* (*oruwo-Yoruba*) leaves; *Azadirachta indica* (*dongo-yaro* Housa) leaves and *Vernonia amygdalina* (*bitter leaf*) leaves. Approximately 100 g of each plant was boiled in 500 ml water for 30 min, filtered and the filtrate was further heated to reduce the volume to 250 ml[9]. The filtrate was sterilized by autoclaving and stored at 4°C for use.

Albino mice of 12-15 wks old (20±2 g) were used in these experiments and 25 mice were used for each plant extract. Approximately 10^5 erythrocytes infected with chloroquine-resistant *Plasmodium yoelii nigeriensis* were inoculated intraperitoneally into each mouse. The day of inoculation was taken as Day 0. Thereafter, the daily parasitaemia was monitored from tail thin blood films which were Giemsa-stained and observed under the microscope.

At 5-7% parasitaemia, the 25 mice were randomly divided into five groups of five mice each. The first four groups were treated with 4, 6, 8 and 10 mg/kg body weight (bw) of each stock solution per mouse for four consecutive days. The different doses of the plant extracts in isotonic sodium chloride were administered intraperitoneally into the mice. The last group was not treated and, therefore, served as control. This was done for all the plant extracts. On Day 4 after start of treatment, the percentage suppression of parasitaemia in relation to the control was assessed using a formula described by Obih *et al.*[10]

$$\text{Average (Av.) \% suppression} = \frac{(\text{Av. \% parasitaemia in control} - \text{Av. \% parasitaemia in test})}{\text{Av. \% parasitaemia in control}} \times 100$$

Thirty-five mice of the type described above were randomly divided into seven groups. The first six groups received 6 mg/kg bw intraperitoneally of stock solution per mouse of each of the plant extracts as well as artemether (0.3 ml containing 150 mg/kg bw) administered intraperitoneally for three consecutive days. The drugs were diluted with freshly prepared sterile physiological (normal) saline. The seventh group was not

treated and, therefore, served as control. On the Day 4 after start of treatment, each mouse was challenged with approximately 10^5 erythrocytes infected with chloroquine-resistant *P. yoelii nigeriensis*. The mice continued to receive the extracts until Day 6. The parasitaemia was monitored daily in Giemsa-stained tail thin blood films to assess the prophylactic activity of the different extracts against chloroquine-resistant *P. yoelii nigeriensis*.

The average chemosuppression for the different plant extracts in the 4-day test is shown in Table 1. *C. giganteus* had the best results with the 6, 8 and 10 mg/kg bw of stock solution per mouse doses having a 100% chemosuppression. The least effective was *V. amygdalina* in which the per cent suppression could not be assessed because none of the mice receiving the extract survived till Day 4 after start of treatment. Patency (presence of parasites) was not established in all the mice receiving artemether, *C. giganteus* and *E. chlorantha*. In the mice receiving *M. lucida*, patency was established a day after that of the controls and the last mouse in the group died on Day 19. In the mice receiving *A. indica*, patency was established on the same day as the controls (Day 9) and the last mouse in the group died on Day 18. All the mice receiving *V. amygdalina* died two days after start of prophylactic treatment.

The results showed that the boiled extracts of *C. giganteus* and *E. chlorantha* have good potentials against chloroquine-resistant *P. yoelii nigeriensis* both as schizontocidal and prophylactic agents when compared to artemether. Such plants will be very helpful in Africa where many people can not afford expensive drugs.

M. lucida and *A. indica* believed to be effective against malaria in western Nigeria showed very little antimalarial activity in the mice. Obih and Makinde[2] reported that the boiled water extract of *A. indica* showed schizontocidal activity against chloroquine sensitive *P. berghei*. It is, therefore, possible that this observation was due to the fact that the strain of parasite in our experiment was resistant to chloroquine or it could have just been due to species differences. It is also

Table 1. Suppressive effect of different plant extracts on approximately 5-7% chloroquine-resistant *P. yoelii nigeriensis* infection in a 4-day test

Dosage of extract of mice (mg/kg bw)	Av. % suppression of parasitaemia of different plant extracts			
	C. giganteus	*E. chlonantha*	*M. lucida*	*A. indica*
4	92.6	74.9	8.5	4.0
6	100.0	98.7	14.6	9.7
8	100.0	100.0	22.9	17.4
10	100.0	100.0	37.2	21.8

possible that *A. indica* functions more as an antipyretic agent than as a schizontocidal agent in malaria therapy[5]. As such, treatment with it would lead to early relief of fever and pyrexia to eliminate the parasite thereby helping the body's immune system.

V. amygdalina was toxic to the mice and this was rather surprising because bitter leaf is commonly eaten in this part of the world. It is also possible that the mice unlike man, lack some enzymes necessary to digest some alkaloids in the bitter leaf. Fagbenro-Beyioku *et al.*[11] found that the fresh water extract prepared with cold water had some prophylactic properties and there was no evidence of toxicity.

Since only the boiled water extract was used in these experiments, it is advisable that other forms of the plants such as fresh water and alcohol forms as well as other parts like roots and bark should be used in future experiments in order to find out their effects on the course of infection of chloroquine-resistant *P. yoelii nigeriensis*. This may likely give rise to other antimalarial drugs.

