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# *INDIAN JOURNAL OF MALARIOLOGY*

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*Note:* The editor assumes no responsibility for the statements and opinions expressed by the contributors. This issue is delayed due to unavoidable circumstances.

REVIEW ARTICLE

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# Malarial Mitochondrial Genome: The 6kb Element

INDU SHARMA[a] and Y.D. SHARMA[a]

## INTRODUCTION

Malaria is re-emerging as the major infectious killer and it is the top priority tropical disease of the World Health Organization. Majority of global malaria cases currently reside in Africa but Asia also harbours a global threat in the form of epicentre of multi-drug resistant *Plasmodium falciparum*, which is gradually encompassing the tropical world. In the last decade enormous interest has been generated in the genomics of malaria parasite. This parasite contains three genomes—the nuclear genome comprising of 14 chromosomes and two extrachromosomal genomes: the 35 kb circular DNA and the 6kb element. The 35 kb circular DNA is known as plastid genome whereas the 6 kb element represents the mitochondrial genome.[1] We will limit our discussion here to the 6kb element also known as mitochondrial genome of malarial parasites.

The history of mitochondrial genome sequencing "project" is a long one. The human and bovine mitochondrial genomes were completely sequenced by 1981 and 1982, respectively.[2,3] Currently more than 150 mitochondrial genome sequences (complete or partial) are available in the mitochondrial genome database (mt Database). These sequences are from diverse groups like vertebrate, protozoan, fungi, plants, algae etc. The interest in mitochondrion/ and its genome is not only due to the fact that it is involved in energy generation but also due to its involvement in pathogenic condition of an organism, apoptosis etc. Mitochondria serve as very good drug targets. Since mitochondrial genome has been completely sequenced from

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various plasmodial species like *Plasmodium falciparum*, *P. vivax*, *P. yoelii*, *P. gallinaceum* and *P. reichnowai*,[4-9] a comprehensive review is warranted.

## MITOCHONDRIA

Each parasite (asexual blood stages) contains only one mitochondrion.[10] Morphological properties of the mitochondrial organelles in the asexual and sexual gametocytic stages of *P. falciparum* have been analysed by various workers and found to be markedly different.[10] From *in vitro* cultures of both stages in human erythrocytes, it has been demonstrated that the asexual stages contained a defined double-membrane organelle having a few tubular-like cristae. The number of mitochondria in the gametocytes were found to be approximately six organelles per parasite, and they showed a greater density of cristae than that of the asexual stage parasite. The functional status of the mitochondrion was probed using the vital dye Rhodamine 123 which gets accumulated in organelle having potential difference.[11-13] This Rhodamine 123 is a cationic fluorescent dye which accumulates specifically in the negatively charged sub-cellular compartment.

Considerable variation in the mitochondrial biochemistry exists between *Plasmodium* species. *Plasmodium*, especially in the erythrocytic stages, rely on the glycolysis for energy production.[14] The acristate morphology and the apparent lack of most of the tricarboxylic acid (TCA) cycle enzymes have left the classical function of the mitochondria of *Plasmodium* in doubt, although avian malarial parasite appears to employ aerobic respiration to some extent. There are many indirect evidences indicating a functional mitochondria in *Plasmodium*, for example Scheibel and Pflaum[15] and Langreth *et al.*[16] have demonstrated cytochrome-c-oxidase (complex III) activity by histochemical staining in all plasmodial species with acristate mitochondria. Cytochrome oxidase activity and cytochrome components of the electron transport chain were detected in *P. falciparum* by Fry and Beesley.[13] One of the reports showed the presence of succinate dehydrogenase complex (complex II) and enzymatic activity in the *P. falciparum* mitochondria.[17] Parasite lacks mitochondrial adenosine triphosphate (ATP) synthase (complex VI) and a rotenone-sensitive complex.[18] Since malaria parasite is dependent entirely on *de novo* pyrimidine biosynthesis, thus a major function of the mitochondrial electron transport chain was proposed as providing a means to dispose electrons generated by dihydroorotate.

The generation of proton motive force, was also suggestive of an important role for the respiration chain and other mitochondrial metabolic reaction. There is conflicting evidence for the presence of a branched electron transport system involving an alternative oxidase in malaria parasite by which electrons are transferred from ubiquinol to oxygen without involving cytochrome-c. It has been demonstrated that the respiratory chain and oxidative phosphorylation are functional in *P. berghei*. Uyemura *et al.*[19] provided the first direct biochemical evidence of mitochondrial function in ATP synthesis and $Ca^{2+}$ transport in malaria parasites, and suggested that the presence of proton ($H^+$) con-

ductance in trophozoites is similar to that produced by mitochondrial uncoupling proteins. It has been suggested that there is a mitochondrial contribution to the parasite ATP pool. It is relatively small and that short fall in this contribution is quickly compensated by ATP from other sources, although this is not necessarily met by increased glycolysis.[20]

## MITOCHONDRIAL GENOME

Protists (most of which are unicellular eukaryotes) represent the bulk of the evolutionary diversity within the eukaryotic domain, and their mitochondrial counterparts are correspondingly diverse. In protists, complete sequence analysis has provided evidence of both circular and linear mtDNAs, with predominant circular DNA. However, a few organisms possess the linear mitochondrial genome — the ciliated protozoans *Paramoecium aurelia* and *Tetrahymena pyriformis*, the algal *Chlamydomonas reinhardtii, Ochromonas danica*, apicomplexan parasite *Theileria parva* and the yeast *Hansenula.*[21,22] In *Plasmodium*, the mitochondrial genome is represented by a tendemly repeated, predominantly linear element with an unit length of 6 kb.[6, 7, 23-27] The mitochondrial genome of *Plasmodium* is uniparentally inherited via the macrogamete and segregated separately.[28] Although there is just one mitochondrion per parasite (asexual blood stage) but the copy number of mitochondrial genome is very high— ~20 copies in *P. falciparum* and *P. gallinaceum*, 8–10 copies in *P. vivax* and ~120 copies in *P. yoelii.*[23,29,30] The mode of replication is not by usual D-loop structure as seen in higher eukaryotes but by recombination dependent replication process as well as by rolling circle method which are similar to that of bacteriophages.[30,31]

