1

Vector Biology and Control

1.1 Vector biology

1.1.1 Computerised key of mosquitoes of India

The information on taxonomy, distribution and biology (breeding habits, biting time, resting habits, etc.) of 270 species of mosquitoes belonging to genus *Anopheles, Culex, Aedes, Mansonia, Armigeres, Toxorhynchites* and *Orthopodomyia* which has been extracted out from the literature is being computerised. Brief biology and distribution maps of 170 species have been completed. Collection of information for rest of the species is in progress.

1.1.2 *Anopheles culicifacies* Complex

Bionomics and distribution pattern

An. culicifaies populations from Chandel, Sarlya, Chikdalyia, Tikri Ghat and Gujar Kheri villages in Khandwa district (Madhya Pradesh) were analysed for sibling species composition and host preference. Results revealed that species A and C, the established vectors of malaria comprised of > 75% of the total *An. culicifacies* population thereby indicating high malariogenic potential of the study villages. A longitudinal study on the bionomics of *An. culicifacies* sibling species in Dindori and Mandla districts (Madhya Pradesh) was continued for the second successive year in collaboration with the Regional Medical Research Centre for Tribals, Jabalpur. Observations revealed predominance of species C (> 80%) in the study villages in all the seasons and confirmed that this species is playing a major role in malaria transmission in both the districts. In all the above mentioned districts, analysis of blood meal source of *An. culicifacies* sibling species using counter current immunoelectrophoresis revealed these to be primarily zoophagic.

1.1.3 *Anopheles fluviatilis* Complex

Distribution, bionomics and biology of sibling species

A longitudinal study on the bionomics of *An. fluviatilis* species V in malarious Laksar PHC of Hardwar district was completed. Results revealed that although species V comprised 20.3% of the total *An. fluviatilis* population, but majority of it (77%) preferred to rest in human and mixed dwellings (Fig. 1.1). The overall anthropophilic index (AI) of species V was 4.13% (Fig. 1.2) whereas the other sympatric species, *An. fluviatilis* species T and U and

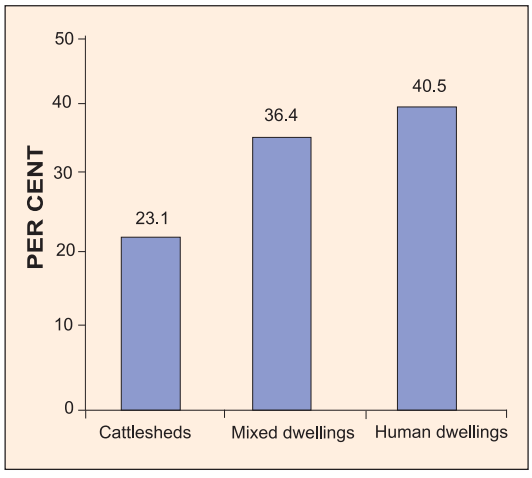

Fig. 1.1: Relative proportion of *An. fluviatilis* species V resting in different types of dwellings in study villages of Laksar PHC (Hardwar)

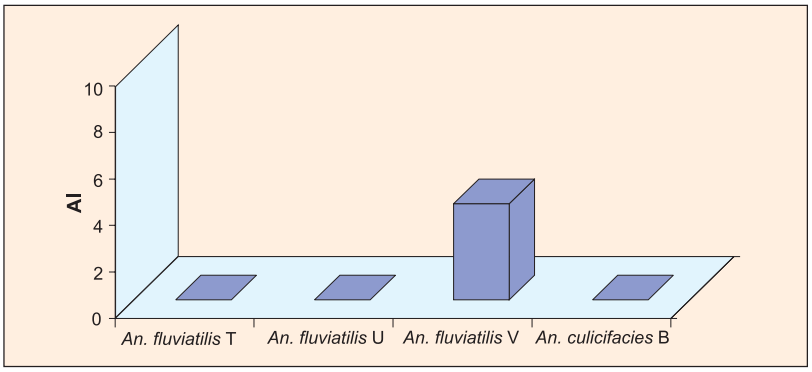

Fig. 1.2: Anthropophilic index (AI) of sibling species in study villages of Laksar PHC (Hardwar)

An. culicifacies species B were found to be totally zoophagic. The proportion of species V was more in the months of October–December when majority of malaria cases were encountered. These observations strongly suggest that species V is playing an important role in malaria transmission in Laksar PHC.

Laboratory rearing experiments were carried out using single female cultures of *An. fluviatilis* species T, U and V. Parameters like number of eggs laid per female, percent hatchability, duration of larval and pupal stages and survival of adult males and females were recorded for each replicate. Observations revealed no significant difference in fecundity, duration of aquatic phase of development and longevity of adults of these sibling species.

Efforts to colonise *An. fluviatilis* species U were successful which is being maintained as a cyclic colony. A study has been initiated to resolve the taxonomic status of members of the Fluviatilis-Minimus group using cytological and molecular tools. Ovaries of *An. minimus* collected from north-eastern states have been processed for polytene chromosome preparations. A photomap of polytene chromosomes complement of *An. minimus* would be prepared and compared with that of *An. fluviatilis* for cladistic analysis.

Taxonomic status of *An. fluviatilis* S as evidenced by molecular characterisation

In a recent taxonomic update by Harbach[1], *An. fluviatilis* S has been regarded as synonym with *An. minimus* species C and thus the later being senior synonym replaced *An. fluviatilis* S. Consequently, he considered the Fluviatilis Complex comprising of two species only—species T and U. Several other recent publications also considered *An. fluviatilis* S as synonym with *An. minimus* species C[2-4]. These reports are primarily based on homology in 335 base pair nucleotide sequence of D3 domain of 28S ribosomal DNA (rDNA) of *An. fluviatilis* S reported by Singh *et al*[5] with that of *An. minimus* species C. In order to resolve the taxonomic status of *An. fluviatilis* S, we determined the nucleotide sequences of a mitochondrial gene cytochrome oxidase II, (COII) and complete internal transcribed spacer 2 (ITS2) region of rDNA of *An. fluviatilis* S and compared these along with already available 28S-D3 rDNA sequence[5] with other members and of Fluviatilis and Minimus Complexes.

A total of seven specimens of *An. fluviatilis* S collected from Sundargarh (22.1°N, 84.0°E) and Koraput (18.8°N, 82.7°E) districts of Orissa state (India), were sequenced for COII and six for ITS2

[1]**Harbach RE (2004).** *Bull Ent Res*, **95:** 537–553.
[2]**Garros C *et al* (2005).** *J Med Entomol*, **42:** 522–536.
[3]**Garros C *et al* (2006).** *Trop Med Intl Hlth*, **11:** 102–114.
[4]**Chen B *et al* (2006).** *Med Vet Entomol*, **20:** 33-43.
[5]**Singh OP *et al* (2004).** *Am J Trop Med Hyg*, **70:** 27–32.

(a)

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An. fluviatilis S  ATGGCAACAT GAGCAAATTT AGGGCTGCAA GATAGATCAT CTCCTTTAAT AGAACAATTA AACTTTTTTC   70
An. fluviatilis T  .......... .......... .......... .......... .......... .......... ..........   70
An. fluviatilis U  .......... .......... .......... .......... .......... .......... ..........   70
An. minimus C      .......... .......... ...AT..... .......... .A........ .......... ..T.......   70

An. fluviatilis S  ACGATCATAC ATTATTAATT TTAACAATAA TTACAATTTT AGTTGGATAT ATTATAGGAA TATTATTATT  140
An. fluviatilis T  .......... .......... .......... .......... .......... .......... ..........  140
An. fluviatilis U  .......... .......... .......... .......... .......... .......... ..........  140
An. minimus C      .......... .......... .......... .......... .......... .......... ..........  140

An. fluviatilis S  TAATAAATTT ACTAATCGAT ATTTACTTCA TGGACAAACT ATTGAAATTA TTTGAACTGT ATTACCAGCA  210
An. fluviatilis T  .......... .......... .......... .......... .......... .......... ..........  210
An. fluviatilis U  .......... .......... .......... .......... .......... .......... ..........  210
An. minimus C      .......... .......... .......... C......... .......... .......... ..........  210

An. fluviatilis S  ATTATTTTAA TATTTATTGC TTTTCCTTCT TTACGATTAT TATATTTAAT AGACGAAATT AATACACCTT  280
An. fluviatilis T  .......... .......... .......... .......... .......... .......... ..........  280
An. fluviatilis U  .......... .......... .......... .......... .......... .......... ..........  280
An. minimus C      .......... .......... ...C...... .......... ....C..... .......... ..........  280

An. fluviatilis S  CTATTACTTT AAAATCAATT GGTCATCAAT GATATTGAAG TTATGAATAT TCAGATTTTT TAAATTTAGA  350
An. fluviatilis T  .......... .......... .......... .......... .......... .......... ..........  350
An. fluviatilis U  .......... .......... .......... .......... .......... .......... ..........  350
An. minimus C      .......... .......... ..A....... .......... C..C.....C .......... ..........  350

An. fluviatilis S  ATTTGATTCA TATATAATTC CAACAAATGA ACTTGAAACA AATGGATTTC GACTATTAGA TGTTGATAAT  420
An. fluviatilis T  .......... .......... .......... .......... .......... ....G..... ..........  420
An. fluviatilis U  .......... .......... .......... .......... .......... .......... ..........  420
An. minimus C      ......C... .........C. .......C.. .......... ........C. ..T....... ..........  420

