

A. Taxonomy

Pictorial Identification Key for Indian Anophelines

The pictorial identification key for 58 species of Indian anophelines has been published. The key was prepared on the request of Defence Research Laboratory, Teipur (Assam) and is meant for researchers, field workers and technicians. The pictorial key comprises an introduction, checklist of Indian anophelines, morphological characters (pictures only) used for identification and guidelines for using the key and pictorial identification. The breeding ecology of each species in brief is also given in the key.

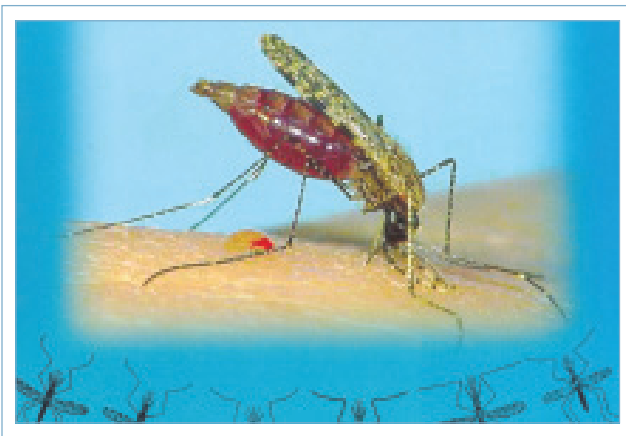


Fig. 1. A mosquito identification key for 58 Indian anophelines

B. VECTOR BIOLOGY

Anopheles culicifacies Complex

Bionomics and Distribution Pattern

An. culicifacies samples collected from Sangrur and Patiala districts (Punjab) were cytologically examined for sibling species composition. There was predominance of species A in Sangrur district and few specimens screened from District Patiala were species B. Examination of *An. culicifacies* population from Betul district (Madhya Pradesh), that witnessed

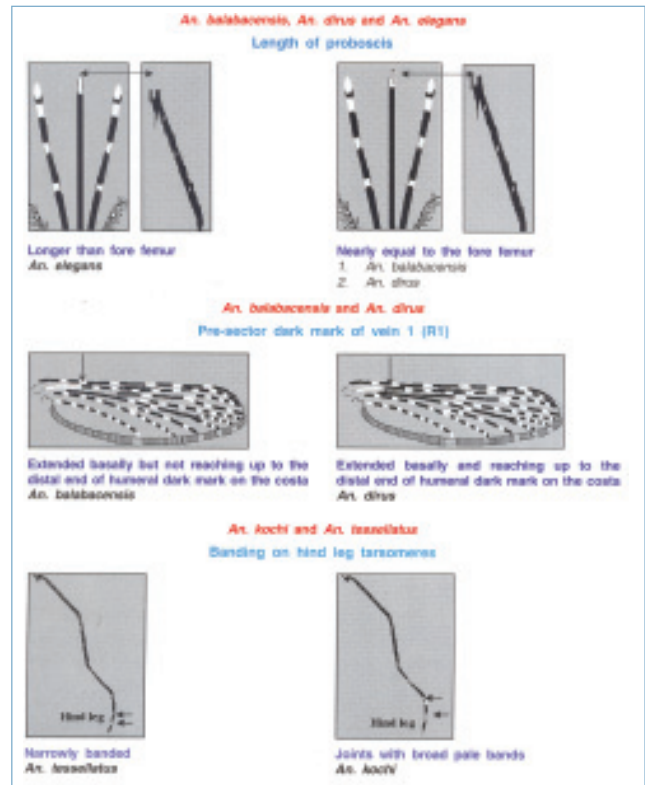


Fig. 2. Mosquito identification is done by nested sequencing through pictorial representation

malaria outbreak in 2000, revealed prevalence of species B and C in the district with predominance of latter. Blood meal source analysis of the cytologically identified specimens using counter current immunoelectrophoresis showed that the *An. culicifacies* sibling species were zoophagic in the above mentioned districts. A longitudinal study on the distribution and bionomics of *An. culicifacies* sibling species in malarious areas of Mandla and Dindori districts (Madhya Pradesh) is being carried

❖ The Pictorial Identification Key for 58 species of Indian anophelines has been published for use by researchers, field workers and technicians





out in collaboration with the Regional Medical Research Centre for Tribals, Jabalpur. Results obtained so far revealed predominance of species C (>80%) in both the districts whereas the relative proportion of species A, B and D was low. These sibling species were found to be primarily zoophagic. The study is in progress.

Studies on Breeding Sites Association of *An. culicifacies* Species A, B and E

A study was carried out in District Mandya of Karnataka state for possible association of *An. culicifacies* species A, B and E larvae to different breeding habitats. Major breeding habitats in the area include tanks, irrigation wells, draw wells, seepage water, riverbed pools and irrigation

channels. Anopheline larvae were collected from breeding habitats and *An. culicifacies* adults emerged were identified to sibling species using AS-PCR assays.

The proportion of breeding of the three sibling species A, B and E varied in the breeding sites examined. The maximum prevalence of species A was in tanks (66%), while species B was found maximum in draw wells and streams (24%), irrigation wells (20%) and riverbed pools (27%), whereas species E was found in riverbed pools (28%), irrigation wells (19%) and tanks (14%). Species A was found in maximum numbers in tanks whereas species B and E are found in different breeding habitats. The data was subjected to statistical analysis to assess sibling species and breeding habitat association. The association of different breeding habitats and sibling species presence was found highly significant ($\chi^2 - 69.04$, $df=16$, $p<0.005$) indicating each sibling species to have specific preference for breeding habitats. The individual sibling species associations with breeding habitats



◆ Association of *An. culicifacies* sibling species A, B, and E to different breeding habitats showed that species B and E have common association and species A showed variation in preference

suggests that among the breeding sites where the sibling species was found there was no specific preference and was found non-significant. Also it was significant for species A and E ($\chi^2 - 60.4$, $df=8$, $p<0.005$), A versus B ($\chi^2-62.8$, $df=8$, $p<0.005$) and A versus B and E ($\chi^2-60.4$, $df=8$, $p<0.005$). Thus, this preliminary study indicated that B and E have common association for breeding habitats while other species have shown variations in breeding.

Anopheles fluviatilis Complex

Distribution, Bionomics and Biology of Sibling Species

Mapping the geographical distribution of *An. fluviatilis* sibling species continued. Samples examined from Dindori and Mandla districts (Madhya Pradesh) revealed prevalence of species S and T in study villages. There was predominance of species T which was found to be totally zoophagic. The proportion of species S was very low thereby indicating that *An. fluviatilis* has limited role in malaria transmission in these districts.