During the last decade, this genome has been well characterised in *P. vivax*, *P. falciparum, P. gallinaceum, P. chabaudi, P. reichenowi* and other murine *Plasmodium* spp. like *P. berghei* and *P. yoelii*. It has been seen that the genome organization of all the *Plasmodium* spp. 6 kb element is conserved. It predominates as linear concatamers.[4, 6-9] The corresponding element in *Theileria* occurs not as tandem repeats but as a single unit flanked by inverted repeat sequences.[32,33]

The size of mitochondrial genome ranges from 2400 kb for Muskmelons to 6kb for *Plasmodium.*[34] The 6kb element of *Plasmodium* is, therefore, the smallest mitochondrial genome known so far among eukaryotes. The small size of the apicomplexan mitochondrial genome makes it comparatively simple to sequence. In fact, the number of plasmodial mitochondrial genome sequences are increasing in the genome database at a faster rate. Size of all plasmodial mitochondrial genomes, sequenced so far, falls in the range of 5948 to 5989 bp; *P. vivax* 6kb element ( 5989 bp) is larger among the plasmodial spp. The sizes of 6kb element of various *Plasmodium* species are as follows: *P. falciparum* (5967), *P. yoelii* (5956), *P. berghei* (5961), *P. chabaudi* (5948 ) and *P. reichnowi* (5966).[5-9] So far, more than 25 complete protist mitochondrial DNA sequences have been determined.[22] The minimally derived primitive mitochondrial genome (~69 kb) of the *Reclinamonas americanus* codes for 67 pro-

teins.[35] On the other hand, the Plasmodial mitochondrial genome contains only three protein coding genes representing components of complex III and IV of the electron transport chain. Blanchard and Lynch[36] hypothesised that the reason for variable mitochondrial genome size could be due to the transfer of genes from organelle to nucleus. This gene transfer is thought to occur through a RNA intermediate. How this happens is presently unclear. Although there are many reports to support the above hypothesis. For example, following the origin of land plants, at least eleven mitochondrial genes (coxII, coxIII, rps2, rps7, rps10, rps11, rps12, rps14, rps19, rpl6, sdhC) and one plastid gene(rpl22) have made a successful trek to the nucleus.[37-39] The rate of transfer of tRNA and cytochrome oxidase subunit II to the nucleus is especially very high. This could have started 60 million years ago.[40,41]

The fact that only very hydrophobic proteins are retained in this mitochondrial genome alone can be explained. If it is assumed that for any gene to be successfully transferred from the mitochondrion to the nucleus, during evolution, it had to code for the proteins that could escape the pre-existing co-translational export mechanisms. That means it should not be very hydrophobic. For example, cytochrome-c-oxidase subunit II (CoII) had only two transmembrane domains whereas CoI, CoIII and Cyb had more than seven transmembrane domains. The CoII was reportedly transferred to nuclear genome but CoI, CoIII and Cyb are invariably retained in most of the mitochondrial genomes. In many mitochondrial genomes, the size is reduced with evolution, the same may be the case with *Plasmodium* mitochondrial genome. However, this can only be ascertained once the complete nuclear genome sequence is known.

Generally, in vertebrate animals, the mitochondrial genome contains genes for rRNAs, tRNAs and inner mitochondrial membrane proteins involved in electron transport and coupled oxidative phosphorylation. With respect to gene content, protist mtDNA generally resembles plant rather than animal or fungal mtDNAs. The *P. vivax* and *P. falciparum* sequences contain open reading frames for three mitochondrial genes — cytochrome oxidase subunit III (CoIII), subunit I (CoI) and Cytochrome b (Cyb). There were a total of 13 different fragmented rRNA genes, scattered through out the 6 kb element. Out of 13 fragments, seven are for large and six for small subunit rRNA genes as shown in Fig. 1. The complete sequence analysis showed that the 6 kb element lacks tRNA genes. The gene content of the 6 kb element of *Plasmodium* spp is very similar to *T. parva* because both contain three open reading frames — Cyb, CoI, CoIII and scrambled rDNA. In addition, both the genomes are linear in nature.

In general, the mutation rate of mitochondrial genome is very high in mammals.[42-44] But the sequence comparison of the 6 kb element showed that the *Plasmodium* mitochondrial genomes are evolving at a much slower pace than the nuclear genome. Overall, the *P. vivax* mitochondrial DNA showed 90.1, 91.7 and 89.9 per cent sequence identity with *P. falciparum* *P. yoelii* and *P. reichenowi*, respectively.[6,7,9] Such high homology, indicates that this genome is highly conserved across the species and among

