

1. Entomology

VECTOR BIOLOGY

Anopheles culicifacies Complex

Bionomics and Distribution Pattern

Anopheles culicifacies population from highly malarious villages in the District Angul (Orissa) was examined for sibling species composition. Results revealed that species B and C were sympatric in the study villages comprising 57.7 and 42.3% respectively of the total identified. Both the species were found primarily zoophagic. In Madhya Pradesh, cytological examination of *An. culicifacies* samples from hilly-forested areas of District Dindori revealed predominance of species C, an established vector of malaria. In study villages of Kutch and Surendranagar districts (Gujarat) species A was predominant and polymorphic for i¹ inversion. Predominance of species A correlated well with high malaria incidence in study areas of these districts.

Studies on detection and characterisation of organophosphate-resistance in *An. culicifacies* sibling species in Madhya Pradesh

Surveys carried out in villages of Mohkher and Pandhurna blocks in District Chhindwara (M.P.) in November and December 2001 and March 2002 on the M.P.– Maharashtra border revealed that the *An. culicifacies* population was resistant to DDT (36–84%) and malathion Mohkher population was 14–40% resistant, while in Pandhurna the resistance was in the range of 36–63%.

Further studies were carried out in October 2003 on this species to assess the susceptibility status to different insecticides, biochemical resistance mechanisms, differential susceptibility status of sibling species to malathion and synergist studies to determine resistance mechanisms. Complete mortality was observed in *An. culicifacies* against organophosphate-insecticides fenitrothion and fenthion, carbamates, propoxur, bendiocarb and to deltamethrin, a synthetic pyrethroid insecticide in insecticide susceptibility tests. To malathion the species was 50% susceptible and confirmed earlier results.

Synergist exposures with a carboxylesterase inhibitor, triphenylphosphate (TPP) followed by malathion have indicated continued synergism with different concentrations (5–25%) of TPP impregnated papers. The observed mortalities were in the range of 69–88% and with malathion alone it was 50%. While exposures with a mixed function oxidases (MFOs) inhibitor, piperonyl butoxide (PBO) followed by malathion indicated continued antagonism against different concentrations (5–25%) of synergist impregnated papers and non-involvement

Mapping the geographic distribution of *An. culicifacies* species revealed prevalence of sibling species B and C in hilly-forest areas of District Angul (Orissa); predominance of species C in District Dindori (M.P.) and species A in Kutch and Surendranagar districts (Gujarat)

Biochemical studies on malathion resistant *An. culicifacies* population from District Chhindwara indicated involvement of carboxylesterase as the major mechanism for conferring resistance. PCR assays indicated differential susceptibility status to malathion in *An. culicifacies* species B & C

Standardised PCR assays could differentiate so far reported all five sibling species of *An. culicifacies* complex. Results of molecular assays correlated with cytological identification

of MFOs as a mechanism to confer malathion resistance. The observed mortalities were in the range of 27–38% and were less than the observed mortality (50%) with malathion alone.

Microplate assays for determining resistance mechanisms indicated non-involvement of general esterases and insensitive acetylcholinesterase in conferring malathion resistance. These assays have supported the observations made with synergist (TPP)-malathion bioassays indicating the involvement of carboxylesterase for conferring malathion resistance.

PCR assays were carried out to assess the prevalence of sibling species and species B and C were found sympatric in respective proportions of 73% (n=101) and 27% (n=37). The observed percent mortalities against malathion in species B and C were respectively 68 and 13.5% ($p > 0.001$) indicating increased resistance in species C. Similar observations were made in our earlier studies in Andhra Pradesh and Gujarat states where these two species are prevalent and sympatric.

Molecular diagnostic assays for the identification of members of *Anopheles culicifacies* complex

The two regions, inter-transcribed sequence 2 (ITS2) of rDNA and cytochrome oxidase II (COII) of mitochondrial DNA were analysed to find species-specific variations to differentiate the so far reported five sibling species of *An. culicifacies* complex. The sequence alignment of COII was utilised to design primers that could differentiate all the five species in two PCR assays on the pre-grouped A/D and B/C/E species by D3/D2-PCR assay. The approach followed for differentiating all the five members of *An. culicifacies* complex was—first, D3/D2 PCR assay to differentiate A/D from B/C/E; second, A-D-PCR to differentiate species A from species D; and third, B-C-E-PCR to differentiate the three species, B from C from E. These PCR assays were validated for field use on about 250 mosquitoes collected from five districts in five states having different sympatricities and the results of molecular assays well correlated with cytological identification.

Population Genetic Analysis of *An. culicifacies* Species A

In addition to the existing 17 microsatellite markers, 14 new markers have been isolated during the year. Primers have been designed for these markers and were tested on positive controls (plasmids) for assessing the amplicon sizes. The size ranged from 104 to 184 bps.

Genotyping was done on two more *An. culicifacies* species A populations from Allahabad (n=24) and Udaipur (n=14) for eight markers. These populations have been found polymorphic for these markers.

In situ hybridization is being done with these markers to construct the physical map of the species A using biotin labelled probes of the clones on the polytene chromosomes. These studies are being done on laboratory reared and field collected species A females.

***Anopheles fluviatilis* Complex**

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Distribution, Bionomics and Biology of Sibling Species

Mapping the geographical distribution of *An. fluviatilis* sibling species continued. Samples examined from Kutch, Narmada, Vadodara (Gujarat) and Mysore (Karnataka) districts revealed prevalence of only species T whereas in district Bhopal (Madhya Pradesh) species T and U were found sympatric. *An. fluviatilis* collected from Iran Shahr, Baluchistan (Iran) were also examined and species T was found prevalent in that area. In contrast only species S was prevalent in Nuapada district (Orissa) which was found highly anthropophagic.

In order to resolve taxonomic status of the new cytological variant observed in *An. fluviatilis* complex, a longitudinal study was carried out in Laksar PHC of District Hardwar (Uttaranchal). Cytological examination of *An. fluviatilis* samples collected from villages Dargahpur, Auspur and Ismilepur during pre-monsoon, monsoon and post-monsoon months revealed that the new cytotype was prevalent in all the seasons and in sympatric association with species T and U. Detailed examination of ovarian polytene chromosomes revealed yet another inversion on chromosome arm 3 of the new cytotype. Thus, presence of two fixed paracentric inversions on polytene chromosomes with total absence of inversion heterozygotes unequivocally establish this cytological variant as new species provisionally designated as species V in the *An. fluviatilis* complex. Morphological identification of cytologically identified specimens confirmed them as *An. fluviatilis*. Species V was found sympatric with species T and U in all the study villages and majority of its specimens were found resting in human dwellings or mixed dwellings. Studies on the bionomics and vectorial potential of species V have been initiated. In addition, efforts are being made to colonise this new species for various laboratory studies.