An. fluviatilis S  CGAATTGTTT TACCTATAAA TAATCAAATT CGAATTTTAG TTACAGCAAC TGACGTATTA CATTCTTGAA  490
An. fluviatilis T  .......... .......... .......... .......... .......... .......... ..........  490
An. fluviatilis U  .......... .......... .......... .......... .......... .......... ..........  490
An. minimus C      .......... .......... .......... .......... .......... ...T...C.. ..........  490

An. fluviatilis S  CAGTTCCTTC TTTAGGAGTA AAGGTTGATG CAACACCAGG ACGATTAAAT CAAATTAATT TTTTAATTAA  560
An. fluviatilis T  .......... .......... .......... .......... .......... .......... ..........  560
An. fluviatilis U  .......... .......... .......... ....C..... .......... .......... ..........  560
An. minimus C      .......... ......G... .......... .......... ....C..... .......... .......C..  560

An. fluviatilis S  TCGACCAGGA TTATTTTTTG GACAATGTTC AGAAATTTGT GGGGCAAATC ATAGATTTAT ACCAATTGTA  630
An. fluviatilis T  .......... .......... .......... .......... .......... .........C ..........  630
An. fluviatilis U  .......... .......... .......... .......... ..A....... .......... ..........  630
An. minimus C      .......... .......... .......... .......... ..A....... .......... ..........  630

An. fluviatilis S  ATTGAAAGAA TTCCTATAAA TTATTTTATT AAATGAATTA CTTCTATAAC TAATT  685
An. fluviatilis T  .......... .......... .......... .......... .......... .....  685
An. fluviatilis U  .......... .......... .......... .......... .......... .....  685
An. minimus C      .......... .......... .......... .......... .......... .....  685
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(b)

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An. fluviatilis S  AATTCCTTGT TACACAATAT TCTAACTACA TGGCGCCCGT GTACGGACGG CATCATGGCG AGCAGCCCGC   70
An. fluviatilis T  .......... ......C... .......... .......... .......... .......... ..........   70
An. fluviatilis U  .......... ......M... .......... .......... .......... .......... ..........   70
An. minimus C      .......... .......... .......... .......... .......... .......... ..........   70

An. fluviatilis S  CTTCTGATGT TGCTGAATGA ACACGTGAGC GCACTGTGCA TCATTGCGTG CAGGGCCCGT CTCCTACCGG  140
An. fluviatilis T  .......... .......W.. .......... .......... .......... .......... ..........  140
An. fluviatilis U  .......... .......A.. .......... .......... .......... .......... ..........  140
An. minimus C      ..A....... .......... .......... .......... .......... .......... ..........  140

An. fluviatilis S  GAACCTTGGG CGCTGAAA-A GGTAAGGCAG TACAGTTCCA CTGTACAATT TGGGGG-TGC AGCGTCAAGT  208
An. fluviatilis T  .......... ........C. .......... .G....GTT. .......... ....K.G... ..........  210
An. fluviatilis U  .......... ........A. .......... ......GTT. .......... .....G.... ..........  210
An. minimus C      .......... ........A. .......... ......GT.. .......... ......-... ..........  209

An. fluviatilis S  CGCACGGGTC GAACTTCGGC TATGGACGAC CTGAGATACC CGGCAGCCTA CTAACACCAG GCTTGTCGAC  278
An. fluviatilis T  .......... .......... .......... .......... .......... .......... .........T  280
An. fluviatilis U  .......... .......... .......... .......... .......... .......... .........T  280
An. minimus C      .......... .......... .......... .......... .......... .......... ..........  279

An. fluviatilis S  CAGGTTCCAG GGGTTACGAA TCATCCGGCC GAGTCGTGTA ACGCGTG-CG ACCCATACGG TGCACCCATG  347
An. fluviatilis T  A......... .......... .......... .......... .......-.. .......... ..........  349
An. fluviatilis U  A......... .......... .......... .......... .......-.. .......... ..........  349
An. minimus C      .......... ........C. .......... .......... .......A.. .......... .......G.A  349

An. fluviatilis S  TTTAGATGGC AACCTACCTT CATAA  372
An. fluviatilis T  ....AT.... .......... T.C..  374
An. fluviatilis U  W...AT.... .......... T.C..  374
An. minimus C      ..G..TG... .....T.... TC..C  374
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Fig. 1.3: (a) Aligned nucleotide sequence of cytochrome oxidase II; and (b) internal transcribed spacer 2 from members of the Fluviatilis Complex and *An. minimus* C. The dot (.) represents similarity of nucleotide with *An. fluviatilis* S and dash (-) missing nucleotide. The *An. minimus* C nucleotides that differ from *An. fluviatilis* S are highlighted

regions. The COII region was PCR-amplified using primers Leu (5′-TCT AAT ATG GCA GAT TAG TGC A-3′) and Lys (5′-ACT TGC TTT CAG TCA TCT AAT G-3′). The ITS2 region of rDNA was amplified using primers ITSA (5′-TGT GAA CTG CAG GAC ACA T-3′) and ITSB (5′-TAT GCT TAA ATT CAG GGG GT-3′). For each PCR assay, we used 50 µl PCR reaction mixture which contained 0.5 µM of each primer, 200 µM of each dNTP, 1.5 mM of $MgCl_2$ and 1.25 unit of Taq Polymerase. The conditions for both PCRs were—an initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min followed by a final extension at 72°C for 7 min. The PCR products were purified using Quiaquick PCR Purification Kit to remove unincorporated primers and dNTPs prior to sequencing. The sequencing was done on both strands of amplified DNA using BigDye v3.1 Terminator Cycle Sequencing Ready Kit following manufacturer's protocol. The primers used for sequencing were same as those used in the original PCR amplification. The other sequences of members of the Minimus and Fluviatilis Complexes for COII, ITS2 and 28S-D3 rDNA were downloaded from GenBank.

DNA sequences were aligned using the ClustalW method implemented in software Molecular Evolutionary Genetics Analysis version 3.1 (MEGA 3.1). The ITS2 and COII sequences were used for paired distance analysis independently for each locus as well as together along with 28S rDNA sequences. Genetic distances were estimated by the Kimura 2–parameter model using software MEGA 3.1. Sites containing alignment gaps were not used in the distance analyses and were treated as missing information. The Neighbour-Joining (NJ) and Maximum Parsimony (MP) trees were constructed using Kimura 2-parameter model including transitions and transversions. The strength of internal nodes was estimated by 1000 bootstrap replicates. The sequences of *An. aconitus* and *An. varuna* were taken as outgroups during construction of the trees.

The DNA sequence data of *An. fluviatilis* S for COII and ITS2 are shown in Fig. 1.3 along with the

Table 1.1. Pair-wise distance (below diagonal) and number of nucleotide difference (above diagonal) among members of the Fluviatilis Complex and *An. minimus* C

	An. fluviatilis S	*An. fluviatilis* T	*An. fluviatilis* U	*An. minimus* C
(a) COII				
An. fluviatilis S	–	2	2	22
An. fluviatilis T	0.003 [0.002]	–	4	24
An. fluviatilis U	0.003 [0.002]	0.006 [0.003]	–	22
An. minimus C	0.033 [0.007]	0.036 [0.008]	0.033 [0.007]	–
(b) ITS2				
An. fluviatilis S	–	10	9	13
An. fluviatilis T	0.028 [0.009]	–	1	15
An. fluviatilis U	0.025 [0.008]	0.003 [0.003]	–	14
An. minimus C	0.036 [0.010]	0.042 [0.011]	0.039 [0.011]	–
(c) Combined (COII, ITS2 and 28S-D3 rDNA)				
An. fluviatilis S	–	15	14	35
An. fluviatilis T	0.011 [0.003]	–	6	42
An. fluviatilis U	0.010 [0.003]	0.004 [0.002]	–	39
An. minimus C	0.026 [0.004]	0.031 [0.005]	0.029 [0.005]	–

Figures in bracket indicate standard error.

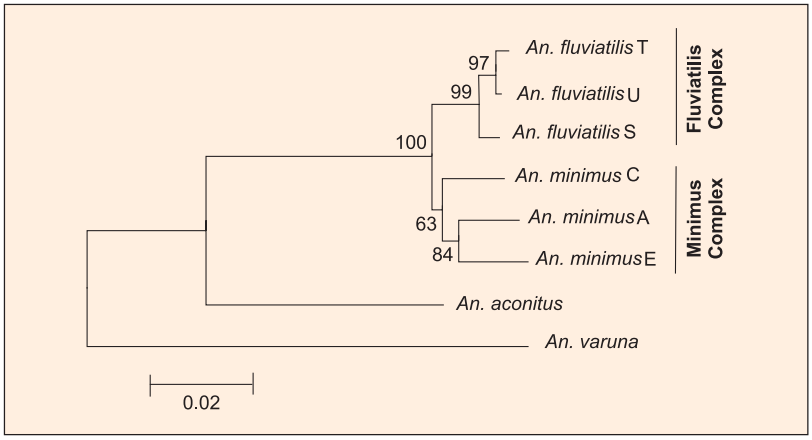


Fig. 1.4: Neighbour-Joining tree inferred from combined sequences data of the three loci (COII, ITS2 and 28S-D3) from members of the Fluviatilis and Minimus Complexes. Numbers above the branches are bootstrap values

sequences of *An. minimus* C and other members of the Fluviatilis Complex. There were two haplotypes of COII in *An. fluviatilis* S (GenBank accession numbers: DQ383278 and DQ383279) differing by one base pair substitution, however, there was no difference in deduced amino-acid sequence. The data shown in Fig.1.3 belong to majority haplotype (representing six out of seven samples sequenced). The ITS2 sequences of all the six specimens of *An. fluviatilis* S sequenced were identical (GenBank accession number DQ345964).