Consequent to the discovery of new species in *An. fluviatilis* complex, a longitudinal study has been initiated on the bionomics of species V and its role in malaria transmission in District Hardwar (Uttaranchal). Three villages namely Dargahpur, Purwala and Auspur have been selected in malarious Laksar PHC and monthly entomological and parasitological surveys are being carried out in these villages to study various parameters like seasonal prevalence, resting and feeding behaviour, vector potential, etc. with respect to new species. Observations made so far revealed that more than 70% of species V population was found resting in human and mixed dwellings thereby having greater chance to come in contact with humans. Blood meal source analysis using counter current immunoelectrophoresis showed species V with anthropophilic index (AI) around 4% whereas the other two sympatric species (T and U) were found to

be totally zoophagic. It is noteworthy to mention that so far only species B, a non vector of *An. culicifacies* complex has been found in study villages along with *An. fluviatilis* sibling species. Therefore, ascertaining the role of species V in malaria transmission assumes greater importance in order to resolve the epidemiological paradox in this area. Efforts are also being made for laboratory colonisation of species V to study its phylogenetic relationship with other sibling species by cross-mating experiments and its susceptibility to plasmodial infection under laboratory conditions.

Molecular Assay for the Differentiation of Members of the *An. fluviatilis* Complex

Identification of sibling species is important in any vector control programme as they differ in biological characteristics such as vectorial competence, host preference or response to insecticides. *Anopheles fluviatilis*, which is second most important malaria vector in India, has now been recognised as a complex of four sibling species— species S, T, U and recently discovered species V. Earlier MRC has reported a molecular technique (PCR) for the

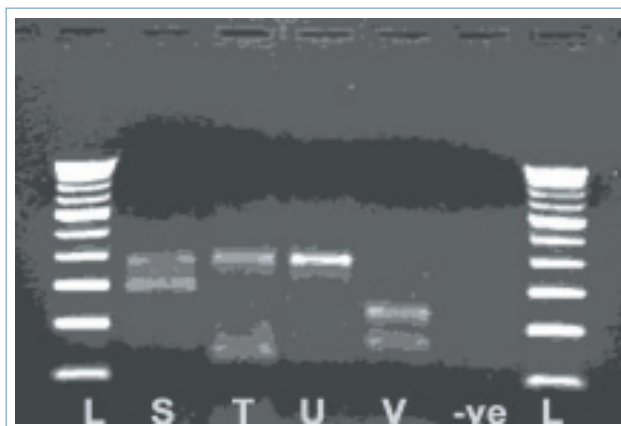


Fig. 3. PCR-RFLP assay for the identification of members of *An. fluviatilis* complex. (L= 100bp ladder; S= species S; T= species T; U= species U; V= species V; -ve= negative control)

differentiation of three known members of the complex, species S, T and U. Due to recognition of new species V, the existing species-diagnostic PCR

◆ **Predominance of species T in Dindori district (M.P.) suggests limited role of *An. fluviatilis* complex in malaria transmission in this area**

◆ ***An. fluviatilis* species V which was found resting in human and mixed dwellings showed an Anthropophilic index of 4% indicating its importance in malaria transmission**

was modified into PCR-RFLP which can differentiate all four members of the complex. The assay was validated by comparing the results of with that of cytological method of species identification based on species-specific diagnostic inversions found in polytene chromosome. Over 500 specimens of *An. fluviatilis* were assayed with PCR-RFLP, out of which 98 samples were validated by comparing results with that of cytological technique. The PCR-RFLP unambiguously differentiates all the members of the complex and is ready for use in research laboratories/vector control programme.

C. Vector Control

Bio-efficacy of New Vector Control Agents

Efficacy of Vectron® 20 WP against Mosquitoes

Vectron® 20 W.P (etofenprox) was evaluated for its indoor residual efficacy in Shapur and Khandera villages of District Ghaziabad (Uttar Pradesh); Basantpur and Agwanpur villages in District Faridabad (Haryana) and Rajeev Nagar and Deep Vihar in NCT area of Delhi. The experimental and control villages were selected on the basis of similar malaria incidence and vector productivity. Different wall surfaces such as brick, mud, cement and thatch walls were sprayed with Vectron® @ 0.05, 0.1 and 0.2 g/m². Results of bioassays tests revealed 100% corrected mortality against *An. culicifacies*, *An. stephensi* and *Cx. quinquefasciatus* @ 0.2 g/m² on different wall surfaces. Persistence of the insecticide was directly proportional to the dosages used. Field evaluation revealed drastic reduction in mosquito densities (MHD) after the spray and the impact was noticed up to a period of six months in the sprayed villages (Figs. 4–6). The results of the trial indicated that IRS of Vectron® 20 WP @ 0.1 g/m² can be used for malaria control and @ 0.2 g/m² can be used for comprehensive vector control.

❖ **A PCR-based assay was developed for the differentiation of all 4 members of the *An. fluviatilis* complex. The assay was field validated using classical cytotaxonomy and is ready for application in the field**

Bio-efficacy of Olyset® Nets Against Mosquitoes in Beel Akbar Village, District Ghaziabad (U.P.), India

Cone bioassays were performed by exposing 3-

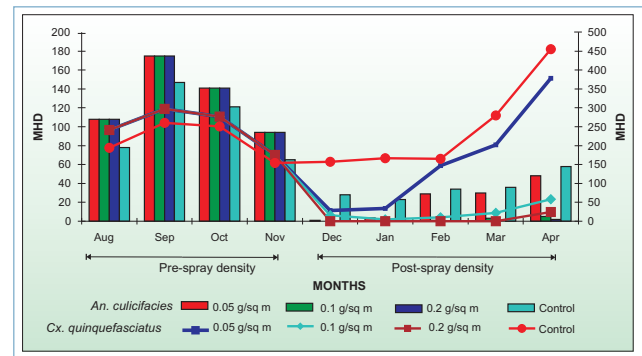


Fig. 4. Man hour densities of *An. culicifacies* and *Cx. quinquefasciatus* in experimental and control areas in District Ghaziabad

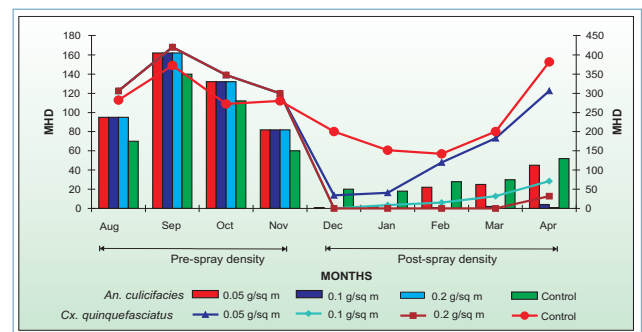


Fig. 5. Man hour densities of *An. culicifacies* and *Cx. quinquefasciatus* in experimental and control areas in District Faridabad

day old fulfed mosquito species such as *An. culicifacies*, *An. stephensi*, *An. subpictus*, *Ae. aegypti* and *Cx. quinquefasciatus* at fortnightly intervals to test the efficacy of Olyset® nets. Results revealed variable degree of susceptibility of different species of mosquitoes. Cent percent mortality was observed in field collected *An. culicifacies*, *An. stephensi* and *An. subpictus* in three minutes exposure time, while only 36% mortality was observed in case of *Cx. quinquefasciatus* in three minute exposure, however, 100% mortality was observed against this species when exposure period was extended up to 30