Development of Comprehensive PCR-based Assay for the Identification of all the Members of *An. fluviatilis* Complex in Consequence of Discovery of New Species

Previously an allele-specific PCR assay was developed for the identification of all three known members of the *An. fluviatilis* complex (species S, T and U), which is based on D3 domain of 28S rDNA (Singh *et al*, *Am J Trop Med Hyg* 2004; 70: 27). The discovery of new species—species V, in *An. fluviatilis* species complex necessitated the development of a comprehensive molecular assay for the identification of all members of the complex. When new species was tested with the existing species specific diagnostic PCR assay, it was not possible to differentiate species V from species U.

To develop comprehensive PCR-based assay, D3 domain of 28S rDNA of new species was sequenced and aligned with the sequences of other three members

Population cytogenetic studies established the cytological variant observed in *An. fluviatilis* population in District Hardwar (Uttaranchal) as new species provisionally designated as species V

Fourteen new microsatellite markers were isolated in addition to existing 17 markers for population genetic analysis of *An. culicifacies*

of the complex. The sequence of species V was found to differ from rest of the three species by at least four base pairs. However, these differences were not suitable to design species V-specific primer, therefore the sequences of all the four species were screened for presence of unique restriction sites. Species V was found to have three unique restriction sites which were absent in all other species. Thus, a PCR-RFLP assay was developed which can differentiate all the members of the complex (Fig. 1.1). The assay is to be validated using cytologically-identified specimens.

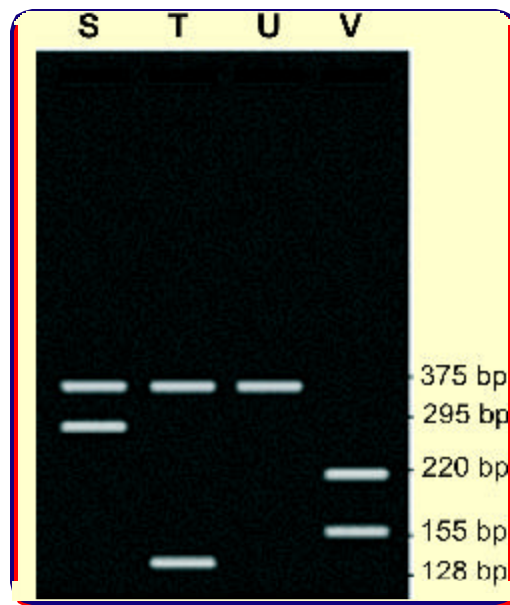


Fig. 1.1: Diagrammatic representation of PCR-RFLP assay for differentiation of all members of *An. fluviatilis* complex (S—Species S; T— Species T; U— Species U; V—Species V)

A new PCR assay was developed to differentiate *An. fluviatilis* species S, T, U & V. The newly identified species V has three unique restriction sites which the others do not possess

***Anopheles minimus* Complex**

Morphologically identified specimens of *An. fluviatilis* collected from Assam were sequenced for D3 domain of 28S rDNA. Alignment of these sequences with database of GeneBank using BLAST and alignment with *An. minimus* sequence generated by MRC revealed complete homology with *An. minimus* species A. Keeping in view that the *An. fluviatilis* is sympatric with *An. minimus* species A in this area, investigation is on whether morphologically identified *An. fluviatilis* from Assam are *An. minimus* species A. Other variable regions of rDNA are also being sequenced for divergence and phylogenetic studies.

***Anopheles dirus* Complex**

Identification of Members of *An. dirus*

Species distribution of *An. dirus* complex in Arunachal Pradesh and Assam was established by PCR assays using published primers designed from ITS2 region (Walton *et al*, *Med Vet Entomol* 1999; 13: 24–32). The assays were carried out on few field-collected mosquitoes (n=11). Results indicated prevalence of species D (diagnostic fragment of 306 bp size) in the two collections made from Arunachal Pradesh and Assam states (Fig. 1.2).

Mosquito Fauna Survey and Identification Key

Mosquitoes of Deciduous Dry Area (Bhopal, Madhya Pradesh)

A mosquito fauna survey was carried out in deciduous dry area of Bhopal district during February 2003. A total of 23 villages were surveyed covering

the entire topography—hilly, plain, irrigated and urban areas of the district. A total of 6927 mosquitoes belonging to six genera—*Anopheles*, *Aedes*, *Culex*, *Armigeres*, *Mansonia* and *Toxorhynchites* were collected using the standard

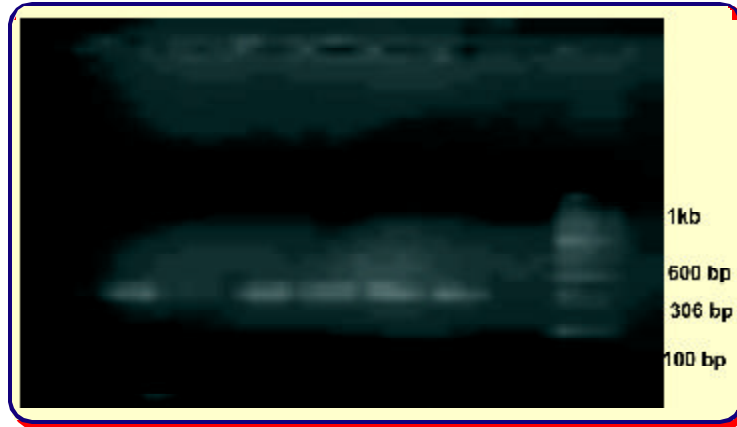


Fig. 1.2: Diagram showing diagnostic fragment for the identification of *An. dirus* species D

WHO techniques. The most dominant genus was *Anopheles* (5231 specimens) followed by *Culex* (933 specimens) and *Aedes* (247 specimens). In genus *Anopheles* 11 species were collected. The most dominant species was *An. culicifacies* followed by *An. subpictus* and *An. annularis*. Out of 11 species three vector species—*An. culicifacies* from rural areas, *An. fluviatilis* from foothill areas and *An. stephensi* from urban areas were collected. The larvae of eight anopheline species were collected from different habitats—ponds, streams, canals, wells, storage tanks, etc. Maximum number (37) out of 46 *An. fluviatilis* were collected during the night collection. All other species were found resting indoor and maximum number of specimens were collected during the day time. This is the first detailed report of mosquito fauna of Bhopal district.