The pair-wise distances among *An. minimus* C and members of the Fluviatilis Complex, as computed separately for COII and ITS2 locus and combined for 28S-D3, CO II and ITS2 loci are shown in Table 1.1. Comparison of sequences revealed that *An. fluviatilis* S differs from *An. minimus* C by 22 base substitutions in COII and by 13 substitutions and two indels in ITS2 region. However, this species differs from other members of Fluviatilis Complex (species T and U) by only 2 and 9–10 base pair substitutions, respectively, for COII and ITS2 regions. In combined analysis, a total of 1388 aligned base pairs data were used including 328 base pair 28S-D3 sequence data where *An. fluviatilis* S and *An. minimus* C have identical sequences. In combined data analysis, *An. fluviatilis* S differs from *An. minimus* C by 35 base pair substitutions (2.6%), while it differs from species T and U by 15 (1.1%) and 14 (1%) base pairs only indicating that *An. fluviatilis* S is closely related to members of the Fluviatilis Complex as compared to *An. minimus* C.

The NJ tree for members of Fluviatilis and Minimus Complexes derived from combined set of data (COII, ITS2 and 28S-D3) shows that the Fluviatilis Complex (along with species S) forms a monophyletic taxon which is distinctly separated by Minimus Complex with nodal support of 100% bootstrap value (Fig. 1.4). Similar trend was obtained when we used MP method for phylogenetic reconstruction (data not shown). These results clearly establish the specific status of *An. fluviatilis* S as one of the member of Fluviatilis Complex and revokes it's synonymy with *An. minimus* C.

1.2 Insecticide-resistance

1.2.1 Insecticide-resistance studies in Surat district (Gujarat)

Field studies were carried out in the villages in Ukalda PHC area, District Surat in November 2005 to assess the status of insecticide-resistance in *An. culicifacies*. Earlier studies indicated development of

resistance to deltamethrin in 2002 in addition to the existing resistance to DDT and malathion. In these villages insecticide indoor residual spray is not being carried out for the past 5–6 years. This study was conducted to assess the present status of resistance to the insecticides used in public health. Studies included determination of base line resistance to different insecticides, synergistic studies, sibling species composition and differential insecticide-susceptibility in sibling species.

Base line resistance

This was determined using the standard WHO method and kit by exposing the mosquitoes to the designated WHO diagnostic doses of different insecticides. In susceptibility tests *An. culicifacies* was found 40% susceptible to DDT (n = 93); malathion—95% (n = 142); fenitrothion—97% (n = 146); propoxur—95% (n = 133); bendiocarb—100% (n = 107); deltamethrin—98% (n = 97); lambda-cyhalothrin—97% (n = 116; cyfluthrin—96.8% (n = 116) and to permethrin it was 99.2% (n = 140). Thus, the species was found resistant to DDT and malathion and was susceptible to other organophosphate, carbamate and pyrethroid insecticides.

Synergistic studies

Synergistic bioassay studies with tri phenyl phosphate (TPP), a specific inhibitor of carboxyl esterase indicated complete synergism with TPP (10%). This indicated the involvement of carboxyl esterase for conferring malathion resistance in this species. The recorded mortality for malathion alone was 66%, while exposure to TPP (5%) followed by exposure to malathion (5%) impregnated papers the mortality increased to 95%. Similarly, with pre-exposure to TPP (10%) followed by malathion it was 100%.

Susceptibility in sibling species

Female *An. culicifacies* mosquitoes and the dead and alive mosquitoes in the insecticide exposures in WHO susceptibility tests were stored in isopropanol and were later identified to sibling species using PCR assays. The species composition of *An. culicifacies* indicated major sympatricity of species B and E. The proportion of species E was 59.5%, species B was 39.3% and species C was only 1.2%. Prevalence of species E from this area is being reported for the first time. Identification of dead and alive mosquitoes in the insecticide bioassays to sibling species indicated differential susceptibilities to DDT and malathion. To DDT, species B was 31.5% susceptible, while to malathion it was 64% and species E was 56.2 and 75% susceptible to DDT and malathion respectively (Table 1.2). Thus, species B was relatively less susceptible to DDT and malathion. Susceptibility in species C could not be determined due to its very low prevalence.

Table 1.2. Susceptibility status of *An. culicifacies* species B and E to different insecticides in District Surat

Species	Insecticide, time	% Mortality (n)
An. culicifacies s.l.	DDT (4%), 1 h	40.0 (93)
Species B	DDT (4%), 1 h	31.5 (57)
Species E	DDT (4%), 1 h	56.2 (32)
An. culicifacies s.l.	Malathion (5%), 1 h	66.0 (142)
Species B	Malathion (5%), 1 h	64.0 (51)
Species E	Malathion (5%), 1 h	75.0 (12)

n: Number exposed.

1.3 Vector control

1.3.1 Evaluation of PermaNet® 2.0 against malaria vectors in India

PermaNet, a long-lasting insecticide treated net (LLIN) treated with deltamethrin @55 mg/m^2 was evaluated for bioefficacy against malaria vectors and other mosquitoes in malaria endemic areas of Uttar Pradesh. For it, the study was conducted in Nawada, Harampur and Dugrawali villages from April to November 2005. Percent corrected mortality of *An. culicifacies* and *An. stephensi* in three minute contact bioassays performed on unwashed and washed PermaNet 2.0 are depicted in Fig. 1.5. The results showed high efficacy of PermaNets against both the

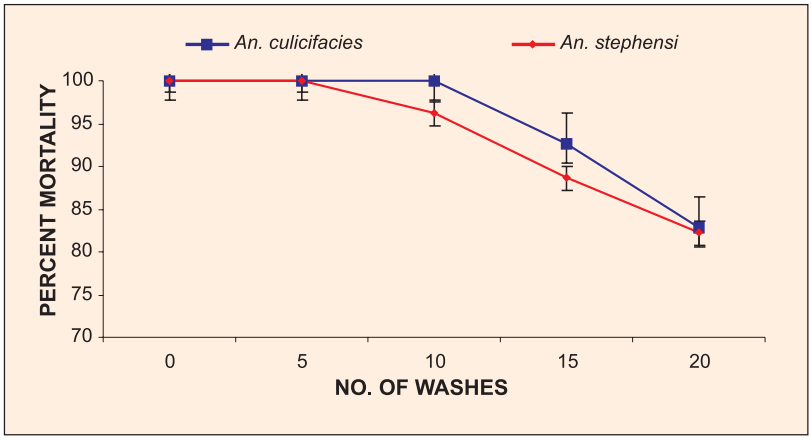


Fig. 1.5: Percent mortality (24 h holding) of mosquitoes exposed to unwashed and washed PermaNet 2.0 in three min contact bioassays

species as evidenced by the fact that even after 20 washes the mortality in both the species remained >80%. Further, there was no significant difference between the mortalities of the two species ($p > 0.05$). In ring net bioassay, the base line median knockdown time (MKDT) against *An. culicifacies* was 390 sec. However, after progressive washings the MKDT was also increased. The MKDT of *An. culicifacies* was less than that of *An. stephensi* and the difference was statistically significant ($p < 0.05$) (Table 1.3).

Use of PermaNets also reduced the man hour densities (MHD) of different mosquito species. The results revealed that the MHD of *An. culicifacies* and *An. stephensi* in the structures selected for PermaNet, plain net and without net during pre distribution period was 27 and 53, 22 and 32 and, 20 and 22 respectively. After the distribution of nets the densities reduced gradually in PermaNet used structures. Though there was reduction in density in structures where the plain nets were used, the impact was more pronounced in PermaNet used structures. PermaNet showed high excito repellent activity (82–97%) against *An. culicifacies*, 77–97% against *An. stephensi*. However, the repellent action was below 30% against all the mosquito species. PermaNet has high killing activity as evidenced by the fact that all the landed mosquitoes on the net died after 24 h of observation. About 20 to 40% mortality was observed in mosquitoes that entered the room and were accidentally exposed to the net and rested on the walls. The results clearly demonstrated that PermaNet showed high efficacy in producing mortality in the mosquitoes, which were exposed to the net.

Table 1.3. Medium knockdown time (in sec) of *An. culicifacies* and *An. stephensi* exposed to unwashed and washed PermaNets

Type of net	*An. culicifacies*	*An. stephensi*
Unwashed PermaNet	390 ± 13	480 ± 22
5 times washed	397 ± 18	548 ± 32
10 times washed	415 ± 16	602 ± 26
15 times washed	448 ± 21	727 ± 17
20 times washed	472 ± 22	968 ± 28

Each value is mean ± S.D. of four replicates.

Safety of PermaNets usage was studied by interviewing the users of the nets through structured questionnaires. Results revealed that eye and skin irritation were the main complaints reported by the inhabitants. Further the inhabitants also mentioned that these symptoms were not persisted after washing the affected areas with clean water. This may be due to high dose of deltamethrin on the PermaNet. Symptoms like headache, vomiting, nausea, etc. were not reported.

1.3.2 Phase III evaluation of Olyset nets for malaria control

The operational feasibility and efficacy of Olyset net (LLIN) was studied against *An. culicifacies*, a principal malaria vector that transmits 65% of total malaria cases in rural areas of India. Three villages in District Gautam Budh Nagar (Uttar Pradesh), India were selected for the trial and Olyset nets were distributed in one village, in other village plain nets were distributed and the third village was kept as control where nets were not used. Entomological and epidemiological data were collected using standard methods. Altogether 1203 Olyset nets were distributed among 701 males and 502 females aged above 10 years in Khandera village and 1289 untreated nets among 719 males and 570 females aged above 10 years in Beel Akbarpur village.