❖ **Vectron® 20 WP spray @ 0.2 g/m² resulted in drastic reduction in mosquito densities and the impact was observed up to six months**

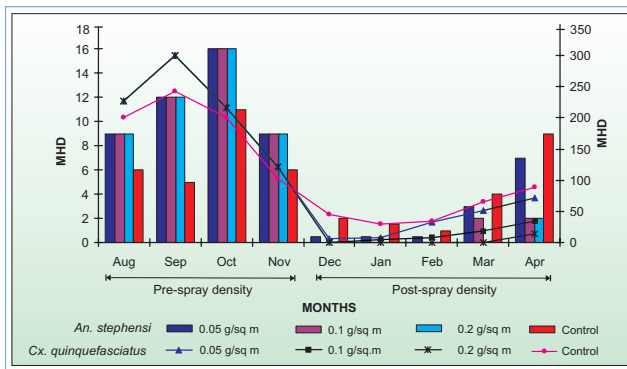


Fig. 6. Man hour densities of *An. stephensi* and *Cx. quinquefasciatus* in experimental and control areas in MCD area of Delhi

minutes, while 100% mortality in *Ae. aegypti* was obtained at five minutes exposure.

Field Evaluation

Results of field evaluation revealed that the average MHD of *An. culicifacies* in structures having Olyset® nets drastically reduced in comparison to that in structures where plain nets were used. The percent reduction in *An. culicifacies* density was 94% based on density in control and 47.2% in case of *Cx. quinquefasciatus*. It was further revealed that landing rate of female mosquitoes on Olyset® nets was drastically reduced and 100% corrected mortality was observed in those mosquitoes which landed on the Olyset® nets (Fig. 7).

Repellent action and excito repellent action of Olyset® nets are presented in Fig. 8. Results revealed



that Olyset® nets produced strong repellent action and it was more pronounced in *An. culicifacies* as compared to total anophelines and *Cx. quinquefasciatus*. The repellent action of the Olyset® nets was 55.2% in *An. culicifacies* as against 38.6%

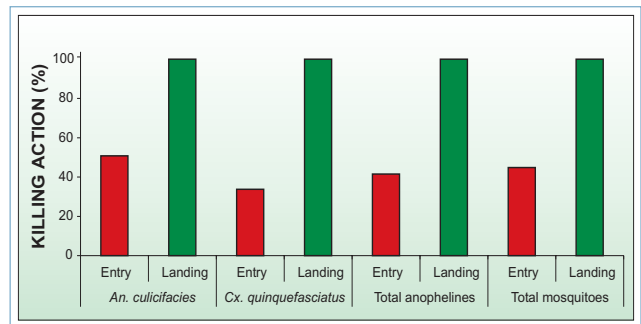


Fig. 7. Average killing action of Olyset® nets against different mosquitoes in field conditions

in *Cx. quinquefasciatus*. Results also revealed that excito repellency action (ERA) was almost 100% against all the mosquito species over a period of six months. Pilot studies are indicated to evaluate its impact on vector borne diseases particularly malaria and its cost-effectiveness in comparison to conventional indoor residual spraying.

Phase II Evaluation of Gokilaht®-S 5EC (Cyphenothrin) Space Spraying against Mosquitoes

Laboratory bioefficacy studies were carried out in a mosquito-free room against *An. culicifacies*, *An.*

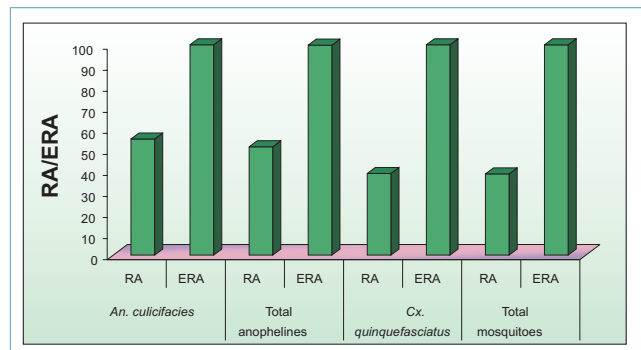


Fig. 8. Average repellent (RA) and excito repellent action (ERA) of Olyset® nets on different mosquito species in field conditions

stephensi, *Cx. quinquefasciatus* and *Ae. aegypti*. About 25 mosquitoes were released into cages of 12 cm³. Four cages were kept in different corners of the room prior to fogging. Fogging was done with the help of Vanfog machine for one minute with the selected dose dissolved in kerosene. For control only kerosene was used. After 30 minutes, mosquitoes

◆ **Olyset® nets showed high repellency and excito-repellency action against different mosquito species**

knocked-down in the cages were scored and immediate mortality, if any, was recorded. Collected mosquitoes were kept for 24 h observation to record delayed mortality. Results showed that 100% mortality was observed @ 0.5 mg/m³ in *An. stephensi*, *An. culicifacies* and *Cx. quinquefasciatus* and only 86.5% in *Aedes aegypti*, whereas 100% mortality was observed in all the four species @ 1.0 mg/m³.

Field evaluation was carried out in selected urban localities of Ghaziabad, Faridabad and Delhi. Two doses of Gokilaht®-S 5EC—1.0 and 0.5 mg/m³ were used for indoor evaluation and 1 and 3.5 g/ha were used for outdoor evaluation. Twenty-five female mosquitoes were exposed in separate cylindrical cages (12 X 18 cm) made of galvanised wire mesh of 20-mesh size. These were kept in different structures both in control and experimental areas prior to fogging. Cages were placed at different heights. All cages were collected after 30 minutes of fogging and brought to the laboratory. Total number of target and non-target species knocked-down during the indoor fogging was also collected from each structure. Mortality of the mosquitoes and non-target species were recorded after 24 h.

Results of indoor evaluation revealed that 100% mortality was recorded in *An. culicifacies* in Ghaziabad and Faridabad, and *An. stephensi* in Delhi @ 1.0 mg/m³ and *Cx. quinquefasciatus* in all the three localities at this dose. However, in case of

Ae. aegypti the average mortality was 99.5%. Almost similar results were obtained in outdoor evaluation against the test species in all the three study areas and the results demonstrated that 3.5 mg/m² is highly effective than 1.0 mg/m² in outdoor conditions. The results clearly indicate that



Gokilaht-S 5EC is effective against *An. culicifacies* and *An. stephensi* @ 0.1 g/m³ in indoors and 3.5 g/ha outdoors.

Efficacy and Persistence of Dimilin GR-2 (2% Granule formulation), 25% WP and Dimilin TB-2 (2% Tablet formulation) for the Control of Mosquito Larvae under Clear and Polluted Water Conditions

Dimilin GR-2, 25%WP and TB-2 formulations were evaluated in laboratory conditions against III instar larvae of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*. Results revealed high degree of larvicidal activity of three formulations of Dimilin and inhibition of the development of pupae and adult mosquitoes at lower dosages. Cumulative mean percent mortality of larvae at different intervals was by and large directly proportional to the dosage. Complete inhibition of emergence of *An. stephensi* and *Ae. aegypti* was observed at 0.008 ppm up to five and six weeks respectively. EC₅₀ and EC₉₀ values for Dimilin 25 WP, GR-2 and TB-2 were 0.0012 & 0.0026; 0.0014 & 0.0095 and 0.0014 & 0.0052 ppm against *Cx. quinquefasciatus*; 0.0015 & 0.0052, 0.0018 & 0.0072 and 0.0013 & 0.0045 ppm against *Ae. aegypti*; and 0.001 & 0.0034, 0.0012 & 0.0061 and 0.0012 & 0.0035 ppm against *An. stephensi* respectively. Field trials are still in progress.