Anopheles Identification: Field Key

With the financial support of Defence Research Laboratory, Tezpur preparation of a pictorial key to identify the 58 species of Indian anophelines in the field by researchers, field workers and technicians is in progress. Drawings of 40 anopheline species have been completed and identification table for the species is also in progress.

VECTOR-PARASITE INTERACTIONS

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

Serine protease in recalcitrant and susceptible strains of *An. culicifacies*

Structure of gene: Earlier the cloning of serine protease gene from refractory and susceptible strains of mosquito was reported. A comparison of cDNA sequence from these strains did not reveal any difference. Nevertheless the northern blot analysis and quantitative RT-PCR clearly revealed differences in the abundance of the transcript, which co-related with the observed differences in the catalytic activity of serine proteases in susceptible and recalcitrant strains. To account for such an observation we isolated genomic clone from susceptible and recalcitrant

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A pictorial key for the identification of all 58 anophelines was developed for use in the field

Molecular studies on *P. vivax*-refractory *An. culicifacies* revealed quantitative difference in serine protease transcript in refractory and susceptible strains

strains and screened for the presence of any intron. A 78 bp intron was detected at the 5' end of the serine protease. Junction and body sequence of intron from either strain did not reveal any difference, which could account for the reduced catalytic activity in the susceptible strain. We now propose to isolate promoter for serine protease from both the strains of mosquito and examine its efficiency using reporter genes. It is likely that a mutation in the promoter is responsible for reduced serine protease activity in the susceptible strain.

Regulation of serine protease and expression in *E. coli*

Serine protease is synthesised as a zymogen and is activated upon removal of propeptide located at its N-terminal end. It is generally believed that the propeptide inhibit their cognate enzyme specifically. We synthesised a peptide corresponding to the pro-region. Titration of serine protease with various concentrations of propeptide exhibited a strong inhibition by the propeptide. The K_i for the propeptide was calculated by plotting double reciprocal plots and compared with a routinely employed serine protease inhibitor, leupeptin. The K_i for the propeptide was $0.17\mu\text{M}$ and for leupeptin was $2\mu\text{M}$. We are now exploring the possibility if the observed difference in the catalytic activity of serine protease is a consequence of differential release of propeptide from the zymogen.

Phenol oxidase activity in *An. culicifacies*

The 2.4 kb cDNA ORF of the proPO was sub cloned in a pET32a expression vector between NOT1 sites and was successfully expressed in BL21DE3 host strain of *E. coli*. The protein product of cloned proPO ORF corresponds to 97 kDa. Ac-proPO is expressed as a Trx fusion protein in pET32a vector. The expression

In northern blot analysis it was revealed that upon blood meal the transcript abundance increased significantly in the recalcitrant females, while it was barely found in the susceptible females of *An. culicifacies*

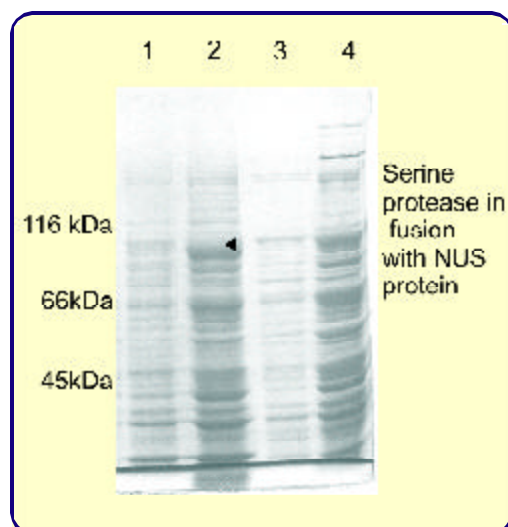


Fig. 1.3: Expression of 30 kDa serine protease in fusion with 65 kDa NUS protein using pET43a expression vector. Inclusion bodies prepared from uninduced culture (lane 1) and induced culture (lane 2). Supernatant prepared from uninduced culture (lane 3) and induced culture (lane 4)

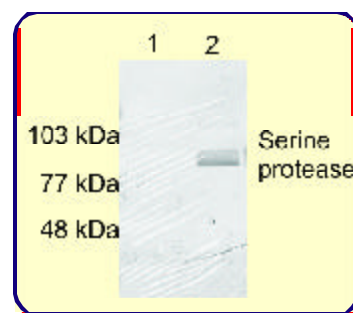


Fig. 1.4: Western analysis of recombinantly expressed serine protease using anti-His antibodies. Lane 1—Inclusion bodies prepared from uninduced culture and lane 2—induced culture

product was found to be present in inclusion bodies. At several growth regimen or IPTG induction conditions expression was detected in the soluble fraction only. To obtain the recombinant prophenol oxidase in catalytically active form, the 2.4 kb Ac-proPO was re-cloned in pET43a vector and expression was studied in BL21DE3 *E. coli* strain. In pET43a, a phenol oxidase is expressed as a NUS fusion protein at an expected size of 130 kDa. Expression was observed in the soluble fraction of the total *E. coli* proteins. On an incubation of soluble protein containing 30–80% phenol oxidase, with Dopamine or 4-Methyl catechol, we could not observe any activity of the phenol oxidase either before or after activation with trypsin (Figs. 1.3 & 1.4).

Northern blot analysis of Ac-proPO expressions

Using a 2.4 kb P³² labelled Ac-proPO as a probe, a transcript of expected size was detected in a Northern blot experiment of total body RNA from recalcitrant strain whereas a low expression was also observed in a susceptible strain. Adult males, which do not feed on blood, did not show any sign of proPo expression. To see the stage-specific expression of the prophenol oxidase, total RNA from different developmental stages of the mosquito was probed with the same probe as above. Result showed a high expression of Ac-proPO in the IV instar larvae, as it was low in the II and the III instar larvae. Upon blood meal the transcript abundance increased significantly in the recalcitrant females, while it was barely detectable in the susceptible female mosquitoes.

VECTOR CONTROL

Evaluation of VectoBac Tablets (Formulation of *Bti* H-14) against Larvae of Mosquito Vectors (Contract Research Project with M/s. Sumitomo Chemicals India Pvt. Ltd., Mumbai)

A new anti-larval product VectoBac tablet was evaluated in small-scale field trials in specified breeding habitats of *An. stephensi* and *Ae. aegypti* in NCT of Delhi, Chennai and Nadiad.

In natural field conditions the testing was carried out at different doses in water storage cement tanks, iron drums, desert coolers and mud pots to ascertain the efficacy of the test larvicide which was assessed by measuring the larval density. VectoBac tablets were used at the dosage of $1/2$, 1 and 2 tablets per sq m and density was monitored up to 3 days and after an interval of 7 days.