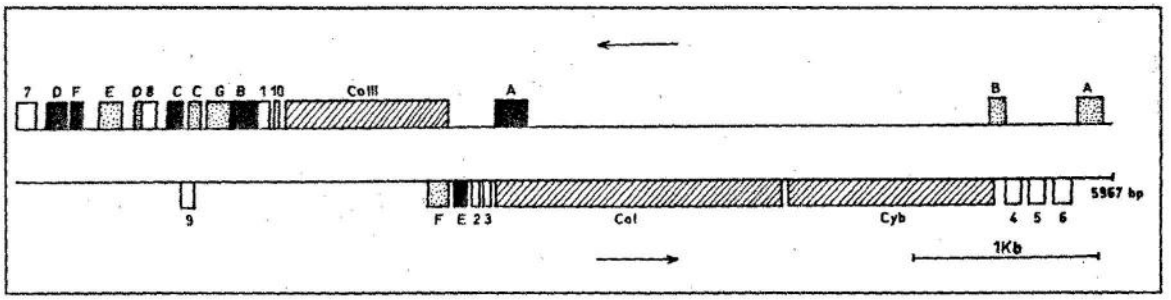


*Fig. 1:* Genome organization of *Plasmodium* spp. mitochondrial 6kb element. Genes encoding mitochondrial proteins (CoIII, CoI and Cyb) are shown as striped box. The direction of arrows indicate the direction of transcription for mitochondrial and fragmented ribosomal RNA genes. The fragmented ribosomal RNA genes for large subunits are shown as checked box and that for small subunit as solid box

different geographical isolates. There are several possible reasons for this conserved nature of the 6 kb element: (i) enzymes of TCA cycle are almost missing; (ii) absence of complex I of respiratory chain; and (iii) lack of mitochondrial ATP synthase and the rotenone-sensitive complex.[18] Indirect evidences indicate functional role for many mitochondrial genes of *Plasmodium*—presence of membrane potential[11-13] and cytochrome oxidase activity.[13,15,16] Thus asexual parasite mitochondrial activities appear to be primarily anabolic rather than serving as a significant source of energy generation.[20] In particular, the respiratory chain is essential for *de novo* pyrimidine biosynthesis.[45] It provides a sink for electron transferred from dihydroorotate dehydrogenase.[46] Therefore, the effective concentration of the reactive oxygen intermediate and free radical formation will be very less and so is the damage to the mitochondrial DNA. The second reason could possibly be the proof reading activity of the mitochondrial DNA polymerase-γ which participate in replication of mitochondrial genome. It also may be due to the low mis-incorporation rate.[47] In case of yeast, the mitochondrial DNA polymerase-γ possess 3'–5' exonuclease proof reading and mis-match repair activity.[48] The other possible reason could be that they are present in multiple copies. If any error is incorporated during mitochondrial DNA replication, it will be diluted out or will be insignificant because the copy number is high.

## Fragmented rRNA

Fragmented rRNA, although uncommon, have been described from other eukaryotic, prokaryotic and organelle genomes. In some cases fragmentation is minor—*Tetrahymena pyriformis* has two fragments only. More complex fragmentation is seen for the nucleo-cytoplasmic large subunit rRNA of Trypanosomatids, a protozoan which has seven fragments and that of *Euglena gracilis* has 14 fragments. However, all the fragments are in expected linear order. In case of *Theileria parva*, *T. pyriformis* and *Chlamydomonas* species, the fragmented rRNA genes are encoded out of expected order—

scrambled and interspersed with open reading frames coding for proteins and tRNA genes on both the strands.[49-53] The occurrence of bacterial endosymbiont in protozoa has been known for nearly a century. The fragmentation of 16S-rRNA has been seen in the obligate bacterial endosymbiont *Caedibacter caryophila* of *Paramoecium caudatum*.[54] But nothing is known about the process that resulted in such discontinuities and scrambled rRNA coding regions. In all the above mentioned cases, the fragmented rRNA may have the potential to form secondary structure much like those proposed for continuous rRNAs.[55] In *P. vivax*, similar fragmentation was observed. The mitochondrial genome of *P. falciparum* encodes unusually highly fragmented rRNA. Small regions (40-190 nt) of sequences similar to rRNA are encoded in scrambled order by both strands of DNA.[56,57] However, they are smaller than the algal rRNA sequences. The fragments are transcribed as small RNAs which have the potential to be assembled through secondary structure interactions forming functionally important domains of the rRNA.[8, 26, 56, 57] These regions are very well conserved between *Plasmodium* species, corresponding to highly conserved and functional regions of rRNA from other species. These fragmented ribosomal RNA genes of *P. vivax* showed minimum variation (deletion, addition and point mutation) when compared with that of *P. falciparum*. This extreme fragmentation and high homology at nucleotide level within genus suggest that they may retain function and assemble into functional ribosomes despite their small size and extreme fragmentation. Still the exact role of these fragmented ribosomal RNA genes is not known. However, this can only be confirmed after purification and characterization of functionally active ribosomes from pure mitochondrial preparation.

## Respiratory chain proteins encoded by the 6kb element

### *Cytochrome b*

The sequence of the Cyb gene of *P. gallinaceum*[25] provided the first evidence of a mitochondrial identity for the tendemly repeated 6kb element. The size of Cyb gene is similar in all the plasmodial spp and also the sequence conservation among the species is quite high — ~90 per cent identity.[4-7, 9, 25] This gene seems to be important as a target for mitochondrial complex III inhibitors like antimycin A, diuron, myxothiazol, stigmatellin, mucidine and inhibitors of 8-aminoquinolines group.[24,58,59] The new antimalarial atovaquone disrupts the mitochondrial membrane potential.[60] It has potent blood schizontocidal activity and is also effective against the pre-erythrocytic and sexual stages of the parasites.[61-65] It has recently been shown that antimalarial activity of atovaquone involves interaction with cytochrome b encoded by the 6kb element of the mitochondrial DNA. Amino acid residues involved in drug-resistance have been identified, as they are located at putative drug-binding site.[66-68] Atovaquone is also drug of choice for toxoplasmosis. The characterization of Cyb gene of *Toxoplasma gondii* also demonstrated the mutations in Qo domain and its correlation with atovaquone drug-resistance.[69] This finding is in agreement with mutations observed in Cyb gene of *P. falciparum*,