Evaluation of VectoBac® WT, a Tablet formulation of *Bacillus thuringiensis* var. *israelensis* H-14 against Larvae of Mosquito Vectors at three different sites in Delhi, Chennai (Tamil Nadu) and Nadiad (Gujarat)

A multicentric trial of VectoBac, a tablet formulation of *Bacillus thuringiensis* var. *israelensis*, strain AM 65-52 having ITU 3000/mg was completed this year at three sites— Delhi, Nadiad and Chennai. The efficacy of VectoBac tablets used at different doses against two mosquito species, which were found to be breeding in a variety of habitats, showed that application of 2 tablets (0.76 g)/m² of water surface area resulted in very high to

- ◆ **Gokilaht® space spraying was found highly effective @1mg/m³ in indoors and 3.5 g/ha in outdoors**

complete control of late instars and pupae of *An. stephensi* and *An. subpictus* up to two weeks period. Similarly, with a dose of 2 tablets/habitat caused cent percent reduction of late instars and pupae up to two weeks period against *Ae. aegypti* and *Cx. quinquefasciatus* in desert coolers, iron drums and mud pots. Application of 1–2 VectoBac tablets/50L water in domestic containers with mainly *Ae. aegypti* breeding caused high control of larvae up to nine days.

An important factor in using VectoBac tablet was the ease of its application in the containers, particularly those which generally remain inaccessible. The tablet formulation was found to be very useful particularly in desert coolers which are the main breeding sites for *Ae. aegypti* in urban areas. High rise buildings in urban areas, where a large number of desert room coolers are found fixed on the windows, VectoBac tablets will be very suitable for mosquito control during summer and monsoon. Moderate alkalinity of water in certain tanks did not have any significant adverse effect on the bio-efficacy of the tablet formulation. VectoBac tablets were found to be safe against non-target species, such as larvorous fishes (*Gambusia affinis*) and notonectid bugs, *Anisops* spp. VectoBac tablet formulation would be very useful specially in water storage tanks and desert coolers for the control of *An. stephensi* in tanks and *Ae. aegypti* in coolers.

Evaluation of VectoBac® WDG, a Granular formulation of *B. thuringiensis* var. *israelensis* H-14 against Larvae of Mosquito Vectors

Trials in Delhi

Application of VectoBac WDG in paddy fields against breeding of *Anopheles* spp. mainly *An. culicifacies* gave high reduction in larval densities for one week at the doses of 0.5 and 1.0 g/m². Against *An. stephensi* breeding in cement tanks, there was 100% control up to one week with all doses applied. Efficacy was >77% at the doses of 0.2, 0.5 and 1.0 g/m² up to three week period. Against *Cx. quinquefasciatus* breeding in pools in vacant

residential plots, the reduction of late instar larvae was 100% at the dose of 0.5 and 1.0 g/m² up to one week period.

Trials in Nadiad

VectoBac WDG was highly effective (80.2–100%) up to three weeks when applied at 0.5 and 1.0 g/m² doses in industrial tanks against late instars of *An. stephensi* and *Culex* breeding in industrial cement tanks and fabrication units. Treatment of domestic tanks with VectoBac WDG formulation gave 100% control of *Ae. aegypti* larvae up to one week. Application in rice-fields and waste water pools gave >80% control of anophelines and *Cx. quinquefasciatus* breeding at all four dosages up to one week and >75% control up to three weeks.

Trials in Bangalore

In stone quarry pits the efficacy of VectoBac WDG applied at 0.2 g and 1.0 g against mixed breeding of anophelines (mainly *An. culicifacies*) and culicines lasted up to three weeks. There was 96.2–100% control of breeding of *Culex* and *Aedes* species up to one week when applied at the dose of 0.2 g/m². The effectiveness of VectoBac WDG in ring well against *An. stephensi* larvae was 95.6–100% for one week period. VectoBac WDG was found safe to non-target species, *G. affinis* and notonectid bugs, *Anisops*.

Thus it is concluded that VectoBac WDG showed moderate to high control of mosquito larvae for 1–3 weeks in different situations when applied at the rate of 0.5 – 1.0 g/m². However, at lower doses varying degree of control was observed in different habitats and against different species. As the formulation had no adverse impact on non-target organisms it can be used as an additional tool in an integrated control approach.

Evaluation of Pyriproxyfen (Sumilarv 0.5% Granule) on Larvae of Mosquito Vectors at Nadiad (Gujarat), Haldwani (Uttaranchal) and Shahjahanpur (Uttar Pradesh)

Pyriproxyfen (Sumilarv 0.5G) (S-71639), a juvenile hormone mimic (JHM), was field tested at three

◆ **Dimilin GR-2, 25% WP and TB-2 formulations showed high degree of larvicidal activity against mosquitoes at low dosages**

◆ **VectoBac tablets were found to be useful in controlling mosquito breeding in storage tanks and desert coolers**



different sites. Pyriproxyfen was evaluated in different breeding habitats against various mosquito species—*An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* at Nadiad (Gujarat), Haldwani (Uttaranchal) and Shahjahanpur (Uttar Pradesh).

Based on the evaluation, following conclusions were made:

- Pyriproxyfen applied at 0.01 to 0.05 ppm a.i. caused produced 100% inhibition of adult emergence of *An. stephensi* and *Cx. quinquefasciatus* IV instar larvae for up to four weeks under laboratory conditions.
- Pyriproxyfen was found to be quite effective against malaria vector *An. culicifacies* even at the lowest dose of 0.01 ppm a.i. and inhibited 100% adult emergence up to four weeks equally at 0.01 and 0.02 ppm a.i. doses.
- In heavily polluted habitats against *Cx. quinquefasciatus* the same formulation at 0.01 to 0.05 ppm a.i. dose produced 100% inhibition of adult emergence for 1–6 weeks in a variety of habitats in different field trial sites.
- Under field conditions, application of pyriproxyfen at different doses, reduced the larval and pupal abundance in various habitats for a significantly longer duration. Its application also controlled *Ae. aegypti* breeding in domestic water storage tanks for an appreciable time although efficacy varied

against mosquitoes particularly against malaria vectors was continued in collaboration with other institutes. The study is focused to test larvicidal, insecticidal and mosquito repellent properties of the plant extracts/fractions/formulations against *An. stephensi*. Bioactivity of various herbal extracts/fractions/formulations received from five extracting laboratories was determined against mosquitoes particularly the malaria vector *An. stephensi* using standard protocol which included larvicidal, adulticidal and mosquito repellent activities. Preliminary screening of plant extracts was done at 250 ppm. The sample was considered to have larvicidal activity if it caused > 70% larval mortality within 24 hours of exposure. The samples causing 70–100% larval mortality in preliminary bioassays at a concentration of 250 ppm were tested further for determining LC₅₀ and LC₉₀ values.