Results of field testing in NCT of Delhi showed that dose of 2 tablets (0.76 g per sq m) in cemented tanks gave complete control of late instars and pupae of *An. stephensi* and *An. subpictus* up to 2 weeks period. Cent percent reduction in the densities of immatures was also achieved up to 2 weeks period against *Ae. aegypti* and *Cx. quinquefasciatus* in iron drums, desert coolers and mud pots when treated @ 2 tablets per habitat. VectoBac tablets were safe to non-target species *Gambusia affinis*, a larvivorous fish and notonectid bug *Anisops sordae*.

VectoBac tablets formulation of *Bti* H-14 provided complete control of immature & stages of vector mosquitoes up to two weeks.

The results of this study would help in selection of appropriate dosage and frequency of application and the use of VectoBac tablets can be an additional tool for control of mosquito larvae and could be one of the choices in the larval control programmes in urban areas.

Laboratory and Field Evaluation of VectoBac WDG (*B. thuringiensis* var *israelensis*) Formulation against Immatures of Mosquitoes (Sponsored Research Project by M/s. Sumitomo Chemicals India Pvt. Ltd., Mumbai)

A study to evaluate the bioefficacy of a new formulation of *Bti*—VectoBac WDG provided by M/s. Sumitomo Chemicals Pvt. Ltd., was carried out in the laboratory against immatures of *An. culicifacies*, *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*. The biolarvicide formulation was most effective against larvae of *Cx. quinquefasciatus*, followed by *Ae. aegypti*, *An. stephensi* and *An. culicifacies*. The LC_{50} values against these four mosquito species were determined as 0.025, 0.046, 0.245 and 0.35 mg/l, respectively.

In field conditions the trial was carried out in breeding habitats ranging between 50 and 500 m² for the breeding of *An. culicifacies* and smaller breeding habitats such as cement tanks for the breeding of *An. stephensi* and desert coolers for the breeding of *Ae. aegypti*. Observations were made by counting immatures of target mosquito species in the experimental and control habitats, before and after the treatment up to 3 days and then after an interval of 7 days. Four doses were applied in the field @ 0.05, 0.1, 0.2 and 0.5 g/m² with the help of sprayer after dilution. Trials are in progress and initial results indicated that VectoBac application produced 100% control of L-3 and L-4 stages of *An. stephensi* up to Day 5 at all the dosages except 0.05 g/m². Around 80% reduction was however maintained up to two weeks at 0.05 g/m². The impact on *Ae. aegypti* breeding in coolers was 100% up to one week.

Bio-efficacy of Pirimiphos-Methyl 50% EC against Immatures of *Anopheles* and *Culex*

Results of laboratory evaluation of pirimiphos-methyl 50% EC against the larvae of *An. culicifacies*, *An. stephensi* and *Cx. quinquefasciatus* are shown in Table 1.1. The larvicide formulation was relatively more effective against immatures of

Table 1.1 Bio-efficacy of pirimiphos-methyl against late III instar mosquito larvae in the laboratory bioassay test

Mosquito species	Lethal concentration (ppm a.i.)		χ^2 (df)
	LC_{50} (95% Confidence limit)	LC_{90}	
<i>An. culicifacies</i>	0.032 (0.027–0.037)	0.057	1.79 (2)
<i>An. stephensi</i>	0.023 (0.019–0.027)	0.045	1.22 (2)
<i>Cx. quinquefasciatus</i>	0.040 (0.035–0.045)	0.114	10.54 (2)

VectoBac WDG application @ >0.1 g/m² could produce 100% larval mortality in *An. stephensi* and *Ae. aegypti* up to five days and one week respectively

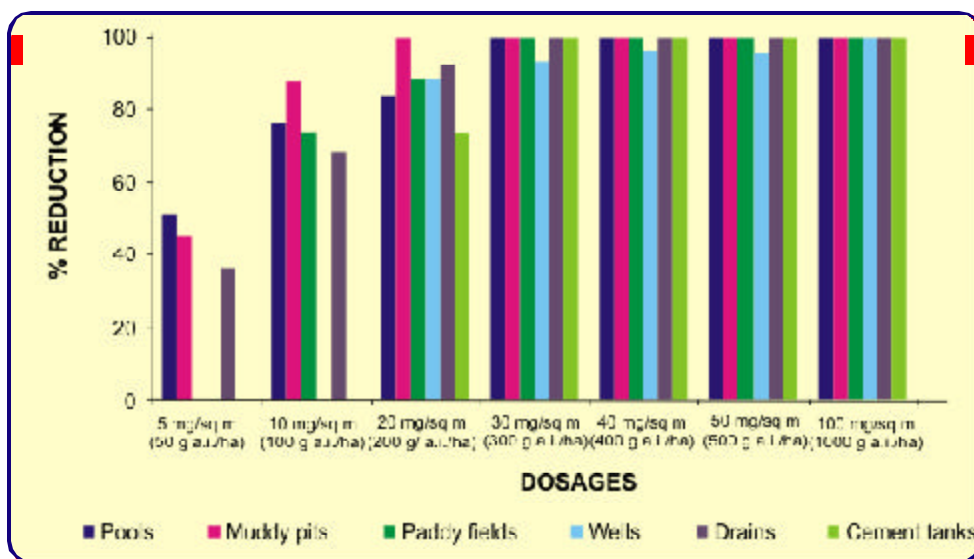


Fig. 1.5: Percent reduction in larval density of anophelines within 24 h after treatment

Anopheles species than *Culex* species. The LC_{50} and LC_{90} values against the larvae of *An. culicifacies* and *An. stephensi* were 0.032 and 0.05 ppm; and 0.023 and 0.045 ppm, respectively, as against 0.04 and 0.114 ppm for *Cx. quinquefasciatus*.