Usage pattern of nets by the inhabitants in the study villages is shown in Fig. 1.6. It was found that the inhabitants were regularly using nets as there was always > 98% usage during transmission season (August to November), and also in the months of March and April when the *Culex* mosquito densities are generally high. In this area in winter season (January and February) the density of *Anopheles* mosquitoes is generally very low due to extremely low temperatures and people generally do not use nets. About 2.5% Olyset nets were lost as against 1.04% untreated nets.

Entomological results revealed that use of Olyset nets has led to significant reduction in mosquito densities in general and particularly *An. culicifacies* ($p < 0.05$). There was a significant difference in the densities of *An. culicifacies* after distribution of nets when compared to those prior to distribution ($p < 0.05$) in both Olyset and untreated net distributed villages. Whereas in the control village the difference between pre-densities and post-densities was statistically insignificant ($p > 0.05$). In case of total mosquitoes, significant difference was observed in pre-densities and post-densities in Olyset net village only. When the post-densities were compared in between the villages, no statistically significant difference was observed in all combinations except between control and untreated nets.

The entry of *An. culicifacies* substantially reduced in Olyset net distributed village in comparison to untreated net village ($p < 0.05$). This was also reflected when the landing rate of *An. culicifacies* on both types of nets was compared in different villages. The mean landing rate on the Olyset net was nil whereas on the untreated net it varied from 8 ± 1.2 to 16.5 ± 2.5 mosquitoes/bait/night. Similarly, the mean landing rate of total mosquitoes was nil on the Olyset net as against 62 ± 9.8 to 125 ± 23.9 on plain net suggesting thereby that Olyset net has either airborne action or strong repellent action against mosquitoes in general and against *An. culicifacies* in particular.

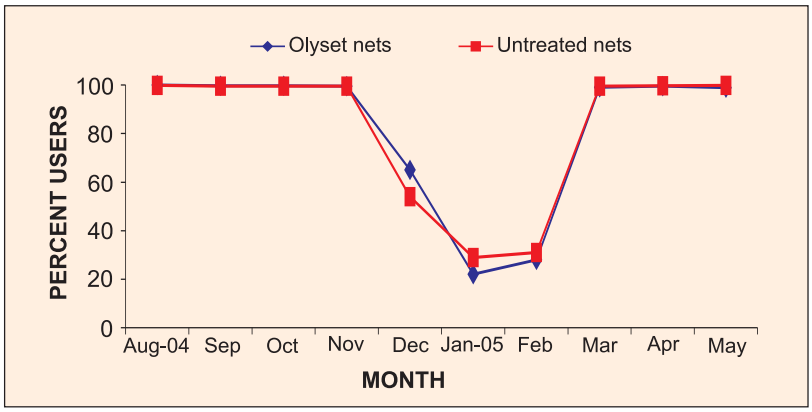


Fig. 1.6: Usage pattern of nets in the study villages

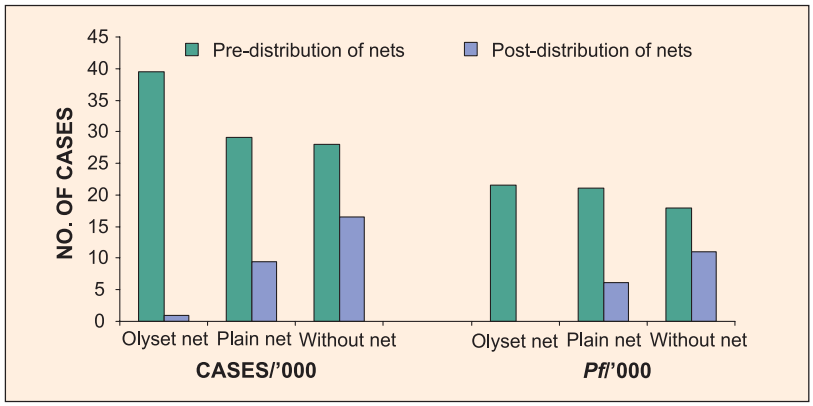


Fig. 1.7: Impact of Olyset nets on malaria incidence

There was a considerable reduction in the parity and sporozoite rates of *An. culicifacies* in the Olyset net distributed village in comparison to plain net distributed and control villages. There was statistically significant difference when the parity rates of *An. culicifacies* in both Olyset net and untreated net villages were compared. This clearly indicates that longevity of vector mosquitoes reduced drastically in the Olyset net distributed village. Further nil sporozoite rates in vector species indicate interrupted transmission in the villages where Olyset nets were used, whereas in the control village malaria transmission continued. These results clearly showed that use of Olyset nets protected the community and reduced the longevity of vectors.

Epidemiological results revealed that after introduction of Olyset nets in Khandera village in the month of August 2004, malaria incidence started declining and impact on SPR and SFR was quite high. There was no significant difference in SPR and SFR in all the villages prior to distribution of nets ($p > 0.05$). However, the SPR reduced drastically in the Olyset net distributed village after introduction of nets ($p < 0.1$ & > 0.05). Similarly significant difference at 0.1 level was observed only in between Olyset net and control village. At 0.05 level of significance the comparisons were insignificant.

Results of mass blood surveys carried out in these villages clearly demonstrated that malaria incidence was completely reduced when compared to that in pre-distribution mass blood survey in the Olyset net distributed village, whereas in the plain net and without net villages malaria cases were reported in both surveys after the distribution of nets (Fig. 1.7). These results further substantiated that Olyset nets are quite effective in controlling malaria. No adverse effect such as itching, nausea, vomiting, etc. was reported by the inhabitants using Olyset nets.

1.3.3 Evaluation of Dimilin (Diflubenzuron) GR-2 (2% granules) and TB-2 (2% tablets) for control of mosquito larvae

Two formulations of Dimlin (N-4 chlorophenyl amino carbonyl-2-6 difluro-benzamide)—GR-2 (2% granules) & TB-2 (2% tablets) supplied by M/s. Crompton Uniroyal Chemicals Asia Pacific Pvt. Ltd., Mumbai were tested for their bioefficacies against immatures of *An. stephensi, Ae. aegypti* and *Cx. quinquefasciatus* in their respective breeding habitats.

Field evaluation was carried out in specific breeding places of target species in and around Delhi. Habitats with predominant breeding of a particular species were selected for these trials. Since it was not possible to observe mortality of larvae, pupae and adults in natural habitats, therefore, these water bodies were initially treated with required con-

Application of Dimilin granule formulation in pits

Application of Dimilin tablet formulation in cooler

centration on volume basis for tablet formulation in contained water habitats such as tanks, and on area basis for granule formulation in other habitats. Water samples from treated habitats were brought to the laboratory on the same day and Day 3 and later at an interval of one week. Laboratory colonised late III instar larvae were introduced in subsequent weeks till the complete pupation or adult emergence under controlled conditions. Also late instar larvae collected from the treated habitats were observed till the complete pupation or adult emergence under controlled conditions. Percent inhibition was calculated on the basis of untreated control run concurrently.

Results of field evaluation revealed that application of Dimilin TB-2% @ ½ tablet and 1 tablet per 40 litre (L) water (equivalent to 0.5 & 1 ppm) produced complete inhibition of the development of pupae and emergence of adult mosquitoes in tanks with clear water against *An. stephensi, Ae. aegypti* and also *Cx. quinquefasciatus* up to 4 weeks of observation. When used @ 1 tablet in 400 L of water (equivalent to 1 ppm) 100% inhibition of adult emergence was observed against *An. stephensi* up to 7 days and against *Ae. aegypti* and *Cx. quinquefasciatus* up to 14 days and 98–99% inhibition was recorded up to 4 weeks. The efficacy of Dimilin GR-2 formulation in cement tanks when treated @ 1.5 kg/ha also showed 100% inhibition of adult emergence in case of *An. stephensi, Ae. aegypti* and *Cx. quinquefasciatus* up to 4 weeks (Figs. 1.8–1.10). However, at lower dose—1 kg/ha, 100% inhibition