Adulticidal activity was determined by exposing adult mosquitoes on impregnated paper with 10% solution (2.5 ml/paper of 180 cm² or 0.25 g/paper of 180 cm²), equivalent to dose of 1.38 mg/cm². Samples giving 70–100% mortality were considered as positive for adulticidal activity. Repellent activity was determined by applying 0.25 g sample mixed with equal amount of coconut oil on hands. The sample was considered to have repellent activity if no confirmed bite was received within one hour of exposure. The positive control, DEET solution 0.25 ml when used gave complete repellent activity for more than three hours.

Since beginning of this project a total of 625 coded samples of different plant extracts/fractions/formulations have been received at MRC for bioassays against mosquito species *An. stephensi* (Table 2).

Of these 99 samples showed larvicidal activity, 29 showed adulticidal activity and five showed repellent activity. However, during last one year 294 samples were tested. Of these 51 samples showed larvicidal activity and 15 samples showed adulticidal activity.

Table 1. Habitat-wise effective doses of pyriproxyfen

Breeding habitats	Target species	Effective dose range
Waste water pools	<i>Cx. quinquefasciatus</i>	0.25 kg/ha
Sewage drains	<i>Cx. quinquefasciatus</i>	0.4-1 ppm a.i.
Domestic ditches, pits	<i>Cx. quinquefasciatus</i>	0.05-0.2 ppm a.i.
Disused wells	<i>Culex</i> spp	0.25-0.5 kg/ha
River-bed pools, clean	<i>An. culicifacies</i>	0.2 ppm a.i.
Tyres with water	<i>Aedes</i> spp	0.02 ppm a.i.
Domestic cemented tanks	<i>An. stephensi</i> , <i>Ae. aegypti</i>	0.1 ppm a.i.
Industrial curing tanks	<i>An. stephensi</i>	0.1 ppm a.i.

according to the use of water from such tanks.

- No adverse effects against non-target organisms were observed during the study period.

Prospecting for Botanical Pesticides: Screening of Plant Extracts for Insecticidal and Repellent Activity against *An. stephensi*

Screening of plant extracts for their bioactivity

◆ **Pyriproxyfen (Sumilarv) was found very useful in the control of mosquito larvae in different breeding habitats**

Larvicidal Activity of *Zanthoxylum alatum* against *An. stephensi*

This study was undertaken in collaboration with IIT, Delhi. Essential oil from *Zanthoxylum armatum*, DC syn. *Z. alatum* Roxb (timur) an evergreen tree in the subtropic Himalayas, was extracted from its seeds using hydrodistillation and liquid CO₂ technique. Hydrodistillation gave 0.8% of oil (v/w) gas chromatographic analysis of the hydrodistilled oil resulted in the identification of 20 constituents, as compared to the 24 constituents by liquid CO₂ extraction technique. Linalool, limonene, terpene-4-

species A and C, *An. stephensi*, *Cx. quinquefasciatus* III/IV instars larvae in bioassays following standard WHO method for a range of concentrations (0.0025 to 0.3% in water). The calculated LC₅₀ values (lethal concentration for killing 50% of treated larvae) for different species were: *An. culicifacies* species A–0.022%, *An. culicifacies* species C–0.028%, *An. stephensi* –0.023% and *Cx. quinquefasciatus* –0.017%. Studies will be carried out with different solvent extracts of different plant parts.

Biocontrol Agents

Effect of Formulation and Encapsulation on the Efficacy of Microbes against Mosquito Larvae

The bio-efficacy of three *Metarhizium anisopliae* strains MRCD-1, MRCD-2 and MRCD-3 was tested against II instar larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* (Table 3). The spores of *M. anisopliae* after 21 days growth in Emerson’s YpSs medium were tested against the larvae. MRCD-3 was found relatively more effective than the other two strains and will be formulated to assess the effectiveness against different mosquito species.

Table 2. Status of screening of of plant extracts/fractions/formulations of samples received at MRC

Laboratory/Institute	No. of samples received	No. of samples screened	Positive results		
			L	A	R
RRL, Trivandrum	105	105	8	3	1
FRI, Dehradun	103	103	26	16	4
IIT, Delhi	101	101	12	2	-
RRL, Jammu	170	170	28	6	-
EID Parry, Bangalore	146	146	25	2	-
Total	625	625	99	29	5

L– Larvicidal; A–Adulticidal; R–Repellent activity.

ol, phellandrene and (Z)-methylcinnamate were the major components obtained from hydrodistillation method, whereas liquid CO₂ showed Linalool, limonene, methylcinnamate, palmitic acid and oleic acid as the major components. The essential oils obtained from both the techniques were tested for their larvicidal activity against *An. stephensi*. The essential oil obtained by using hydrodistillation technique was found to show better larvicidal activity than that obtained from liquid CO₂ extraction technique. These oils were fractioned to obtain linalool which is the major component of the essential oils and tested for the larvicidal activity. The activity of linalool against the larvae of *An. stephensi* was negligible which indicates that the activity shown by the essential oils is due to the presence of some other active component.

Studies on Larvicidal Properties of Aqueous Leaf Extract of *Trianthema portulacastrum* (Family : Aizoaceae)

Larvicidal effect of crude aqueous extract of the leaf of a medicinally important plant *Trianthema portulacastrum* was assessed against *An. culicifacies*



◆ The essential oil obtained from *Z. alatum* using hydrodistillation showed better larvicidal activity than that obtained from liquid CO₂ extraction



Bio-efficacy of Culture Filtrates of *M. anisopliae* against *An. stephensi* and *Cx. quinquefasciatus*

Toxicity of cell-free culture filtrates of *M. anisopliae* (MRCD-1) was tested against larvae of *An. stephensi* and *Cx. quinquefasciatus*. Metabolites derived from the culture broth of Emerson's YpSs and chitin broth after 21 days of growth were tested. The metabolites derived from the chitin broth were relatively more effective than those from the Emerson's YpSs broth. The respective calculated LC₅₀ values for different species are given in Table 4. The specific activity of chitinase from the dialysed culture filtrate of chitin broth was 6-fold more than the chitinase activity observed in dialysed Emerson's YpSs broth (Fig. 9).

of serine protease (AcSp30 accession No. AY995188) and prophenol oxidase (AcPPO6A accession No. AF466196) gene from refractory strains of malaria vector *An. culicifacies*. Both the semi-qualitative and quantitative RT-PCR studies showed inherently higher levels of both these genes in refractory strains as compared to susceptible strains. To characterise the role of serine protease and prophenoloxidase in mounting immune response, the expression pattern of both the genes in adult naïve female refractory mosquitoes was temporally studied following injury and bacterial infection (Fig. 10). The mosquitoes were injured using a capillary glass needle and infected with gram-positive bacteria, *Micrococcus luteus*. The