Fields trials were carried out in muddy pits and pools, irrigation channels, unused wells, cement tanks, waste commodes, polluted drains and paddy fields to determine the optimum dosage against immatures of *Anopheles* and *Culex* spp. Application of pirimiphos-methyl @ 100 g a.i./ha and above resulted in 100% reduction in the larval density of anophelines within 24 h after the treatment (Fig. 1.5). However, the bio-efficacy (percent reduction in the larval density on

In laboratory and field trials, Pirimiphos-methyl 50% EC was found to be more effective against immature stages of anophelines in fresh water than culicines in polluted water

Table 1.2 Bio-efficacy of pirimiphos-methyl 50% EC against larvae of anopheline immatures

Habitats	% Reduction after one week						
	5 mg/ m ² (50 g a.i./ha)	10 mg/ m ² (100 g a.i./ha)	20 mg/ m ² (200 g a.i./ha)	30 mg/ m ² (300 g a.i./ha)	40 mg/ m ² (400 g a.i./ha)	50 mg/ m ² (500 g a.i./ha)	100 mg/ m ² (1000 g a.i./ha)
Pools	51.2	76.3	83.4	100	-	-	-
Muddy pits	45.1	87.6	100	-	-	-	-
Paddy fields	-	74	88.2	100	-	-	-
Wells	-	-	88	93	96.5	96	100
Drains	36	68.4	92.3	100	-	-	-
Cement tanks	-	-	73.3	100	-	-	-

Surface area—Pools (8 to 15 m²); Muddy pits (3.6 to 4.2 m²), Paddy fields (542 to 916.6 m²); Wells (2.4 to 3.6 m²); Average area of waste commodes₂ was 5.9 m² and depth was 6.45 inches. Average area of cement tanks in Goa was 5.6 m² and depth was 17 inches. Drains (14 to 106.2 m²) and Cement tanks (5.5 to 8.6 m²).

Day 7 after the treatment) of pirimiphos-methyl against immatures of anopheline species in different types of breeding habitats ranged between 64 and 100% @ 100 g a.i./ha and > 80% @ 200 g a.i./ha (Table 1.2). In pits and pools with *Anopheles* breeding consisting mainly of *An. culicifacies*, 100% reduction in immature density was obtained with a dosage of 200 and 300 g a.i./ha respectively. At lower doses @ 100 g a.i./ha, 76 to 87% reduction was observed on Day 7 in small shallow water bodies. At higher doses @ 400 and 500 g a.i./ha, 100% reduction was recorded up to three weeks. In paddy fields and irrigation channels, with anopheline breeding consisting mainly of *An. culicifacies* and *An. subpictus*, 100% reduction of immature density was observed @ 300 g a.i./ha. In wells used for irrigation purpose, supporting breeding of *An. culicifacies* and other species, 93 to 96% reduction of anopheline immatures density was observed @ 300 to 500 g a.i./ha, while cent percent reduction was observed @ 1000 g a.i./ha. In cement tanks where *An. stephensi* and *An. subpictus* breeding was observed, 100% reduction in density/dip was observed @ 100 to 200 g a.i./ha.

An. stephensi breeding was also commonly observed in waste commodes (WCs) in Goa. The efficacy of pirimiphos-methyl against breeding of *An. stephensi* in WCs, was also determined @ 100 and 200 g a.i./ha. Results revealed > 80% reduction @ 100 g a.i./ha as against 100% reduction @ 200 g a.i./ha up to one week (Table 1.2).

These trials clearly indicate that in fresh water habitats of *Anopheles* species pirimiphos-methyl @ 200 g a.i./ha will be required to obtain 80 to 100% reduction invariably in all the habitats at weekly intervals.

Results of field trials against *Culex* species, carried out in polluted pools and muddy pits, unused wells and drains are given in Fig. 1.6 and Table 1.3. The efficacy of pirimiphos-methyl against immatures of *Culex* species was determined at different doses ranging from 50 to 1000 g a.i./ha. Results revealed almost 100% reduction in the density of immatures @ 200 g a.i./h with in 24 h after the

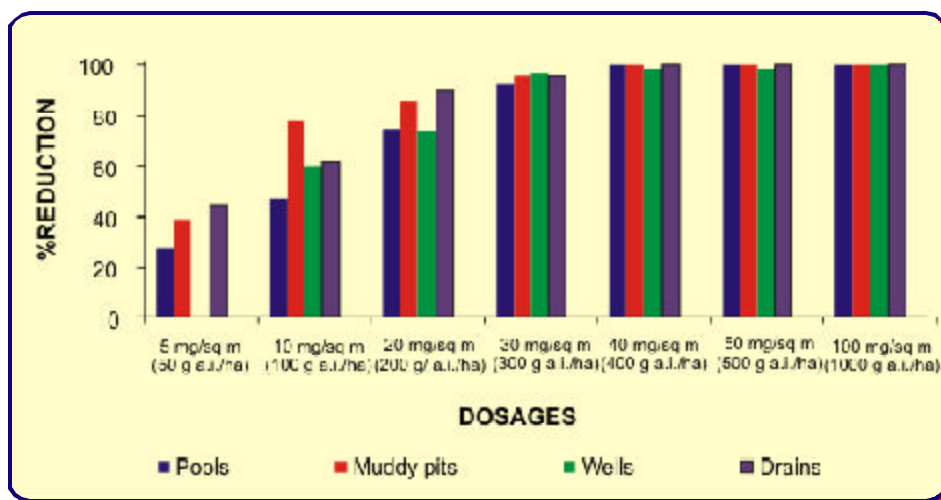


Fig. 1.6: Reduction in larval density of *Cx. quinquefasciatus* within 24 h after treatment

Table 1.3 Bio-efficacy of pirimiphos-methyl 50% EC against larvae of culicine immatures

Habitats	% Reduction after one week						
	5 mg/ m ² (50 g a.i./ha)	10 mg/ m ² (100 g a.i./ha)	20 mg/ m ² (200 g a.i./ha)	30 mg/ m ² (300 g a.i./ha)	40 mg/ m ² (400 g a.i./ha)	50 mg/ m ² (500 g a.i./ha)	100 mg/ m ² (1000 g a.i./ha)
Pools	27.6	47.3	74.6	93.1	100	–	–
Muddy pits	39.2	78.4	86	95.4	100	–	–
Wells	–	59.7	74	96.9	97.9	98.4	100
Drains	44.1	61.4	90.4	95.8	100	–	–

Note: Surface area—Pools (8 to 21.1 m²); Muddy pits (3.4 to 4 m²); Unused wells (1.69 to 3.3 m²); and Polluted drains (79 to 110 m²).

treatment in different habitats (Fig. 1.6). However, the reduction @ 200 g a.i./ha was even < 80% after one week but > 80% reduction was obtained at the dose of 300 g a.i./ha and above in all the habitats (Table 1.3). In unused wells supporting *Culex* breeding, 100% reduction in the density of immatures was observed at a dose of 300 g a.i./ha. At higher doses similar impact, however, persisted for three days to four weeks. In polluted drains supporting *Culex* breeding, 90% reduction was observed on Day 4 @ 200 g a.i./ha and higher doses. These trials clearly indicate that in case of culicine species in different types of polluted water habitats, 90–100% reduction in the density of immatures can be obtained @ 200–400 g a.i./ha by weekly application of the larvicide. These results also suggest that pirimiphos-methyl is more effective against anopheline immatures in clear water than culicine immatures in polluted water habitats.