*P. berghei* and *P. yoelii.*[66-68,70] However, in one of the study, which involved sequencing of entire Cyb gene from eight clinical isolates of *P. falciparum* (from India) and five parasite lines did not show any mutation in these residues.[4] This is expected since the drug has not yet been used in India. Indeed 12 of 14 Indian isolates have identical nucleotide and amino acid sequence of Cyb gene. The Cyb gene from these isolates did not show much variation as seen in the isolate *Pf*PH10 in comparison to Gambian isolate *Pf* C10.[4] The sequence pair distance analysis showed that except isolate *Pf*PH10 all other isolates from India were identical.[4] Furthermore, there was no sequence divergence in those amino acids which are involved in providing the resistance against various other drugs. The sequences of Qi and Qo centres were particularly conserved. The point mutations in these centres have been found to be associated with the drug-resistance.[24] The Cyb gene sequence of all the chloroquine resistant and sensitive lines was also same as wild type thereby indicating that chloroquine resistance does not involve this gene.[4] Highly conserved sequence of Cyb gene among majority of isolates was surprising since more variations in nuclear encoded proteins (CSP, MSA-1, MSA-2, TRAP) have been observed among Indian isolates.[71, 72]

The expression studies of cytochrome b gene indicate that this gene is expressed in all the asexual blood stages of the parasite. This is in accord with many reports which indicate the cytochrome oxidase activity in the parasite. Thus suggestive of the active mitochondria in the plasmodial species.[4, 73] It is a probable target for antimalarial drugs like primaquine, ascofuranone, atovaquone and the rest.[17, 24, 74]

### *Cytochrome oxidase subunit I*

It is one of the largest subunit of cytochrome-c-oxidase complex also known as complex IV. On an average the size of this protein ranges from 510 to 554 amino acid residues. The size of plasmodial CoI (514 amino acid residues) is comparable to the size of CoI from bacteria or eukaryotic organism. It catalyses the terminal act of respiration by delivery of electrons derived from the stepwise oxidation of foodstuffs to molecular oxygen. This subunit is present in all cytochrome-c-oxidase of bacterial or eukaryotic origin studied to date. It also has large number of conserved residues within the known species. However, the total number of evolutionary conserved residues is limited. Despite this, the general pattern of protein folding appears to be following a common pattern. Holm *et al.*[75] have proposed a model of the 12 transmembrane domains containing CoI polypeptide. The subunit I of *E. coli* was predicted to have nine transmembrane domains.[76] It is one of the important subunit, which forms the catalytic core of cytochrome-c-oxidase. CoI has been proposed to contain three of the four redox centres. These centres are heme a, heme $a_3$ and CuB. These redox centres are involved in the electron transfer reaction. The CoI gene of *P. falciparum* was studied in detail by Vaidya *et al.*[7] They showed that all the histidine residues involved in ligand binding are conserved. The overall primary structure is found to be conserved in all the plasmodial species along with the histidine residues involved in ligand binding. The presence of transcripts of CoI during asexual stages and cytochrome-c-oxidase activity is suggestive of an active mitochondrion in the parasite.[4, 73]

### *Cytochrome oxidase subunit III*

CoIII is one of the major subunits in the mitochondrial and bacterial cytochrome oxidase. It does not contain any of the enzyme redox active metal centres. Since mitochondria are originated from endosymbiotic organism so the CoIII gene in prokaryotic and eukaryotic organisms show high level of similarity at the structural level—seven transmembrane domains.[77] The exact role of this subunit is still not clear. The isolation of active cytochrome oxidase complex without CoIII suggested that this may not be involved in proton pumping.[78-79] The other possible roles of this subunit could be in the assembly of cytochrome oxidase complex. This is supported by the fact that the deletion of CoIII gene from the bacteria *Paracoccus denitrificans* leads to defective assembly of bacterial cytochrome oxidase. It reduced the cytochrome oxidase activity by 50–80 per cent.[80] Furthermore, a pool of bound lipid in CoIII has been proposed to provide an oxygen reservoir in oxygen pathway.[81] The sequence analysis of cytochrome oxidase subunit III of all the plasmodial species showed that the critical glutamate residue is conserved. It seems to be important in proton translocation and its modification by dicyclohexyl carbodiimide (DCCD) inhibits the proton pumping in *Paracoccus denitrificans* cytochrome-c-oxidase.[82, 83] On the contrary, crystallographic studies suggested the role of this residue may be structural.[84] The CoIII gene seems to be expressed in all the asexual blood stages of the parasite.[4, 73] This is in accord with the cytochrome-c-oxidase activity reported by many groups. Thus suggesting a functional/or active mitochondrion.

### MITOCHONDRIA AND NUCLEAR CODED PROTEINS

Strong communication pathways exist between the mitochondrial and nuclear genome. The assembly and function of respiratory component of mitochondria in eukaryotic cells results from collaboration between gene product derived from both mitochondrial and nuclear genomes.[85] Approximately 90 per cent of the proteins present in the mitochondria are nuclear coded. In contrast, mitochondrial genome specifies only a few proteins, which resides mainly in the inner mitochondrial membrane in *Plasmodium* spp. These proteins play an essential role in mitochondrial transcription, translation, TCA, oxidative phosphorylation and the rest. In *Homo sapiens*, the respiratory chain consists of five complexes. Complex I contains seven mtDNA and >41 nDNA coded proteins; complex II does not contain mtDNA coded proteins but only four nDNA coded proteins; complex III contains 10 nDNA coded proteins and one mtDNA coded; complex IV contains 10 nDNA and three mtDNA coded proteins, and complex V contains 14 nDNA and two mtDNA coded proteins (ref: www.mips.biochem.mpg.de), whereas nothing is known about the nuclear coded parts of complex I, III, IV and V in *Plasmodium*. Recently, Takeo *et al.*,[17] have partially characterised two catalytic subunits of complex II also referred to as succinate ubiquinone oxidoreductase or succinate dehydrogenase complex from *P. falciparum*. Both nuclear coded subunit, SDH A and B are single copy genes, present on different chromosomes. The primary substrate for this enzyme is succinate, which under normal circumstances comes from TCA cycle. Since