Table 3. LC₅₀ of *M. anisopliae* strains in spore/ml against II instar larvae of three mosquito genera after 96 hours of inoculation

Name of strains	<i>An. stephensi</i>	<i>Cx. quinquefasciatus</i>	<i>Ae. aegypti</i>
<i>M. anisopliae</i> MRCD-1	6.41 X 10 ⁷ (5.10 X 10 ⁷ – 9.09 X 10 ⁷)	6.91 X 10 ³ (5.16 X 10 ³ – 3.62 X 10 ⁴)	4.12 X 10 ⁴ (2.54 X 10 ⁴ –1.06 X 10 ⁵)
<i>M. anisopliae</i> MRCD-2	1.05 X 10 ¹³ (1.03 X 10 ¹³ –1.42 X 10 ¹³)	2.03 X 10 ⁹ (1.87 X 10 ⁹ –2.90 X 10 ⁹)	6.91 X 10 ¹² (6.69 X 10 ¹² –9.22 X 10 ¹²)
<i>M. anisopliae</i> MRCD-3	2.69 X 10 ³ (2.58 X 10 ³ – 2.80 X 10 ³)	1.34 X 10 ³ (1.25 X 10 ³ – 1.51 X 10 ³)	4.66 X 10 ⁵ (3.45 X 10 ⁵ – 7.39 X 10 ⁵)

Figures in parentheses are 95% fiducial limits.

Further study is in progress to know the biochemical role of chitinase and other proteases in causing mortality.

Vector-Parasite Interactions

Studies on *P. vivax*-refractory *An. culicifacies*

Expression Profiles of Serine Protease and Prophenol Oxidase following injury and bacterial infection

Earlier, we reported cloning and characterisation

mosquitoes were collected at different time intervals and the levels were analysed by realtime PCR. Upto 2.5-fold increase in AcSp30 transcript was observed upon injury in contrast to a 4-fold increase in AcPPO6A transcript under identical conditions (Fig. 10 a & b respectively). The observed increase in the transcript levels of the AcSp30 was maintained throughout the 24-hour duration. A marginal increase in AcSp30 transcript was observed upon challenge with *Micrococcus luteus*. The transcript levels of AcSp30 were much lower than those compared to the levels obtained after sterile injury

Table 4. LC₅₀ of culture filtrates of *M. anisopliae* against I-II & III-IV instars of *An. stephensi* and *Cx. quinquefasciatus* after 72 hours of inoculation

Name of the species	Instar	YpSs µl/ml (95% FL)	Chitin µl/ml (95% FL)	Relative potency (folds)
<i>An. stephensi</i>	I & II	7.23 (6.26–8.15)	3.94 (2.69–5.10)	1.83
	III & IV	5.23 (4.46–6.07)	5.62 (4.31–6.89)	0.93
<i>Cx. quinquefasciatus</i>	I & II	8.15 (7.20–9.06)	2.92 (1.49–4.32)	2.79
	III & IV	41.83 (31.91–59.22)	7.15 (5.49– 8.76)	5.85

Figures in parentheses are 95% fiducial limits.

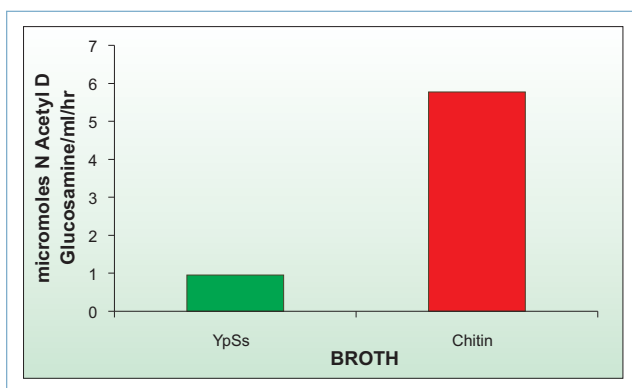


Fig. 9. Specific activity of chitinase present in culture filtrates of *M. anisopliae*

indicating that this serine protease is not involved in triggering the cascade for bacterial elimination. Upon injury, an increase in the *AcPPO6A* transcript levels was observed after two hours (2-fold) and continued to increase up to 12 hours (4-fold) and finally declined to the basal level in 24 hours. The modulation of PPO upon injury is suggestive of its role in wound healing. A temporally divergent regulation of PPO transcription was observed in mosquitoes challenged with gram-positive bacteria *M. luteus*. Interestingly, a 5-fold increase in *AcPPO6A* transcript was observed immediately after two hours of infection and maximum levels were attained six hours post-challenge (9-fold increase). Such a rapid induction of the *AcPPO6A* gene in response to bacterial infection is suggestive of some role in combating bacterial invasion.

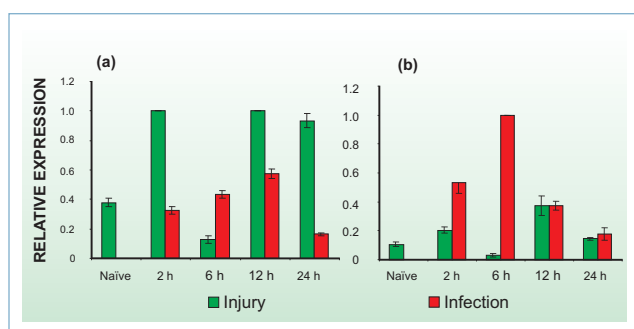


Fig. 10. *AcSp30* and *AcPPO6A* expression profiles following injury and bacterial infection

Refractory adult female mosquitoes were subjected to sterile injury using finely drawn glass capillary needle and to infection with gram-positive bacterium, *Micrococcus luteus* using a needle dipped in bacterial cell pellet. *AcSp30* (a) and *AcPPO6A* (b) transcript levels were measured at different time intervals (2, 6, 12 and 24 hours) post-injury (open bars) or post-infection (shaded bars) by real-time PCR using the Comparative CT Method and were normalised to the internal control transcript for β -actin gene. The data (mean \pm SD) are from three independent experiments.