Laboratory bioassays carried out to determine toxicity of pirimiphos-methyl against *Gambusia affinis* revealed that pirimiphos-methyl is not toxic to fish at a

Table 1.4 Toxicity of pirimiphos-methyl 50% EC against the larvivorous fish *Gambusia affinis* under laboratory condition

Dosage (ppm)	No. of fish in each replicate	% Mortality after (h)		
		24	48	72
1.0	25 x 4	24 (24)	24 (24)	24 (24)
0.5	25 x 4	12 (12)	12 (12)	12 (12)
0.25	25 x 4	4 (4)	4 (4)	4 (4)
0.125	25 x 4	0 (0)	0 (0)	0 (0)
0.0625	25 x 4	0 (0)	0 (0)	0 (0)
0.3125	25 x 4	0 (0)	0 (0)	0 (0)
Control	25 x 4	0 (0)	0 (0)	0 (0)

Figures in parentheses indicate number exposed.

concentration of < 0.25 ppm, as no mortality was observed in any of these concentrations tested up to 72 h. However, at higher concentrations there was some mortality in larvivorous fish in the laboratory bioassays and therefore, doses higher than 0.25 ppm are not safe to this non-target species (Table 1.4). Toxicity test of pirimiphos-methyl to the main non-target organism—*Gambusia affinis* which is being used extensively under EMCP, suggest that this larvicide should not be used at concentration above 0.25 ppm in habitats harbouring the larvivorous fish.

Evaluation of IGR-Triflumuron against Larvae of Mosquito Vectors (Contract Research Project with M/s. Bayer India Ltd., Mumbai)

Field trial carried out in Delhi and Sonapat district (Haryana) to evaluate IGR- Triflumuron was completed this year. In small-scale field trials Triflumuron was applied at doses of 0.25, 0.5 and 1 ppm in breeding habitats of *An. culicifacies* and *Cx. quinquefasciatus*. Results showed that a dose of 1 ppm was most effective up to two weeks against *An. culicifacies* in paddy field.

Prospecting for Botanical Pesticides: Screening of Bio-activity of Plant Extracts against Mosquitoes particularly *Anopheles* spp. (DBT Funded Collaborative Project)

This multi-institutional collaborative project funded by DBT was carried out in collaboration with various laboratories as shown in Table 1.5. Bio-activity of various herbal extracts/fractions/formulations received from five laboratories was determined against mosquitoes particularly the malaria vector *An. stephensi* using standard protocol which included larvicidal, adulticidal properties and mosquito repellency.

Since beginning of this project a total of 331 samples have been received at MRC for bioassays against malaria vector (*An. stephensi*). Of these 48 samples showed larvicidal activity (70–100% mortality) at 250 ppm, while 14 samples

Table 1.5 Activities of various laboratories involved in the project

Activity	Responsible laboratories
Collection, preservation and taxonomic identification of plants	RRL, Jammu; RRL, Trivandrum; NBRI, Bangalore
Extraction and fractionation of herbal products	IIT, Delhi; FRI, Dehradun; EID Parry (India) Ltd, Bangalore; RRL, Trivandrum ; RRL, Jammu
Bioassay testing of the efficacy against agricultural pests	IHBT, Palampur; EID Parry, Bangalore
Bioassay testing of the efficacy against mosquitoes (<i>An. stephensi</i>)	MRC, Delhi & Hardwar
Formulation of the selected samples	IIT, Delhi; IPFT, Gurgaon

showed insecticidal activity against adult mosquitoes and five samples have shown repellent activity for more than one hour (Table 1.6). Tables 1.7 to 1.9 show the results of preliminary screening of larvicidal, adulticidal and repellent activity of different samples received from different laboratories.

Table 1.6 Samples received at Malaria Research Centre and their screening status

Laboratory/ Institute	Received	Screened	Under screening	+ve results		
				L	A	R
RRL, Trivandrum	76	76	0	8	1	1
FRI, Dehradun	77	77	0	16	10	4
IIT, Delhi	56	56	0	5	2	–
RRL, Jammu	66	66	0	11	1	–
EID Parry, Bangalore	56	41	15	8	0	0
Total	331	316	15	48	14	5

L—Larvicidal; A—Adulticidal; R—Repellent property.

Table 1.7 Plant extract samples showing larvicidal activity within 24 hours

S. No.	Sample code number	Larvicidal activity		
		Show	LC ₅₀	LC ₉₀
1.	NBDB (4)-002B-07-P-13a	(+)	*	*
2.	NBDB (4) 022B-08-P-10a	(+)	*	*
3.	NBDB (4) 023B-07-P-10a	(+)	*	*
4.	NBDB (4) 042B-07-P-10a	(+)	*	*
5.	NBDB (2) 008A-06-P-01a	(+)	166	234
6.	NBDB (2) 010A-06-P-10a	(+)	*	*
7.	NBDB (2) 010A-06-P-10b	(+)	153	261
8.	NBDB (4)-001B-08-P-13a	(+)	117	276
9.	NBDB (4)-033B-07-P-10a	(+)	250	450
10.	NBDB (3)-022i-08-P-11e1	(+)	119	182
11.	NBDB (2)-005A-07-P-10a	(+)	138	230
12.	NBDB (2)-005A-07-P-10b	(+)	*	*
13.	NBDB (2)-005A-07-P-10c	(+)	53	266
14.	NBDB (2)-005A-07-P-04a	(+)	58	113
15.	NBDB (2)-005A-07-P-04b	(+)	77	133
16.	NBDB (2)-005A-07-P-04c	(+)	124	250
17.	NBDB (2)-055D-11-P02oil	(+)	87	132
18.	NBDB (4)-022B-08-P-11b	(+)	100	175
19.	NBDB (4)-023B-08-P-11b2	(+)	150	225
20.	NBDB (3)-022I-08-P-11E1	(+)	*	*
21.	NBDB (5) 021D-04-P-11b	(+)	100	150
22.	NBDB (1) 058T-12-P-01b	(+)	150	250

contd...

Of the 331 herbal extracts screened for pesticidal activity, 48 showed larvicidal, 14 insecticidal and five repellent activity when tested against *An. stephensi*

Table 1.7 (contd...)