*Plasmodium* lacks enzymes of TCA cycle, so a possible pathway to produce succinate is phosphoenolpyruvate carboxykinase-succinate pathway (PEPCK-succinate pathway) which is found in many parasites.[17, 86, 87]

Now it is a well-known fact that the mitochondrial coded subunits are responsible for the catalysis and that the nuclear coded subunits have one or two of the following functions. Some of the subunits modulates/regulates catalysis while others are required for the stability of the catalytic subunits and/or the stable assembly of holoenzyme.[88, 89]

## RESPIRATORY CHAIN GENE EXPRESSION

The expression of three mitochondrial encoded protein genes by the parasite was studied by detecting their RNA transcripts by using the RT-PCR, northern blotting and *in situ* hybridization.[4, 73] (Figs. 2 and 3). The data suggest that

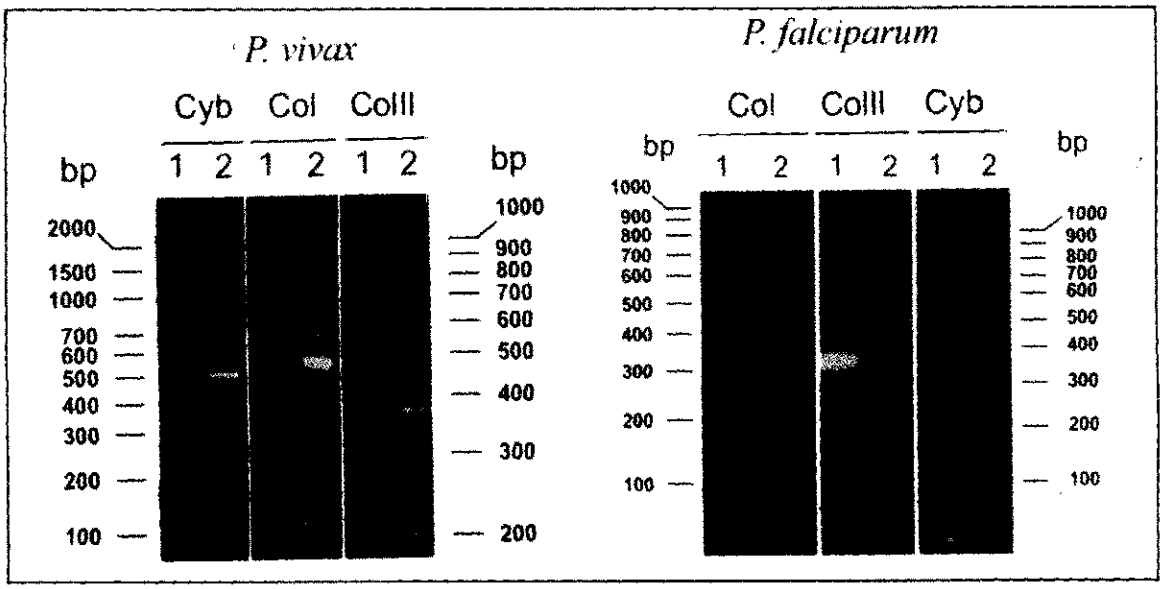


*Fig. 2:* Expression of Col, ColII and Cyb gene in *P. vivax* and *P. falciparum* by RT-PCR: RT-PCR on the RNA of *P. vivax* and *P. falciparum* from erythrocytic stages was carried out for Col , ColII and Cyb genes using specific primers. The sequences of primers used for *P. vivax* RT-PCR is as follows: for ColII gene P101 5'-ACT AGA GAT TTA AAA ACT CAT TC-3'/P102 5'-GTA TCT TAT CCT TCA TTA ACA TCA-3', for Col gene R103 5'-ATC TCC TGC AAA TGT TGG GTC-3'/P109 5'-AAT GCC AGG ATT ATT CGG AGG-3' and for Cyb gene CybF 5'-GTT TGG ATC CAT GAA CTT TTA CTC TAT TAA T-3'/ P106 5'-CCT CCT ATA TGA CAC TCA CTA G-3'. The primer sequence for *P. falciparum* genes were same as described in Sharma *et al.*[4] The size of RT-PCR product for each gene is shown in lane 2 for *P. vivax* and lane 1 in *P. falciparum*, lane 1 in *P. vivax* and lane 2 in *P. falciparum* were without reverse transcriptase. Marker lane for *P. vivax* ColII is shown on right hand side, and for Col and Cyb on left hand side. For *P. falciparum*, the marker lane for ColII and Cyb is shown on right hand side and for Col on left hand side