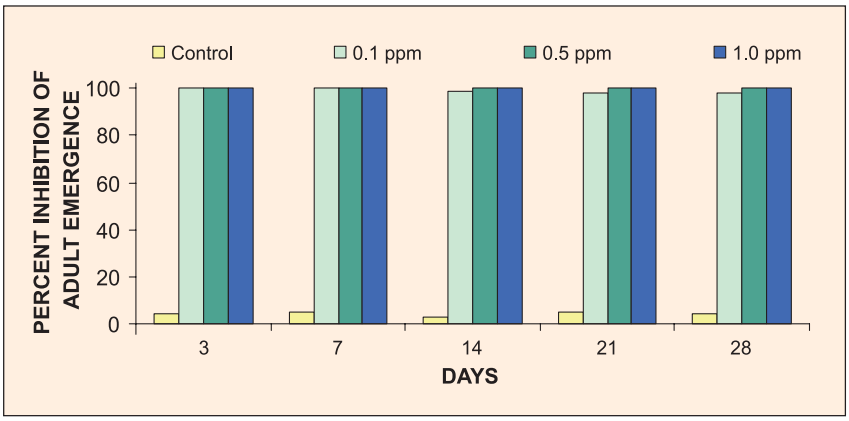


Fig. 1.8: Efficacy of Dimilin TB-2 against *An. stephensi* in cement tanks

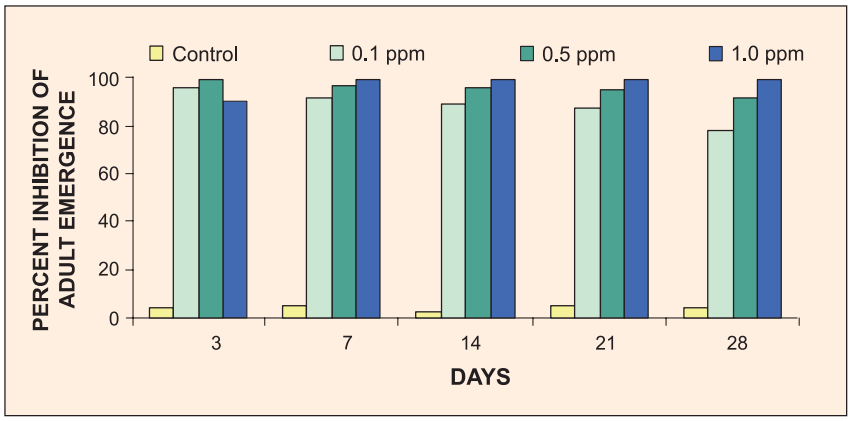


Fig. 1.9: Efficacy of Dimilin GR-2 against *An. stephensi* in cement tanks

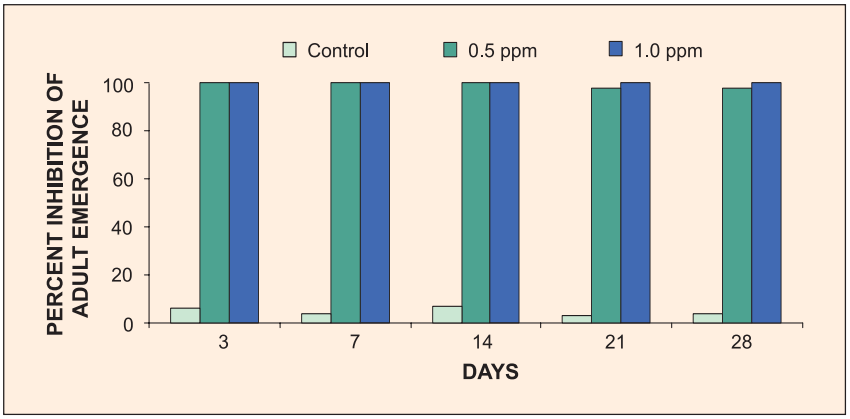


Fig. 1.10: Efficacy of Dimilin TB-2 against *Ae. aegypti* in desert coolers

was observed against *Cx. quinquefasciatus* up to 3 days only, whereas against *An. stephensi* and *Ae. aegypti* only 97 and 96% inhibition was observed on Day 3 respectively. It may be pointed out that only higher dose of granule formulation—1.5 kg/ha was effective in clean water in cement tanks for complete inhibition of adult emergence up to four weeks.

Field evaluation of Dimilin 2% tablet against immatures of *Anopheles* spp and *Cx. quinquefasciatus* in moderately polluted small pools also showed 100% inhibition of adult emergence up to 4 weeks of observation @ 0.5 & 1 Tab/40 L water. However, @ 0.1 Tablet/40 L (1 Tab/400 L) the percent inhibition of adult emergence ranged between 92 and 98 against both the species during the four weeks of observations.

Dimilin 2% Granules against larvae of *Anopheles* spp in pools @ 1.5 kg/ha produced 100% inhibition of adult emergence of *Anopheles* larvae up to 4 weeks of observation but at lower doses—1 & 1.25 kg/ha, the percent inhibition was 86–92 after 3 days and this effect gradually diluted as evidenced by the fact that after 4 weeks 80–84% emergence was recorded. Dimilin GR-2 @ 1.5 kg/ha against *Cx. quinquefasciatus* larvae in pools produced 100% inhibition of adult emergence up to 2 weeks. However, at lower doses only 86–89% inhibition was observed on Day 3 itself which declined to 73–81% after 4 weeks (Fig. 1.11).

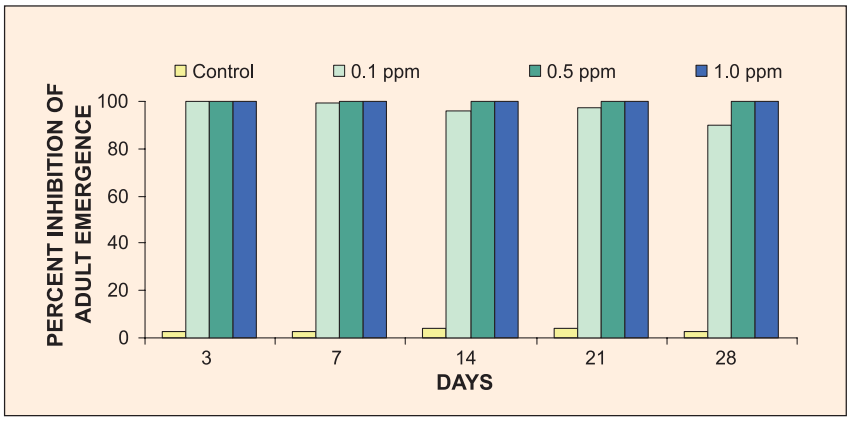


Fig. 1.11: Efficacy of Dimilin GR-2 against *Cx. quinquefasciatus* in cement tanks

1.3.4 Larvicidal activity of extracts of different parts of a tissue cultured plant (code VA-1 of family Astereaceae)

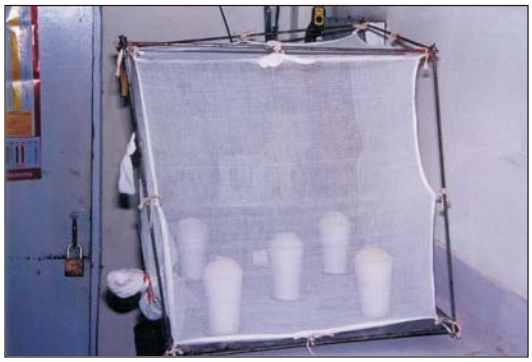
Studies were carried out in collaboration with University of Delhi to assess the larvicidal activity of the hexane extract of root, flower, leaf and stem of the coded VA-1 plant (Family: *Astereaceae*). The efficacy was tested against two mosquito species, *An. stephensi* and *Cx. quinquefasciatus*. The calculated LC_{50} and efficacy of different parts against *An. stephensi* was in the order—root (2.71 ppm) > flower (7.38 ppm) > leaf (61 ppm) > stem (86.92 ppm). For *Cx. quinquefasciatus* the values were: root (1.19 ppm)> flower (8.52 ppm) > leaf (14.54 ppm) > stem (26.33 ppm). The order of efficacy for different parts in the two species was same but the overall efficacy of the formulations of different parts was more against *Cx. quinquefasciatus* (Table 1.4).

1.3.5 Study to assess the repellency of a natural oil formulation (DRP-I) on disease mosquito vectors

Cage studies were carried out to assess the repellency of a natural oil formulation (DRP-1) in five vector species namely *An. culicifacies* species A, and C, *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*. Relative repellency studies were carried out against three concentrations of the natural oil formulation (DRP-1)—2.5, 5 and 10% and a known chemical repellent (DEET) at 2.5%. Observations of the relative repellencies were made at 0 h and subsequently at 1, 2, 4 and 6 h. This was done by recording the number of mosquitoes landing on the impregnated oil and DEET in 10% glucose soaked cotton ball placed in Styrofoam glasses. Glucose alone was the control in the study. A total of 100, 5–6 day old sugar-fed female mosquitoes were intro-

Table 1.4. Larvicidal activity (ppm) of different concentrations of the crude hexane extract of different parts of the tissue cultured plant VA-1 of family Astereaceae against *An. stephensi* and *Cx. quinquefasciatus*

Part	*An. stephensi*			*Cx. quinquefasciatus*		
	Conc. (Range)	LC_{50}	LC_{90}	Conc. (Range)	LC_{50}	LC_{90}
Root	7.5 (0.29–15)	2.71	4.26	3.75 (0.29–7.5)	1.19	2.06
Flower	33.3 (0.26–66.4)	7.38	13.52	16.66	8.52	14.27
Leaf	105 (15–210)	61.0	95.0	71.6 (1.1–142)	14.54	27.7
Stem	220 (50–440)	87.0	110	110 (0.86–220)	26.33	47.83



Mosquito cage showing Styrofoam glasses with control and repellent soaked cotton

Recording of landing rates of mosquitoes on control and repellent soaked cotton

duced into 2 sq ft cloth cage and the Styrofoam glasses with the oil impregnated cotton, DEET impregnated and glucose alone were placed in different corners of the cage. Number of mosquitoes landing on cotton impregnated with different concentrations of DRP-1 and glucose were simultaneously counted. DRP-1 was found effective in repelling the species of mosquitoes in the observations made at different hours of observations (Table 1.5). The percent repellency observed at different observation periods ranged from 80–100% against different concentrations of repellents and different species. *Ae. aegypti* and *Cx. quinquefasciatus* have shown >95% repellency to DRP-1 at all the concentrations—2.5, 5 and 10%. *An. culicifacies* species A, *An. culicifacies* species C and *An. stephensi* have shown 93–100% repellency against both 5 and 10% concentrations of DRP-1. From the observed data on the repellency against the five important disease vectors it can be concluded that a dose of 5% could be used for achieving the desired level of protection against bites of these mosquitoes. However, these results pertain to the effectiveness in cage experiments using only sugar solution and need further confirmation by testing the same on human subjects to testify the observed repellency against the natural host.