Immune Responses to *Plasmodium* Infection in Refractory and Susceptible Mosquito Strains

Further the role of the genes in encapsulation phenotype of the refractory strain was investigated by feeding model rodent malaria parasite *P. vinckei petteri*, using susceptible strain as control (Fig. 11). Both, refractory and susceptible 4–6 day old adult female mosquitoes were separately fed on blood of Balb/c mice, which had been infected with *P. vinckei petteri*. The refractory *An. culicifacies* mosquito strain is partially resistant to the rodent malarial parasite. Mosquitoes fed on un-infected mice served as blood-fed controls. Temporal expression of both the genes was monitored after blood meal, at regular intervals over a period of 24 hours by real-time PCR (Fig. 11). In the refractory strain, although a 68-fold increase in *AcSp30* transcript was observed 24 hours post-blood meal, the invasion of *Plasmodium* resulted in a 300-fold increase in the transcript levels within the same time duration, when compared to transcript levels in naïve unfed female mosquitoes. Similarly, a significant up-regulation of *AcPPO6A* transcript levels was observed in response to parasite invasion. A 22-fold increase was observed in the expression levels of *AcPPO6A*, 6 hours after infective feeding as compared to blood feeding alone (16-fold). These levels almost doubled after 18 hours of infected blood meal thereby demonstrating the induction of *AcPPO6A* in response to parasite. This result is in conjunction with the microscopic observation wherein melanotic encapsulation of *Plasmodium* ookinetes was observed 16 to 24 hours post, infective blood feeding. On the other hand, in the susceptible strain, the expression levels of the *AcSp30* gene remained at the basal level upon blood feeding and parasite-



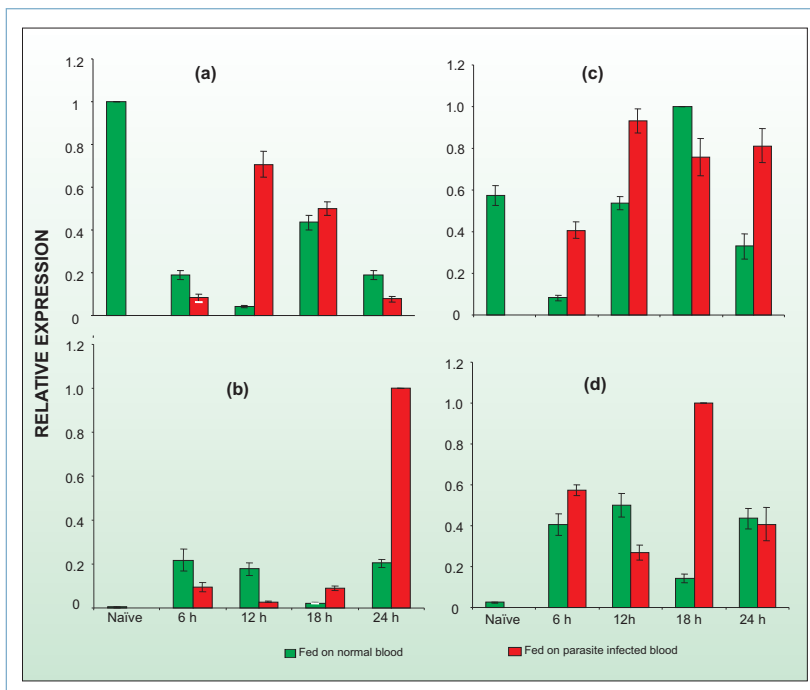


Fig. 11. Temporal induction of *AcSp30* and *AcPPO6A* upon *Plasmodium* infection Susceptible (S) and refractory (R) mosquitoes were fed on uninfected blood (open bars) and on *Plasmodium vinckei* (shaded bars) infected blood and transcript levels of *AcSp30* in S strain (a) and R strain (b) were measured by real-time PCR at different time intervals post-blood meal (PBM). Transcript abundance of *AcPPO6A* was similarly determined in S strain (c) and R strain (d). Transcript levels were normalised to the internal control transcript for β -actin gene. The data (mean \pm SD) are from three independent experiments

infected blood feeding. Unlike the *AcSp30* gene, the *AcPPO6A* transcript levels in the susceptible strain were insignificantly up regulated (1.4-fold increase), in response to blood and parasite. But these induction levels were substantially lower when compared to the refractory strain.

These results demonstrate the inherent high levels of these genes was not sufficient to combat the invading parasite and a tremendous induction was required to block the development of parasite. Noticeably, the up-regulation of *AcPPO6A* and *AcSp30* transcript levels at 18 and 24 hours post-parasite feeding respectively coincided with the appearance of melanotic capsules in the gut of *An. culicifacies* refractory strain. Such a coordinated response suggests that *AcSp30* and *AcPPO6A* enzymes are part of melanisation cascade that is triggered in response to *Plasmodium*-infected blood. A further validation of this fact is that the non-melanising susceptible strain showed no induction of transcript levels when fed on *Plasmodium*-infected blood.

Differential Expression of Serine Protease Gene: Role of Promotor

Earlier, we have reported differential expression of serine protease gene but no structural difference was observed in the gene. The cDNA corresponding to the *AcSp30* was cloned from R and S strains and sequenced. A comparison of the two sequences did not reveal any mutational differences in the two strains. Since the cDNA sequence of *AcSp30* was identical in both the strains, we isolated its genomic clone using PCR. An 887 bp amplicon was obtained in both the strains. The gDNA fragment was slightly bigger than cDNA indicating the presence of an intron. Sequence analysis revealed that the gene has a single intron of 71 bp at its 5' end and is a phase 0 intron. Since the location and the sequence of the intron were identical in both the strains, the upstream sequences of *AcSp30* were isolated and explored for promoter activity.

The upstream region of serine protease gene is trapped using a set of nested gene specific reverse primers and a set of four forward universal walker primers WP1, WP2, WP3 and WP4, which differ from each other at their 3' end. Their 5' end is identical and comprises of the T7 primer sequence. In refractory strain, a 1.4 kb amplicon was obtained using WP4 and in susceptible strain, a 702 bp amplicon using WP2. A 702 bp fragment and a smaller 333 bp fragment of upstream sequences from both the strains were cloned into promoter-less vector pGL3-Basic (Promega) having a firefly luciferase reporter gene. The resulting reporter plasmids were named pGL3-Ref702 and pGL3-Ref333 for the refractory strain and pGL3-Sus702 and pGL3-Sus333 for the susceptible strain.

To understand the functional relevance of the upstream region a failed attempt was made to record the activity of region by cloning into eukaryotic promoter-less vector which was further sub-cloned into mammalian cell line. The inactivity of promoter region in the cell line was thought to be because of distantly related cell line. Relative strength of the

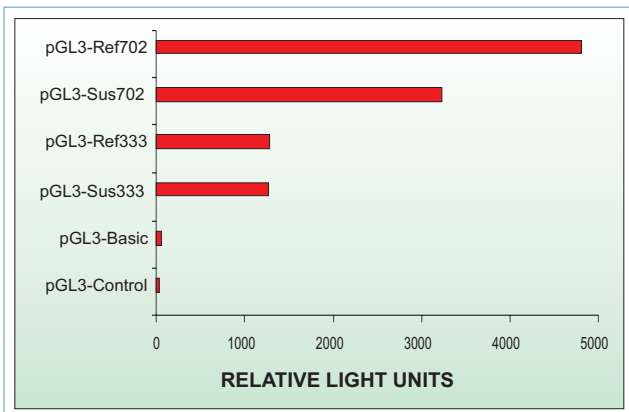


Fig. 12. Assessment of the activity of serine protease promoter from R and S strains of *An. culicifacies* using luciferase-based reporter assay in S2 *Drosophila* cell line. Mean luciferase (\pm S.D.) activities from pGL3-Ref (702 bp and 333 bp) and pGL3-Sus (702 bp and 333 bp) following transfection in the *Drosophila* Schneider (S2) cell line using Lipofectin. These activities were compared to those obtained using promoter-less vector pGL3-Basic and to the pGL3-Control plasmid having the SV40 promoter as controls. Reported activities were based on three independent transfections and the data were recorded in relative light units (RLU)

promoter was evaluated by transfecting these recombinant reporter constructs into *Drosophila* S2 cells. The luciferase activity from all the four constructs was higher than that of the vector (pGL3-Basic) without a promoter and pGL3-Control with an SV-40 based promoter, thereby confirming that these upstream gene sequences, of both the R and the S strains, contain the necessary regulatory elements for their respective promoters (Fig. 12). The two constructs pGL3-Ref333 and pGL3-Sus333 yielded similar levels of luciferase activity. However, pGL3-Ref702 and pGL3-Sus702 displayed difference in luciferase activity. The pGL3-Ref702 from the

refractory strain showed a 1.5-fold increase in luciferase activity compared to the susceptible strain. We can attribute such a differential luciferase activity to differences in the upstream gene sequences between the 333 and the 702 bp regions in both these strains.