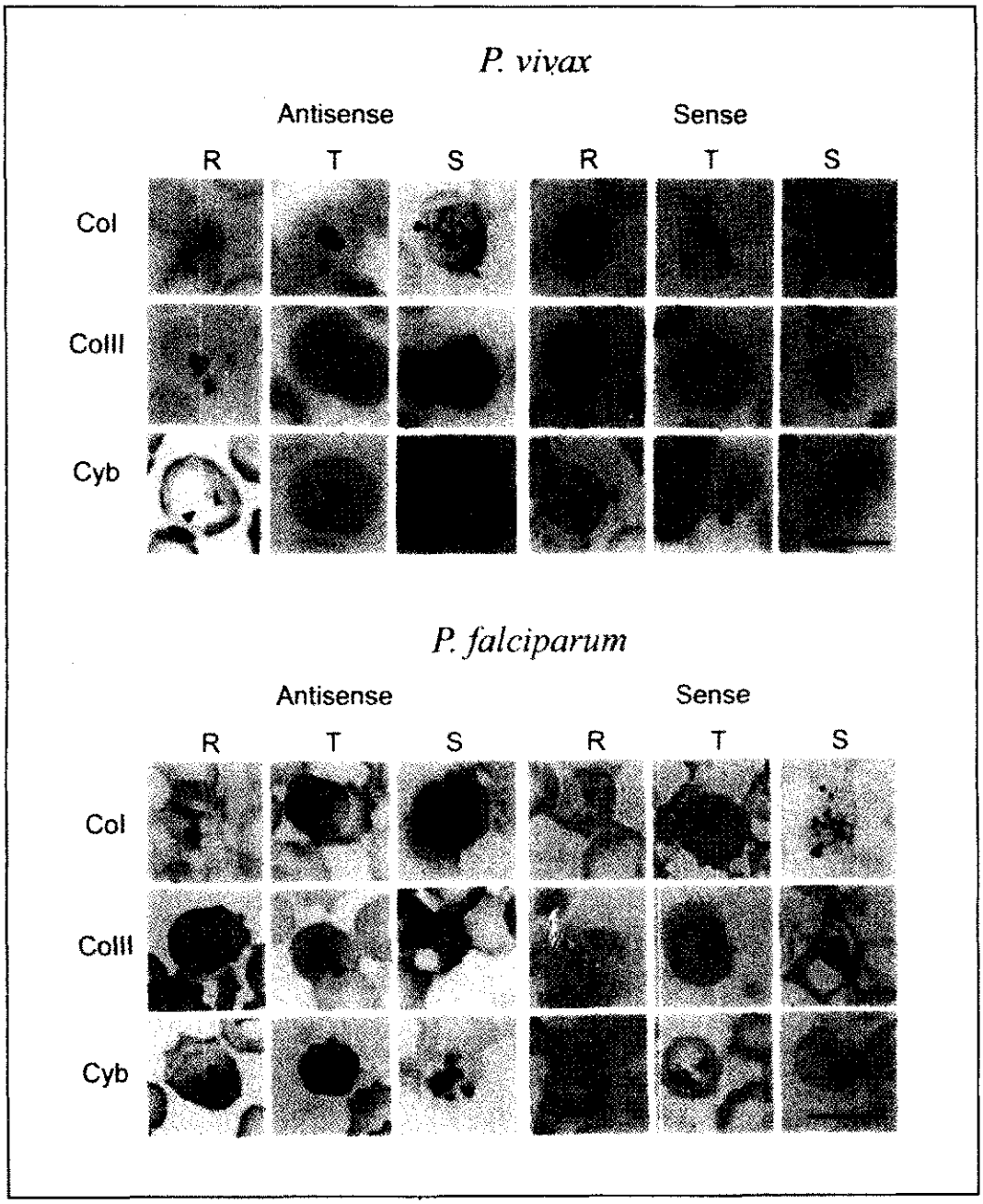


*Fig 3:* Expression of CoI, CoIII and Cyb gene of *P. vivax* and *P. falciparum* by in-situ hybridization: The mRNA in-situ hybridization was carried out on thin smears of the *P. vivax* and *P. falciparum* parasites by using antisense and sense riboprobe for CoI, CoIII and Cyb genes as described in Sharma *et al.*[4]; R—Rings; T—Trophozoite; S—Schizont. Arrowheads indicate the parasite. Scale bar—10 μm.

all the three mitochondrial genes (CoI, CoIII and Cyb) are being transcribed during the erythrocytic stages of both the parasites *P. vivax* and *P. falciparum*. However, this does not confirm that these genes are also translated. If these transcripts are translated *in vivo* by the parasites, then the expression of these genes in mitochondria is suggestive of the presence of active mitochondria in malaria parasite. Mitochondria of malaria parasite generates a membrane potential through an electron transport system/or other pathway. The presence of transcript (mitochondrial proteins) for complex II, III and IV and activity of succinate dehydrogenase complex (nuclear coded, complex II) in the asynchronous erythrocytic stages of the *P. falciparum*[17] are suggestive of the partially active respiratory chain in *Plasmodium*.

## CONCLUSION AND PERSPECTIVE

The mitochondrial molecular biology and biochemistry needs to be explored in greater depth. The efforts taken so far suggest that the 6kb element is the smallest mitochondrial genome known so far. It has been sequenced from various geographically distinct isolates of *P. falciparum* and also from other *Plasmodium* spp. The comparison of all the 6kb element suggested that it is a highly conserved element in the *Plasmodium* genus with ~90 per cent homology. The genome organisation of the 6kb element from all the *Plasmodium* species sequenced so far is the same. These mitochondrial genomes are very economically packed. The rate of sequence variation was found to be more in protein coding region and less in non protein coding region in both *P. vivax* and *P. falciparum*.

Many groups have shown the cytochrome-c-oxidase activity in the parasite. This was also supported by the fact that the three open reading frames of the 6kb element were found to be transcribed during erythrocytic stages of the parasite. If these transcripts are indeed translated *in vivo* then the expression of these genes in mitochondria is suggestive of the presence of active mitochondria in malarial parasite.

Mitochondrion is the power house of a cell. In case of *Plasmodium* it is a probable drug target for many new lines of drugs. It needs to be studied in immense depth. This can be done only if the three proteins encoded by this element are purified and their interaction with nuclear proteins is investigated.