Table 1.5. Repellency rates against different concentrations of repellents in different species

Species	DEET (2.5%)	DRP (2.5%)	DRP (5%)	DRP (10%)
An. culicifacies species A	100	84–100 (6 h)	93–100 (4, 6 h)	96–100 (0, 2, 4 h)
An. culicifacies species C	90–100 (1, 2, 4 h)	83–100 (1, 2, 4 h)	100	100
An. stephensi	85–100 (6 h)	85–100 (6 h)	92–100 (0, 2, 4 h)	97–100 (0,1,4,6 h)
Cx. quinquefasciatus	80–90 (–)	95–100 (0,1 h)	100	100
Ae. aegypti	93–97 (–)	99–100 (0,12 h)	100	100

Figures in parentheses indicate time period.

1.3.6 Antifungal activity of neem oil against entomopathogenic fungi and its possible application in integrated vector control programme

The growth inhibitory action of neem oil against the entomopathogenic fungi *Lagenidium giganteum* and *Metarhizium anisopliae* was studied. The toxicity of the neem oil to mycelia growth of *L. giganteum* and *M. anisopliae* is shown in Fig. 1.12. The IC_{50} of neem oil was compared among the three media namely peptone-yeast extract-glucose (PYG), Emerson's YpSs and Sunflower seed (SFS). It was observed that the growth of *L. giganteum* was 3.9- and 2.4-fold more than the *M. anisopliae* in PYG and Em YpSs media respectively. When grown in SFS media, neem oil did not inhibit the growth of *L. giganteum*. Minimum inhibitory concentration (MIC) of the neem oil against *L. giganteum* and *M. anisopliae* in the three broths are shown in Table 1.6. The MIC of *L. giganteum* was 3- and 2-fold higher than the MIC of *M. anisopliae* in PYG and Em YpSs respectively. In SFS broth, neem oil did not inhibit the growth of *L. giganteum*. However, it was more effective in inhibiting the growth of both the species in Em YpSs than in PYG broth. The minimum fungicidal concentration (MFC) was achieved at much higher concentration than the MIC (Table 1.6). *L. giganteum* growth in SFS was unaffected in the presence of neem oil, whereas the growth of *M. anisopliae* was restricted at various concentrations of neem oil. These studies have indicated that the neem oil inhibits its mycelial growth in PYG and Em YpSs media, while in SFS medium no inhibition of *L. giganteum*

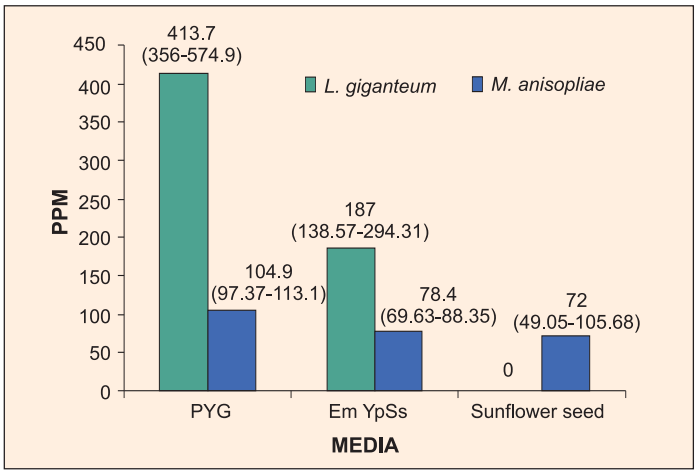


Fig. 1.12: Growth inhibition concentration (IC_{50}) of neem oil in ppm against *L. giganteum* and *M. anisopliae* in three different media (95% fiducial limit in parentheses)

Table 1.6. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of neem oil (µl/ml) against *L. giganteum* and *M. anisopliae* after 24 h of incubation

Strain	MIC			MFC		
	PYG	Em YpSs	SFS	PYG	Em YpSs	SFS
L. giganteum	30	20	–	60	60	–
M. anisopliae	10	10	–	40	60	40

was observed. This study provides a lead to a successful Integrated Vector Control Management with neem oil as a candidate vis-à-vis the fungal strains.

1.3.7 Isolation of *Bacillus* strains from soil and their efficacy against *An. culicifacies*

Soil samples were collected from locations in the vicinity of mosquito breeding habitats of Delhi and Himachal Pradesh in asceptical conditions. Five strains of *Bacillus* were isolated from the Delhi soil and three from the Himachal Pradesh. Out of five strains of Delhi, two were found to be entomopathogenic and coded as MB-1 and HB-1. One strain of Himachal Pradesh was found to be effective against mosquitoes and coded as HP-1. The efficacy of the three strains against *An. culicifacies* species A was tested after growing them for 48 hours in LB + NYSM media. The LC_{50} of the three strains are shown in Table 1.7. Among the three strains, MB-1 was found to be more effective than the other two strains. These coded strains have been sent to MTCC (IMTECH), Chandigarh for identification.

Table 1.7. LC_{50} of coded *Bacillus* strains (in µl/ml) against II instar larvae of *An. culicifacies* species *A* after 24 h of inoculation

Name of coded strain	LC_{50}	LC_{90}
MB-1	28.87 (20.5–34.92)	45.05 (36.92–78.59)
HB-1	62.33 (56.69–67.82)	84.84 (77.03–98.36)
HP-1	56.04 (47.76–65.67)	286.42 (209.65–308.84)

1.3.8 Studies on larvicidal properties of aqueous leaf extract of *Xanthium stramorium* (Family: Aizoaceae)

Larvicidal activity of crude aqueous extract of the leaf of a medicinally important plant *Xanthium stramorium* was tested against III/IV instar larvae of *An. culicifacies* species A and C, *An. stephensi* and *Cx. quinquefasciatus* following standard WHO method for a range of concentrations. The calculated LC_{50} (lethal concentration for killing 50% of treated larvae) for different species were respectively: *An. culicifacies* species A—0.19%, *An. culicifacies* species C—0.22%, *An. stephensi*—0.13%, *Cx. quinquefasciatus*—0.27% and *Ae. aegypti*—0.27% (Table 1.8).

1.3.9 Prospecting for botanical pesticides

The study was undertaken in collaboration with other institutes as a part of the DBT funded project "Prospecting for botanical pesticides". Plant extracts/ fractions/formulations obtained from other collaborating institutes under the project were screened at NIMR for their bioactivity against mosquitoes, particularly against malaria vector, *An. stephensi* using standard protocol.

Since beginning of this project, a total of 732 coded samples of different plant extracts/ fractions/ formulations were received at NIMR for bioassays against *An. stephensi*. Of these, 727 samples were

Table 1.8. Larvicidal activity of different concentrations of the crude aqueous extracts of the leaves of *Xanthium stramorium*

Species (instar)	LC_{50} (% Solution)	LC_{90} (% Solution)	χ^2 (df=6)
An. culicifacies A (III/IV)	0.19	1.3	123.88
An. culicifacies C (III/IV)	0.22	1.5	81.53
An. stephensi (III/IV)	0.13	0.8	33.18
Cx. quinquefasciatus (III/IV)	0.27	1.0	42.93
Ae. aegypti (III/IV)	0.27	1.8	86.95

Table 1.9. Samples and screening status of plant extracts/fractions

Laboratory/Institute	Received samples	Screened samples	Positive results		
			L	A	R
RRL (Trivendrum)	105	105	8	3	1
FRI (Dehradun)	121	121	32	16	4
IIT (Delhi)	187	182	75	2	–
RRL (Jammu)	170	170	28	6	–
EID Parry (Bangalore)	152	152	29	2	–
Total	732	727	172	29	5

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

tested and their bioactivity is shown in Table 1.9. Of the 727 samples tested so far, 172 samples showed larvicidal activity (70–100% mortality) at 250 ppm, while 29 samples showed insecticidal activity against adult mosquitoes and 5 samples have shown repellent activity for more than one hour. Of the various samples tested, 4 plants were identified for further development of plant extract based formulations for testing against mosquitoes and other agricultural pests. About 83 formulated samples were received and tested against larvae of the two mosquito species—*An. stephensi* and *Ae. aegypti*. Most of the formulated samples, produced 100% mortality in the mosquito larvae at 250 ppm, but very low or no mortality was recorded at lower concentrations and, therefore, lethal concentrations could not be determined. None of these formulated samples showed activity against adult mosquitoes.

1.4 Vector immunity

1.4.1 Immune response in the haemolymph of *An. culicifacies* against the challenge of gram-positive bacteria *Micrococcus leutus*

We have earlier studied the immune response in the body tissue of species A, B and C of Culicifacies Complex upon challenge with gram-positive bacteria *M. leutus*. Further, another immune-responsive site—haemolymph which has a direct involvement in wound healing and combating invaders of the body cavity of mosquitoes was used for studying the immune response of species A, B and C upon *M. leutus* challenge. Four to six day old laboratory-colonised *An. culicifacies* mosquitoes were used along with appropriate control. Studies were carried out on three groups of mosquitoes, I group was kept as naïve, II group was given sterile injury and the III group was challenged with bacteria. Haemolymph was harvested after 1, 4 and 8 hours time period intervals post-injury. The tissues were processed for SDS-PAGE and were analysed for the changes in the polypeptide profile using Gene Tool™ software.