The 702 bp upstream sequences obtained from the refractory and susceptible strains were aligned (Fig. 13). A high degree of sequence similarity (94.2%) was observed in the refractory and susceptible sequences up to the 333 bp regions, upstream of the translational start site (ATG). Beyond this region there was a considerable divergence in the gene sequence. This difference could directly account for the observed difference in promoter activity in both these strains (Fig. 13). Analysis of the upstream sequences of serine protease gene revealed characteristics of RNA polymerase II-transcribed promoters.

Using the computer-based promoter prediction tool at <http://www.fruitfly.org/>, two putative transcription start sites were predicted to be located at -31 bp and -40 bp upstream of transcription start

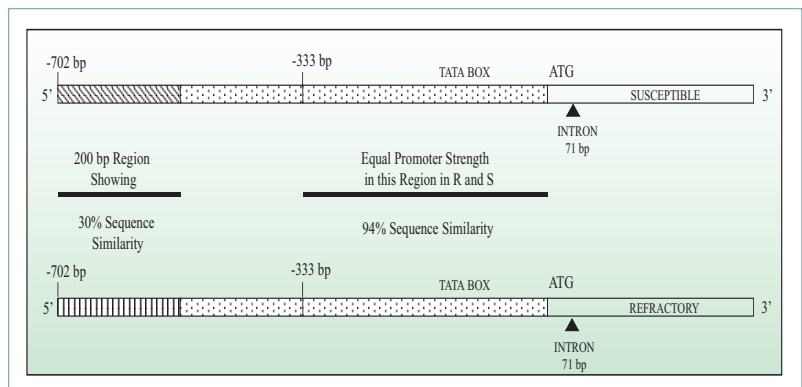


Fig. 13. Schematic diagram depicting the comparative analysis of the *AcSp30* coding and noncoding regions in the refractory and susceptible strain



site in refractory and susceptible strains. The region surrounding this transcription start point corresponded to the arthropod initiator sequence. This reinforced the prediction of this site being the true transcription start site. In both the strains, two

◆ **Serine protease and prophenol oxidase are involved in conferring refractory phenotype to *P. vivax* refractory strain of *An. culicifacies***

TATA motifs were found at position 53 and –189 from the ATG (translation start site), and two arthropod transcription initiator motifs TCAGT were present at position –12 and –152. Using the consensus DCAKTY, two putative capsites were found at position –31 and –152 respectively in both the strains. The sites constituted the putative core promoter elements.

Molecular Characterisation of Nitric Oxide Synthase (NOS) in *An. culicifacies*: Relevance for Refractory Mechanism

Global efforts to control malaria, caused by parasitic protozoa of the genus *Plasmodium*, have been hindered by insecticide resistant mosquitoes, drug-resistant parasites and socioeconomic obstacles. The drive to identify novel control strategies has, in part, focused on identifying mosquito gene products that impart refractory phenotypes. Our aim is to characterise the gene and gene elements that may be associated with NOS biology in *An. culicifacies* sibling species to understand the biochemistry of mosquito-parasite interaction and to evaluate the potential of manipulating AcNOS gene expression as a means of generating the refractory phenotypes. Our goal is to develop tools for altering the vector competence of *An. culicifacies* which requires understanding the mechanism of vector resistance to the malaria parasite including biochemical and molecular studies of vector parasite interactions. In a way that the vertebrate antiparasite immune responses have been used to identify vaccine and transmission blocking targets, we plan to use AcNOS response in mosquito vectors to *Plasmodium* as a tool to explore critical components of parasite development in mosquitoes and correlate it to mechanism of refractoriness.

An. culicifacies sibling species namely A, B were maintained at 27°C and 75% humidity under a 12 h

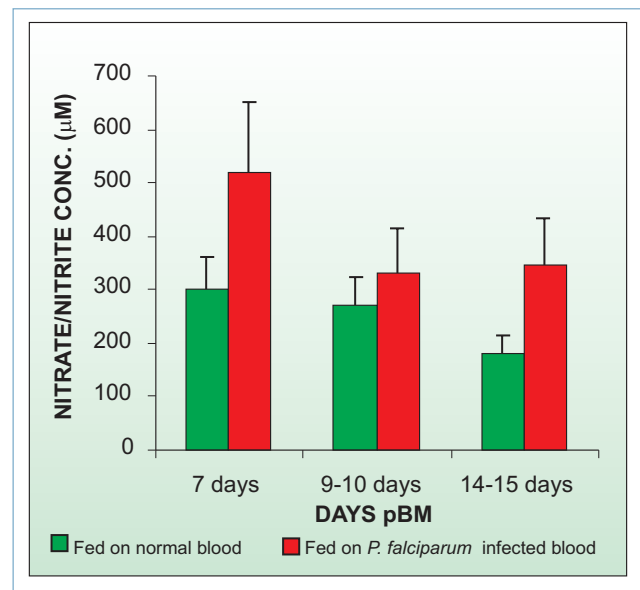


Fig. 14. Hemolymph nitrite/nitrate of blood-fed uninfected and *P. falciparum* infected *An. culicifacies* species B was determined at 7, 9-10 and 14-15 days pBM by using Cadmium reduction/Griess reagent microassay. Means were analysed by using a paired *t*-test. *p*-values are depicted above the bars

light/dark cycle. *An. stephensi* mosquitoes were also treated similarly and were used as controls. Samples (midguts and carcasses without midguts) and haemolymph have been collected from *An. culicifacies* species A and B and *An. stephensi*.

Haemolymph nitrate/nitrite ($\text{NO}_2^-/\text{NO}_3^-$) concentrations were measured using a modified cadmium reduction/griess reagent microassay after collection from the group of 100–200 infected and uninfected mosquitoes at 7, 9–10 and 14–15 days pBM. At day 7 pBM NO levels were higher in *Plasmodium* infected mosquitoes than in uninfected mosquitoes at all time points (Fig. 14). Elevated levels of $\text{NO}_2^-/\text{NO}_3^-$ at this time may be the result of sustained production of AsNOS induction at nine days.

◆ Differences in the promoter sequences of serine protease are responsible for its differential expression in refractory (R) and susceptible (S) strains of *An. culicifacies*