S. No.	Sample code number	Larvicidal activity		
		Show	LC ₅₀	LC ₉₀
23.	NBDB (1) 058T-12-P-01c	(+)	120	190
24.	NBDB (1) 022-T-12-P-01b	(+)	75	175
25.	NBDB (1) 022-T-12-P-01c	(+)	160	200
26.	NBDB (1) N13 positive control	(+)	23	43
27.	NBDB (2) 048-A-10-P-10a	(+)	125	200
28.	NBDB (2) 048-A-10-P-10b	(+)	70	175
29.	NBDB (2) 055-D-12-P-02a	(+)	100	200
30.	NBDB (2) 055-D-12-P-02b	(+)	175	250
31.	NBDB (3) 005-D-10-P-10e1	(+)	72	121
32.	NBDB (3) 005-D-10-P-10a1	(+)	97	278
33.	NBDB (3) 005-D-10-P-10b2	(+)	156	248
34.	NBDB (1) 001-K-03-P-02C	(+)	202	287
35.	NBDB (1) N14-K-03-P-09a	(+)	26	57
36.	NBDB (1) N14-K-03-P-09b	(+)	*	
37.	NBDB (5) 056-A-07-P-04a	(+)	142	232
38.	NBDB (5) 056-A-07-P-04b	(+)	113	250
39.	NBDB (5) 056-A-07-P-04cd	(+)	55	125
40.	NBDB (5) 017-K-08-P-01a	(+)	175	317
41.	NBDB (5) 017-K-08-P-01b	(+)	*	
42.	NBDB (5) 017-K-08-P-01cd	(+)	*	
43.	NBDB (5) 052-Q-09-P-04a	(+)	*	
44.	NBDB (5) 052-Q-09-P-04b	(+)	*	
45.	NBDB (5) 042-E-06-P-08a	(+)	38	68
46.	NBDB (5) 042-E-06-P-08b	(+)	137	155
47.	NBDB (2) 005-A-07-P-10b	(+)	139	264
48.	NBDB (2) 008-A-06-P-01a	(+)	166	234

*To be determined after confirmation; (+) Positive activity.

Table 1.8 Plant extract samples showing insecticidal activity against adult mosquitoes

S.No.	Sample code number	Adulticidal activity
1.	NBDB(2)008A-06-P-01a	(+)
2.	NBDB(2)-005A-07-P-10a	(+)
3.	NBDB(2)-005A-07-P-10b	(+)
4.	NBDB(2)-005A-07-P-10c	(+)
5.	NBDB(2)-005A-07-P-04a	(+)
6.	NBDB(2)-005A-07-P-04b	(+)
7.	NBDB(2)-005A-07-P-04c	(+)
8.	NBDB(2)-055D-11-P02oil	(+)
9.	NBDB(1)- N-13	(+)
10.	NBDB(3)-005-D-10-p-10e1	(+)
11.	NBDB(5)- 042 E06 P08a	(+)
12.	NBDB(3)- 005-D-10P-10a	(+)
13.	NBDB(2)-005-A-07-P-10b	(+)
14.	NBDB(2)-055-D-11-P-02 oil	(+)

Table 1.9 Samples showing mosquito repellent activity

S.No.	Sample code number	Repellency activity
1.	NBDB(2)-008A-06-P-01a	(+)
2.	NBDB(2)-005A-07-P-10b	(+)
3.	NBDB(2)-005A-07-P-04a	(+)
4.	NBDB(2)-055D-11-P02oil	(+)
5.	NBDB (1)-N13 positive control	(+)

Larvicidal Activity of Crude Aqueous Extract of *Tribulus terrestris*

Larvicidal effect of crude aqueous extract of the leaf of a medicinally important plant *Tribulus terrestris* (Family: Zygophyllaceae) was tested against *An. culicifacies* species A and C, *Cx. quinquefasciatus* and *Ae. aegypti*. Third and fourth instar larvae were used for bioassays following standard WHO method for a range of concentrations (0.0025 to 0.3% in water). The calculated LC_{50} (lethal concentration for killing 50% of treated larvae) for different species were respectively: *An. culicifacies* species A–2100 ppm, *An. culicifacies* species C–4200 ppm, *Cx. quinquefasciatus*–3800 ppm and *Ae. aegypti*–3200 ppm. The calculated lethal values were at least 10 x more than the earlier recorded values for larvicidal effect of *Solanum nigrum* (Singh *et al*, *Curr Sci* 2001; 81: 1529–30). It prompted to test extracts from other parts in different solvents.

Effectiveness of Spinosins Mixture DKVR-0001 Mats in Repelling Mosquitoes

Variety of mats and coils are marketed in India to prevent mosquito bites. Mats prepared with spinosins mixture marketed by De Nocil, Mumbai were tested for their efficacy (commercial name–Tracer Cardboard mats 35 x 25 mm). Mats were placed in the electrical device in the room. After putting electrical device a volunteer was asked to place left arm inside the cage covered with nylon net for mosquito feeding. Concurrently control cages were used with simple cardboard soaked in water.

Results revealed that mixture of spinosins mats had strong repellent action against mosquitoes. Against *An. stephensi* a principal vector of malaria, the mats showed 94.7% protection. Similar degree of protection was obtained against *An. culicifacies* (90.6%). However, 91.8% protection was obtained against *Cx. quinquefasciatus* a pest mosquito and vector of filariasis in the country. It was interesting to note that there was low protection against *Ae. aegypti* (21.6%). The results were compared with the results of Good Night mats and there was no significant difference in the efficacy of both mats.

Larvicidal and Mosquito Repellent Activity of Chir (*Pinus longifolia*, Family: Pinaceae) Oil

Results of the larvicidal activity of Chir oil against different species of mosquitoes are presented in Table 1.10. In terms of lethal concentration for 50% mortality (LC_{50})

**Spinosins mixture
DKVR-0001 mats
provided >90%
protection against
An. culicifacies,
An. stephensi and
Cx.
*quinquefasciatus***

Table 1.10 Larvicidal activity of Chir oil against different mosquito species

Concentration (ppm)	No. tested	No. larvae dead		
		<i>An. stephensi</i>	<i>Cx. quinquefasciatus</i>	<i>Ae. aegypti</i>
200	100	84	88	96
100	100	38	50	50
50	100	6	24	24
25	100	8	10	10
12.5	100	2	4	4
6.25	100	0	0	0
Control	100	0	0	0

Chir oil was effective at doses ranging between 80 and 112 ppm against larvae of the three mosquito species. Of the three species tested Chir oil was most effective against *Aedes aegypti* ($LC_{50} = 82$ ppm) followed by *Culex quinquefasciatus* ($LC_{50} = 85.7$ ppm) and *An. stephensi* ($LC_{50} = 112.6$ ppm).