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# Impact of Urbanization on Bionomics of *An. culicifacies* and *An. stephensi* in Delhi

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Study on bionomics of malaria vectors was carried out in riverine and non-riverine areas, on account of tremendous ecological changes in the topography of Delhi. The densities of adult anophelines were estimated by two techniques, hand catch and total catch index. Percentage of *An. stephensi* (15.68) collected by both the techniques was more than *An. culicifacies* (3.16) in both the areas. Day-time resting preferences of vector species in human dwellings and cattlesheds did not differ significantly. Preferred larval habitats of *An. culicifacies* in riverine area shifted to large lakes, channels and ponds. In malaria transmission, *An. culicifacies* played a role only in the northern part of the riverine area where water pollution was at minimal level, while *An. stephensi* played an equal role in the malaria transmission in both the areas. High sporozoite rates were found in type form of *An. stephensi* in localities where its proportion was high, thus confirming its active role in malaria transmission. The overall sporozoite rate of vectors was 0.7 per cent and *P. falciparum* sporozoite infections of the vectors were detected in *An. stephensi* only. *P. vivax* and *P. falciparum* infections were found in the ratio of 68 : 32. The non-riverine area was more malarious than the riverine area.

**Keywords:** *An. culicifacies*, *An. stephensi*, Density, Malaria, Resting preferences, Sporozoite rates

## INTRODUCTION

The Yamuna river area in Delhi has been reported as highly malarious and the non-riverine area less malarious since the beginning of twentieth century.[1] Very high mortality rate (>300/1000) was reported among military troops when stationed near River Yamuna.[2] On the basis of

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child spleen rates, villages were classified as meso- and hyper-endemic for malaria in riverine area where malaria was mainly attributed to River Yamuna. In riverine and non-riverine areas 54 and eight per cent of villages respectively showed a spleen rate of 51 and above.[3] Incrimination studies showed *An. culicifacies* and *An. stephensi* as vector species in Delhi, but subsequently only *An. culicifacies* was considered playing the principal role.[4,5] Later on Dhir[6] pointed out that *An. stephensi* was replacing *An. culicifacies* as a major vector and Pattanayak *et al.*[7] observed *An. stephensi* as the principal vector of malaria in south Delhi. Since then Delhi has undergone rapid ecological changes. The population has increased tremendously from 0.9 million in 1941 to 9.4 million in 1991. Rural areas have been converted into urban and semi-urban areas under the Delhi Development Authority (DDA) for accommodating the population influx through migration. Sewage, sullage and industrial waste in large quantity is being disposed into the Yamuna through 17 major drains. River Yamuna is no more a wild river, it has been tamed on both sides by providing protective embankments. The flooding effect on localities situated nearby has now been neutralised. However, localities of north Delhi near the outer ring road are under the impact of water logged lands due to large borrow pits.

In view of the rapid ecological changes, a thorough knowledge on bionomics of the two known malaria vectors throughout the year, is essential for understanding of malaria transmission. The present paper reports some of the observations on bionomics of malaria vectors in riverine and non-riverine areas of Delhi which would help in devising a better malaria control strategy.

## MATERIALS AND METHODS

### Study area

Study localities were divided into two areas. These are riverine area in the Yamuna river belt of east Delhi and the non-riverine area of northwest Delhi (Fig. 1). Selected localilties of riverine area have become urbanized and non-riverine area industrialised. Embankment of Yamuna has blocked the natural drainage of the area and water stagnated in pools is polluted.

The riverine area is spread about 193 sq km over eastern part of the territory and has an average height of 210 m above the sea level. The sub-soil water level is very high, as high as 0.6 m.[8] The Yamuna river flows through this area from north to south and receives a higher annual rainfall (about 600 mm) as compared to other area. In its flow, the river is joined by 17 major storm water drains which carry sewage and sullage of the city. Four localities—Mukundpur, Kanchanpuri, Gazipur and Madanpur Khadar were selected for entomological and parasitological studies (Fig. 1).

The non-riverine area in northwest Delhi has an area of about 400 sq km and an average height of 215 m above the sea level. Western Yamuna canal traverses through this area and provides irrigation and supplies water to a water treatment plant at Haiderpur. Normal monthly and