In species A, the sterile injury induced the synthesis of seven new polypeptides (220, 200, 198, 180, 176, 152, 120 kDa) and the expression of twelve naïve polypeptides ranging from 20 to 76 kDa was also enhanced (Fig. 1.13, Densitogram 1.1, Table 1.10). One 20.5 kDa polypeptide disappeared from the haemolymph samples after 8 h of sterile injury. *M. leutus* infection induced the synthesis of six new polypeptides (220, 198, 180, 176, 152 and 120 kDa) and activated ten existing polypeptides (76, 71, 56, 45, 37, 34, 32, 26.5, 24 and 22 kDa). In species B, injury induced two new polypeptides (18 and 11 kDa) and enhanced the expression of four existing polypeptides (37, 20.5, 19 and 6 kDa) from their basal level (Fig. 1.14, Table 1.11). In addition to the up regulation of these polypeptides, the injury also resulted in the down regulation of three naïve polypeptides (130, 122 and 10 kDa). In contrast, *M. leutus* infection stimulated the production of a novel

Table 1.10. Injury and bacteria inducible polypeptides of the haemolymph of species A of the Culicifacies Complex

A HI	220	200	198	180	176	152	120	76	71	56	45	43	37	34	32	26.5	24	22	20.5	20
N	–	–	–	–	–	–	–	+	+	+	+	++	+	+	+	+	+	+	+	+
1W	+	++	+	+	+	–	–	+	+	++	++	+	+	+	+	++	+	+	+	+
4W	+	++	++	++	++	+	+	++	++	++	++	++	++	++	++	++++	++	++	+	+
8W	+	–	++	++	++	+	+	++	++	++	++	+++	+	++	++	++++	++	++	–	++
1I	++	–	+	+	+	+	+	++	++	++	++	++	++	++	+	+++	++	++	+	+
4I	+	–	++	+++	+	+	+	++	++	++	++	++	++	++	++	++++	++	++	+	+
8I	–	–	++	+	++	–	–	+	+	++	+	++	+	+	+	+	+	+	+	+

Table 1.11. Injury and bacteria inducible polypeptides in the haemolymph of species B of the Culicifacies Complex

B HI	130	122	92	81	37	26.5	26	20.5	19	18	11	10	6
N	++	++	+	+	+	+	++	+	+	–	–	+	+
1W	+	+	+	+	+	+	++	+	+	–	+	-	+
4W	+	++	+	+	++	+	++	++	++	++	+	+	++
8W	++	++	+	+	++	+	++	+	+	++	+	+	++
1I	++	++	++	++	++	++	+	+	+	+	–	+	–
4I	+	+	+	+	++	+	–	+	+	+	–	+	–
8I	+	++	+	+	++	+	–	+	+	+	–	+	–

Table 1.12. Injury and bacteria inducible polypeptides in the haemolymph of species C of the Culicifacies Complex

C HI	198	180	152	81	56	45	43	32	26.5	24	11
N	+	+	+	++	+	++	++	–	+	+	+
1W	+	+	+	+	+	++	++	+	+	+	+
4W	+	+	+	+	+	+	++	–	+	++	+
8W	+	+	++	+	++	++	++	–	+	+++	++
1I	+	+	+	–	++	+	+	–	+	++	+
4I	+	+	+	+	+	-	+	–	+	++	++
8I	–	++	++	++	+	++	++	–	++	++++	++++

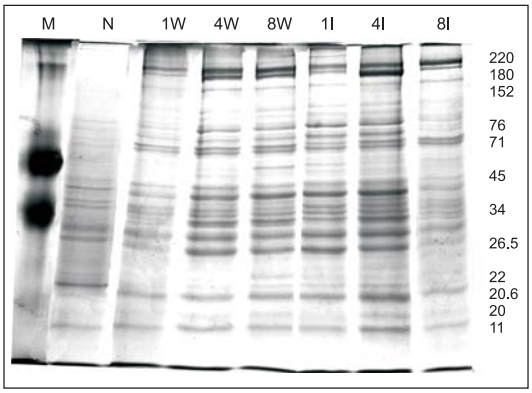


Fig. 1.13: Haemolymph of species A after injury with entomological needle contaminated with or without *M. leutus* (N : Naïve female; W: Sterile injury,1, 4 and 8 : Hours after treatment; I : Infected with *M. leutus* II)

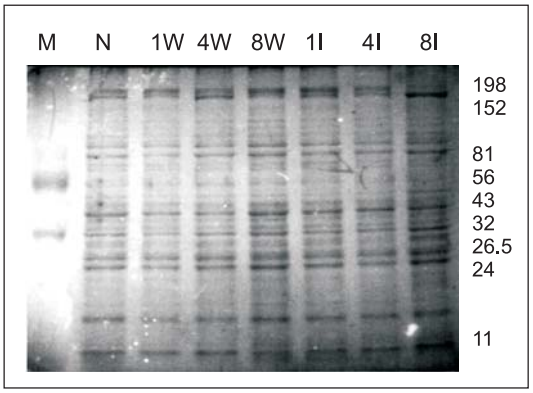


Fig. 1.15: Haemolymph of species C after injury with entomological needle contaminated with or without *M. leutus* (N : Naïve female; W: Sterile injury, 1, 4 and 8 : Hours after treatment; I : Infected with *M. leutus*)

18 kDa polypeptide and up regulated four polypeptides (92, 81, 37 and 26.5 kDa). Among all the up regulated polypeptides, a 26.5 kDa polypeptide was found to be up regulated many fold as compared to sterile injury in naïve mosquitoes. Three polypeptides; 130, 122 and 26 kDa were down regulated in response to bacterial infection. Sterile injury in species C resulted in the synthesis of 32 kDa, a new polypeptide and activated expression of four naïve polypeptides 152, 56, 24 and 11 kDa (Fig. 1.15, Table 1.12). Further, two polypeptides of molecular weight 81 and 45 kDa were found to be down regulated. The bacterial infection up regulated the expression of six naïve polypeptides 180, 152, 56, 26.5, 24 and 11 kDa. Further, four polypeptides of 198, 81, 45 and 43 k Da were down regulated.

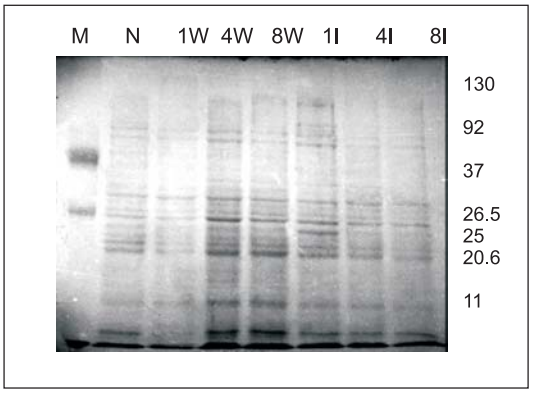
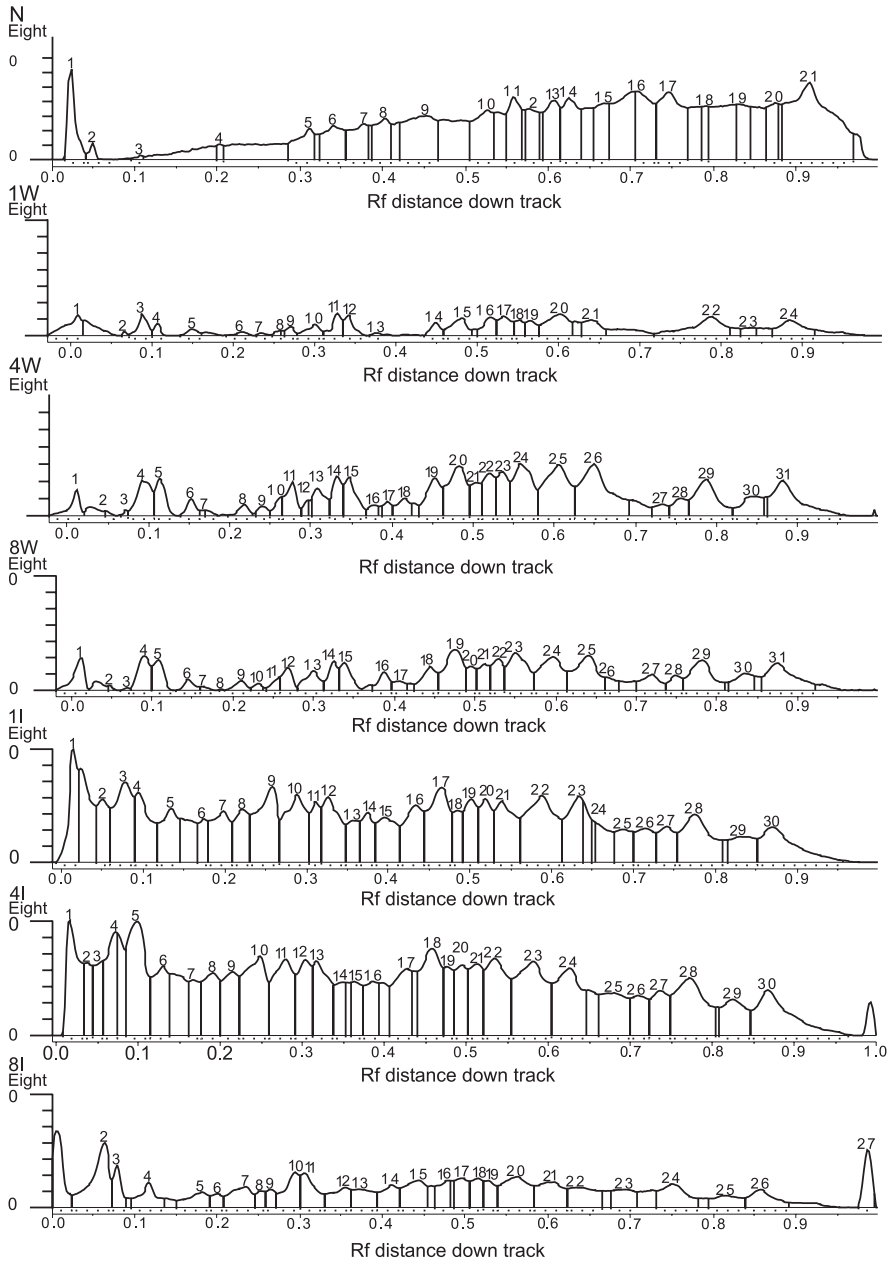


Fig. 1.14: Haemolymph of species B after injury with entomological needle contaminated with or without *M. leutus* (N : Naïve female; W: Sterile injury, 1, 4 and 8 : Hours after treatment; I : Infected with *M. leutus*)

The haemolymph of all these species responded differentially to sterile injury and against gram-positive bacterial challenge. Various injuries and *M. leutus*-responsive polypeptides detected in the haemolymph are candidates for future in-depth studies.