Table 1.11 shows the number of female mosquitoes, which landed on treated and untreated baits in eight night periods from dusk-to-dawn. The Chir oil

Table 1.11 Efficacy of Chir oil and lemon grass oil as mosquito repellent on human volunteers in village Dehra (PHC Dhaulana)

Repellent oil	<i>Cx. quinquefasciatus</i>		<i>An. culicifacies</i>	
	% Protection	Av. Protection time (h)	% Protection	Av. Protection time (h)
Chir oil	97.4 \pm 6.8	9	100	11
Lemon grass	98.5 \pm 5.5	9.6	100	11

provided 100% protection for 11 hours against *An. culicifacies*, a principal vector of the northern rural plain areas of India. Against *Culex quinquefasciatus* it provided 97% protection for nine hours. The results were compared with results of lemon grass oil and there was no significant difference in the efficacy of these two types of oils.

Mats prepared from Chir oil were also tested in the field. These mats provided 94.1% protection against bites of *An. culicifacies* with a protection time of 10.3 hours and 88% protection against *Cx. quinquefasciatus* with an average protection time of 7.6 hours.

Chir oil mats provided ~ 94% protection against *An. culicifacies*. Chir oil provided 100% protection up to 11 h against *An. culicifacies* and > 97% protection up to 9 h against *Cx. quinquefasciatus*

INSECTICIDE RESISTANCE

WHO Collaborative Study for the Establishment of Diagnostic Concentrations for Bifenthrin and Alphacypermethrin for Resistance Monitoring in Malaria and Dengue Vectors

Malaria Research Centre was identified for carrying out the studies to establish the diagnostic doses against two pyrethroid insecticides with six graded doses, alphacypermethrin (0.001 to 0.05%) and bifenthrin (0.01 to 0.5%). *An. culicifacies* species C, *An. stephensi* and *Ae. aegypti* were tested using standard WHO protocols. *An. culicifacies* registered 98% mortality against 0.0025% alphacypermethrin and 0.1% bifenthrin. Similarly for *An. stephensi* 94% mortality was registered against 0.05% alphacypermethrin and 99% against 0.25% bifenthrin. Likewise *Ae. aegypti* registered only 91% mortality against 0.05% alphacypermethrin and 99% against 0.25% bifenthrin. Results have been communicated to WHO for the determination of diagnostic doses against these insecticides.

OTHER STUDIES

Entomological and Epidemiological Field Investigations in Jaisalmer district, Rajasthan

District Jaisalmer of Rajasthan state was visited in the month of March 2004 for carrying out malaria situation analysis and to identify malraia risk factors. Epidemiological data of the district for the last 10 years (1994–2003) indicated that 142 sub centres had API >2 and were recognised as high risk areas.

In parasitological studies carried out during the field study a total of 64 blood smears were collected during active surveillance and 19 were positive giving SPR of 29.68. Out of 267 blood samples collected during mass blood survey, 25 were positive for malaria.

In seven PHCs surveyed two malaria vectors *An. stephensi* and *An. culicifacies* were prevalent. Density of *An. culicifacies* was maximum in canal area. *An. stephensi* was prevalent in high density in almost all the PHCs surveyed. Larval surveys were carried out in selected PHCs. *An. culicifacies* breeding was maximum in Indira Gandhi Canal distributaries, while *An. stephensi* was found breeding in all the PHCs surveyed. The most common breeding sites of *An. stephensi* were underground tanks locally called “tankas”. About 64% breeding of anophelines was exclusively in tankas and rest in other sites such as concrete cisterns and cemented tanks.

Insecticide susceptibility tests were carried out against both the malaria vectors and these were found susceptible to DDT, malathion and deltamethrin insecticides in high risk PHC, Ramgarh. Based on survey, risk factors were identified and recommendations were made to contain malaria in the district.

Entomology

MRC was identified by WHO for determining the diagnostic concentration of synthetic pyrethroids for field testing of susceptibility status of various mosquitoes

Entomological studies in Jaisalmer district showed that *An. culicifacies* and *An. stephensi* the major malaria vector species in this area were susceptible to DDT, malathion and deltamethrin

***Aedes aegypti* Survey in Certain Dengue Affected Localities of Municipal Corporation of Delhi**

As per the request of the Municipal Health Officer (MHO)-cum-Director Health Services (DHS), Municipal Corporation of Delhi (MCD), an entomological survey was carried out from 6–12 November 2003 by a team of Malaria Research Centre, Delhi in five localities reporting dengue fever cases as identified by MCD. These localities, namely Dayanand Colony (Lajpat Nagar Phase IV), Lajpat Nagar (Phase I and II), Kotla Mubarakpur are urban whereas Dayalpur Extension and Harsh Vihar are sub-urban. The survey was carried out during day time for both larvae and adults of *Aedes* mosquitoes in all the five identified localities by using WHO standard techniques. To assess the levels of *Ae. aegypti* infestation in four localities surveyed—Lajpat Nagar, Kotla Mubarakpur, Dayalpur Extension and Harsh Vihar; two indices—container index (CI) and breteau index (BI) were calculated.

A survey of five dengue affected localities in urban and sub-urban areas under MCD revealed maximum breeding of *Ae. aegypti* in overhead cemented tanks

During the survey seven types of breeding habitats—overhead tanks (cement and syntax), underground tanks (cement and syntax), ground cement tanks, ornamental fountains and mud-pots containing drinking water for birds were found supporting the breeding of *Ae. aegypti*. The maximum breeding was recorded in overhead cemented tanks (58.49%) out of 53 tanks checked in five localities. On the other hand, 17.36% of syntax overhead tanks were found positive out of 190 checked. Two ornamental fountains inside the drawing room in Lajpat Nagar Phase I and three mud-pots (two in Kotla Mubarakpur and one in Dayanand Colony) containing the water for birds were supporting heavy breeding of *Ae. aegypti*. It is also noteworthy to mention that in all the four colonies surveyed majority of overhead tanks were found inaccessible because they do not have fixed ladders, therefore, breeding in these tanks could not be checked. The maximum level of infestation of *Ae. aegypti* was found in Lajpat Nagar Phase I, II (CI = 8.93, BI = 17.33) followed by Kotla Mubarakpur (CI = 7.29, BI = 11.29) and minimum was recorded in Harsh Vihar (CI = 0.89, BI = 1.47). A total of 23 adult *Ae. aegypti* were collected from three localities, namely Kotla Mubarakpur (six specimens), Harsh Vihar (seven specimens) and Dayalpur Extension (10 specimens). The species was found resting inside the houses and cattlesheds on floor, under the furniture, cupboards and water storage tanks. The detailed report of the survey was submitted to ICMR (HQ) and MCD.