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Plasmodium malariae — A Case Report from District Nainital, Uttar Pradesh

R.P. SHUKLA and V.K. KOHLI

Keywords: *Plasmodium malariae*, Uttar Pradesh

A female patient named Smt. Jeevanti Devi, aged 40 years, resident of the village Sultan Nagri, located on forest fringe of District Nainital reported to OPD, Malaria Research Centre, Haldwani on 24th April 1997 with complaints of fever followed with severe chills since a week. Examination of blood smear revealed *Plasmodium malariae* infection with characteristic band pattern trophozoites, schizonts containing 10-12 nuclei and gametocytes. The patient was given radical treatment as per NMEP schedule (600 mg chloroquine base and 15 mg primaquine for five days). After the treatment blood smear from the above patient was further prepared and examined which did not reveal any malaria parasite. The patient was enquired about her visit to any malarious area and previous malaria history. The patient informed that she had fever episodes on many occasions in the past but no blood smear was examined for malaria parasite. However, the family is frequently visiting the malarious Chorgalia forest range of District Nainital.

Terai and Bhabar regions of District Nainital was notorious for highly malarious conditions due to the widespread swampy grasslands, high soil water content and submontane tropical vegetation. The region was known as 'death-trap area' before independence[1]. Three species of malaria parasite, viz. *P. falciparum, P. vivax* and *P. malariae* were reported from Terai area[2,3]. Before coloni-

Malaria Research Centre (Field Station), Inderjeet Garden, Bhotia Parao, Haldwani-263 141, India.

zation of the Terai *P. malariae* prevalence was 16.7% in 1949 which declined to 0.6% in 1951[3]. Thereafter, there is no record of its prevalence from this area. Application of DDT was started in Terai and Bhabar area of District Nainital by malaria control team in 1941 and later by establishment of NMCP in 1953 and NMEP in 1958 resulted in drastic decline in malaria incidence with elimination of *P. malariae* from this area. The species distribution pattern of *P. malariae* in this area is in agreement with the view of Yadav *et al.*[4] that it was this species which was expected to die out first under the extensive DDT spray.

Occurrence of *P. malariae* in this area after four decades is a new case report. Recently a case of *P. malariae* infection was also reported from Dooars region of northern West Bengal[5] whereas, earlier in undivided Bengal in 1930 it constituted 10% of the total infections[6]. Hence it is concluded that *P. malariae* focus still exist in Terai area of Uttar Pradesh and we agree with the reports of Beljaev *et al.*[7] that the species should be correctly diagnosed as it is not a rare species and mixed cases are mostly observed in situations of high prevalence of malaria. In context of re-emergence of malaria[8] the diagnostic centres must assess their pathological reports cautiously.

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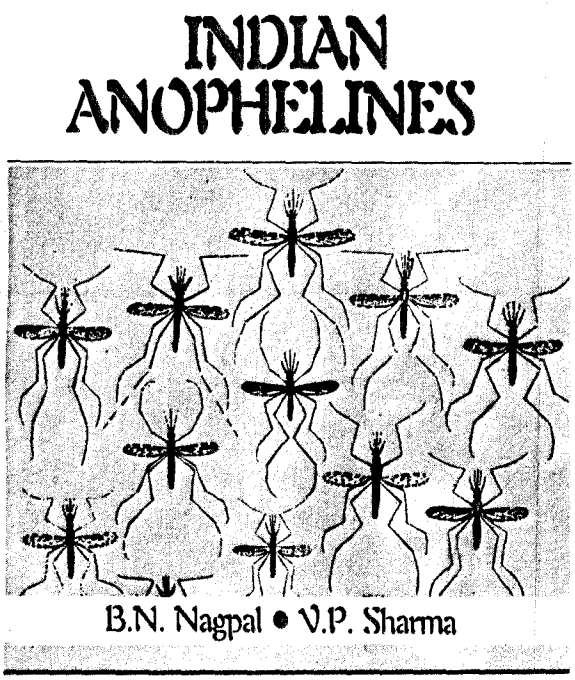

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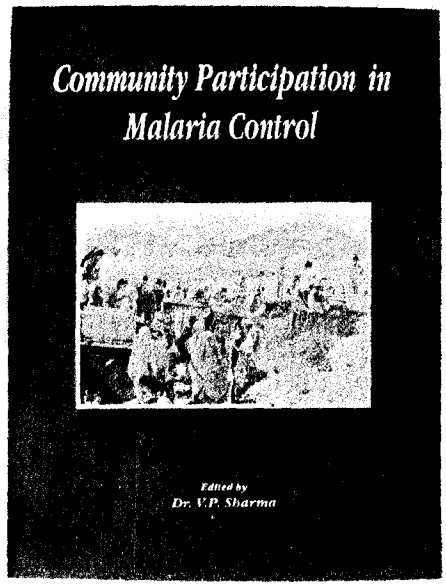

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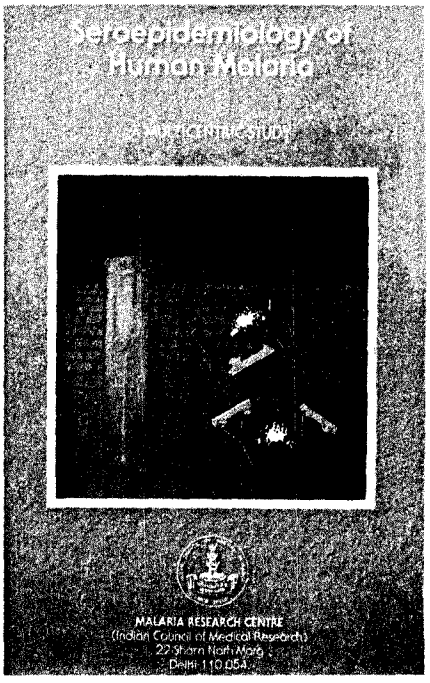

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