1.4.2 Quantitative analysis of enzyme phenoloxidase in *An. stephensi*

Phenoloxidase (PO) is one of the major enzymes of immune system in mosquitoes, whose expression can be seen phenotypically as melanisation—a phenomenon of deposition of melanin on the surface of invading foreign body. The expression of PO was studied in *An. stephensi* upon feeding on normal and *P. vinckei petteri* infected blood meal. For this purpose, 4–6 day old adult female mosquitoes from same batch were fed on healthy and *P. vinckei petteri* infected mice for one hour after nine hour starvation. Only full-fed mosquitoes were considered for the experiment. Body tissue (excluding midgut) was dissected in ice-cold sodium



Densitogram 1.1: Response of the haemolymph of *An. culicifacies* species A to injury and *M. leutus* infection (N : Naïve female; W: Sterile injury, 1, 4 and 8 : Hours after injury; I : *M. leutus* infection)

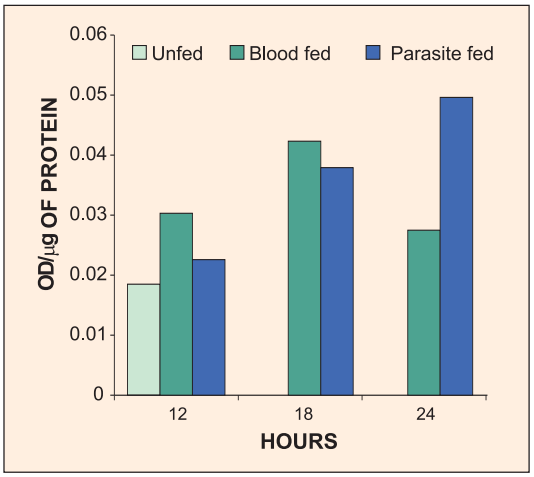


Fig. 1.16: Phenoloxidase activity (OD/µg of protein) in body tissue of *An. stephensi* upon normal and *P. vinckei petteri* infected blood meal

phosphate buffer (0.01 M, pH 7.2) at different time intervals (12, 18 and 24 h) post feeding and stored in liquid nitrogen. Tissues were homogenised in sodium phosphate buffer so as to obtain 25 µl supernatant. Part of the sample aliquot (5 µl) was used for protein estimation using Bradford method. PO assay was carried out by incubating the remaining extract (20 µl) with 500 ml 2 mM tyrosine in sodium phosphate buffer for 2 h at 30°C and absorbance was read at 420 nm. The whole experiment was repeated thrice. Activity of PO after 12 and 24 h of feeding on healthy mice was found to be more or less similar, but the activity at 18 h post feeding was significantly higher. On the other hand, PO activity in mosquitoes fed on *Plasmodium* infected blood, showed significant increase with time (Fig. 1.16) which indicated the probable role of PO in the immune response of mosquitoes against invading foreign body. Comparative immune response studies involving species A, B and C of the Culicifacies Complex are in progress.

1.4.3 Biochemical and molecular characterisation of Nitric Oxide synthase in *An. culicifacies*: relevance for refractory mechanism

The drive to identify novel control strategies has, in part, focused on identifying mosquito gene products that impart refractory phenotypes. Our goal is to develop tools for altering the vector competence of *An. culicifacies* which requires understanding the mechanism of vectorial resistance to the malaria parasite including biochemical and molecular studies of vector-parasite interactions. We have planned to use AcNOS (*An. culicifacies* Nitric Oxide Synthase) responses in mosquito vectors to *Plasmodium* as a tool to explore critical components of parasite development in mosquitoes and correlate it to the mechanism of refractoriness.

Specific activities of AcNOS (sp. B) in lysates of non-blood fed, blood fed, uninfected or *P. falciparum* infected mosquitoes at 6 and 9 days post blood meal activity were measured with or without NOS inhibitor L-NAME (1mM). Difference in AcNOs specific activities was much higher in infected and uninfected mosquitoes on Day 9 than on Day 6 in

Table 1.13. AsNOS and AcNOS specific activity in uninfected and infected mosquitoes gut samples

Sample	*An. stephensi*		*An. culicifacies* A		*An. culicifacies* B	
	Control	L-NAME	Control	L-NAME	Control	L-NAME
Non-blood fed	38.4	13.7	35.8	17.9	36.5	16.8
Day 6 infected	14.4	16.3	18.6	13.7	18.9	12.4
Day 6 uninfected	9.9	10.0	12.6	11.9	13.2	11.0
Day 9 infected	66.0	20.3	68.2	18.6	93.3	30.9
Day 9 uninfected	12.4	8.4	14.8	10.3	25.9	17.8

This data indicate significant differences at Day 6 and 9 parasite infection of NOS activity in control reactions. L-NAME (N-nitro-L-arginine methyl ester) inhibited in the infected mosquitoes guts.

Table 1.14. Primer details (Exon size, features , sequence, etc)

Exon no.	Exon size (bp)	Features	Amplimer size (bp)	Sequence(5'–3')
1	402	Haeme	200	ATGAGGACCAACTATCGGG GCCTTGGTGACAATGCTC
7	183	Haeme	163	AAGATGGGACTGGACACGC TGGTTTCGTTCTCAAAGTGC
15	170	FAD Ppi	162	GTGTCTACAAATCTTGGGAACC GCATCAGAAGCCTTTCCTCA
17	138	FAD NADPH	108	ATGGCTCTTCTTTGGCTGTC CGTGAAAGTGCCAGGAAAAC

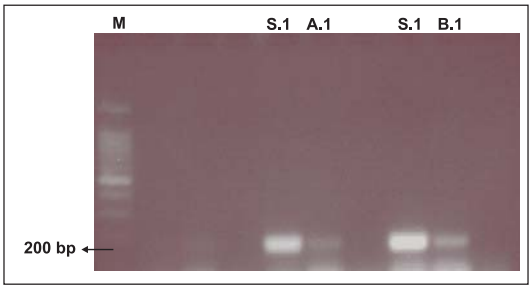


Fig. 1.17: PCR amplification of NOS gene using Primer 1 and 2 in *An. culicifacies* species B

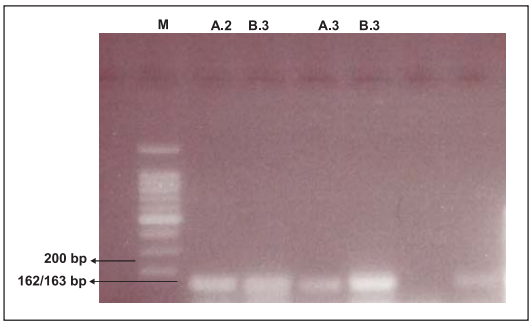


Fig. 1.19: PCR amplification of NOS gene using Primer 2 and 3 in *An. culicifacies* species A and B

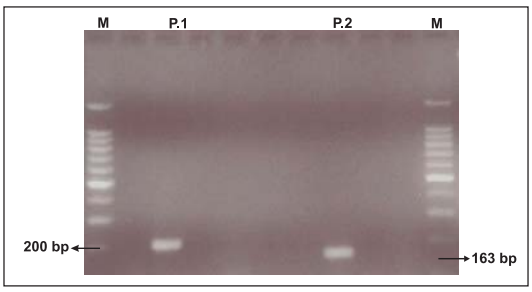


Fig. 1.18: PCR amplification of NOS gene in *An. culicifaces* species A and B using Primer 1

control reactions. The effect of L-NAME on AsNOS activity in infected mosquitoes was relatively unchanged by L-NAME at 6 days, whereas activity in infected mosquitoes was significantly inhibited by the inhibitor three days later on 9 Day (Table 1.13).

Four Primers were designed complementary to the *An. stephensi* exonic regions 1, 7, 15 and 17 encoding for the co-factors haeme, FAD PPi, FAD NADPH respectively (Table 1.14). Three primers P1, P2 and P3 primers have shown the amplification of the NOS gene by PCR (Figs. 1.17 and 1.18). Amplification of 200 base pairs against Exon 1 and Exon 7 and 15 have also shown the amplification of 163 base pairs (Figs. 1.18 and 1.19). This understanding is critical in evaluating the potential for manipulating *Anopheles* NOS gene expression to generate a refractory phenotype and this work will also reveal novel aspects of *Plasmodium* physiology for transmission blocking strategies.