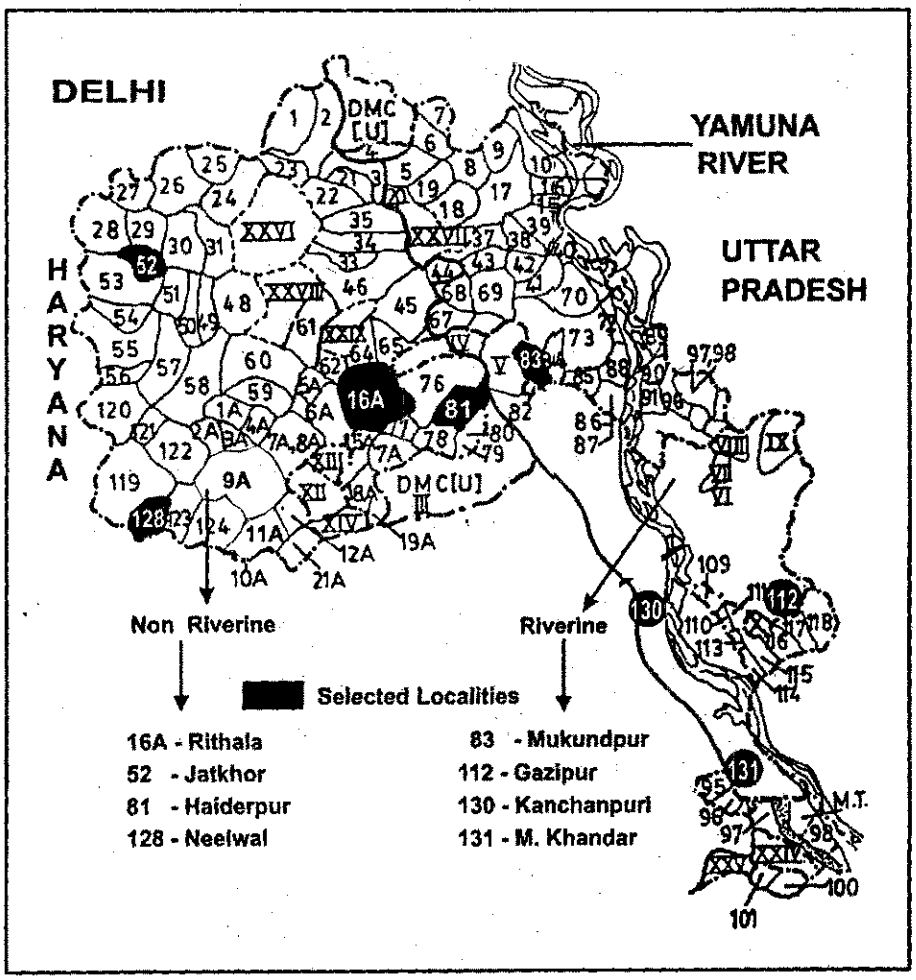


*Fig. 1:* Map of selected localities in riverine and non-riverine areas of Delhi

annual rainfall (500 mm) is greater on the eastern part as compared to 300 mm in the western part.[9,10] The four localities selected in this area were Haiderpur, Rithala, Neelwal and Jatkhor (Fig. 1).

## Methodology

Regular entomological surveys were carried out at fortnightly intervals from August 1989 to July 1990 and later at monthly intervals up to July 1991.

Sampling of adult mosquito populations was carried out by man hour density and pyrethrum spray collection using standard sampling techniques.[11,12] Indoor resting mosquitoes were collected between 0530 and 0830 hours in summer and 0600 and 0900 hours in winter months. Collections were made in eight structures rep-

resenting human dwellings and cattlesheds in equal proportion (four fixed sites and four randomly chosen sites) in each selected locality. In each structure collection was done for fifteen minutes. Field collected mosquitoes were identified to species following identification keys[13] and species-wise man hour density was calculated. Vector mosquitoes were processed for various studies.

Gut and gland infection was examined by classical dissection method as well as by immunoradiometric assay (IRMA).[14] A WHO susceptibility test kit was used for the determination of susceptibility of mosquito to insecticides following standard WHO procedure.[15] Immunoelectrophoresis technique[16] was used for the identification of blood meal source. Identification of *An. culicifacies* sibling species was done by examining species specific paracentric inversions in polytene chromosomes[17] and seasonal prevalence of sibling species A and B was compared in between riverine and non-riverine areas.[18]

Identification of ecological races of *An. stephensi* was done following the method of Subbarao *et al.*[19] All permanent breeding places were surveyed at fortnightly intervals for two years. Larval collections were made using a standard dipper made of aluminium bowl with nine cm diameter and 300 ml capacity. For sampling in wells an iron bucket was used. A fixed number of dips were taken according to the habitats. Samples of immatures collected were reared in the laboratory for species identification at the adult stage.

Active surveillance and mass blood survey were carried out as per National Anti Malaria Programme (NAMP) procedure. Presumptive treatment consisting of 600 mg chloroquine phosphate as a single adult dose was given to all fever cases as per drug policy of the national programme.[20]

## RESULTS

### Entomological studies

Seven anopheline species were encountered in the indoor resting collections from both the areas. Among anophelines, *Anopheles subpictus* was most prevalent (78.6 per cent), followed by *An. stephensi* (15.68 per cent), *An. culicifacies* (3.16 per cent), *An. annularis* (2.21 per cent), *An. pulcherrimus* (0.24 per cent) and *An. barbirostris* (0.03 per cent). *An. culicifacies* was the third most prevalent species in non-riverine and fifth in riverine area.

In the riverine area, a higher peak of *An. culicifacies* was observed in April followed by an another peak in October and in the non-riverine area, the peak density was observed in May and August. Peak density of *An. stephensi* was observed in May, July and October in the riverine area and three peaks in June, October and December in the non-riverine area (Figs. 2 and 3).

Inter-zonal results of the estimates of mosquitoes per structure were tested by analysis of variance (Table 1). No significant difference between the capture of mosquitoes in cattlesheds and human dwellings was observed. The interaction between species and type of structure was also insignificant ($p = 0.327$).

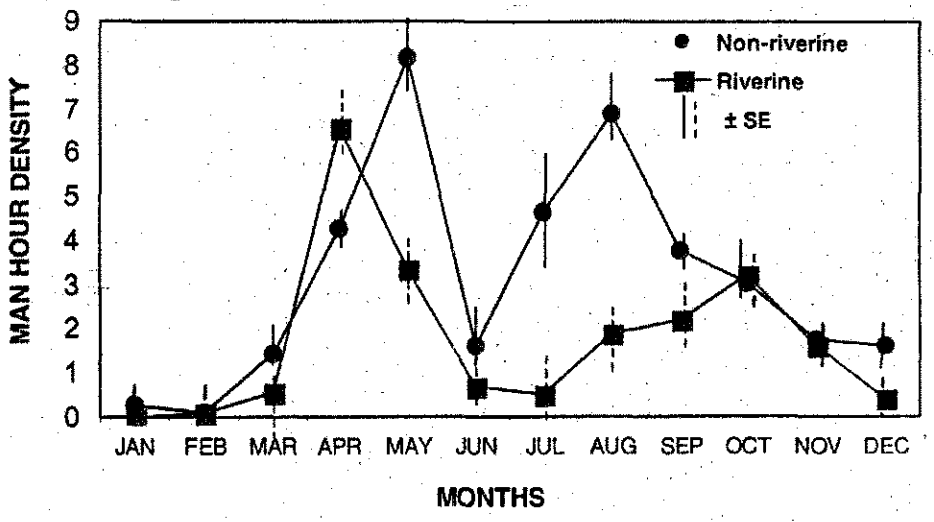


*Fig. 2:* Man hour density of *An. culicifacies* in riverine and non-riverine zones

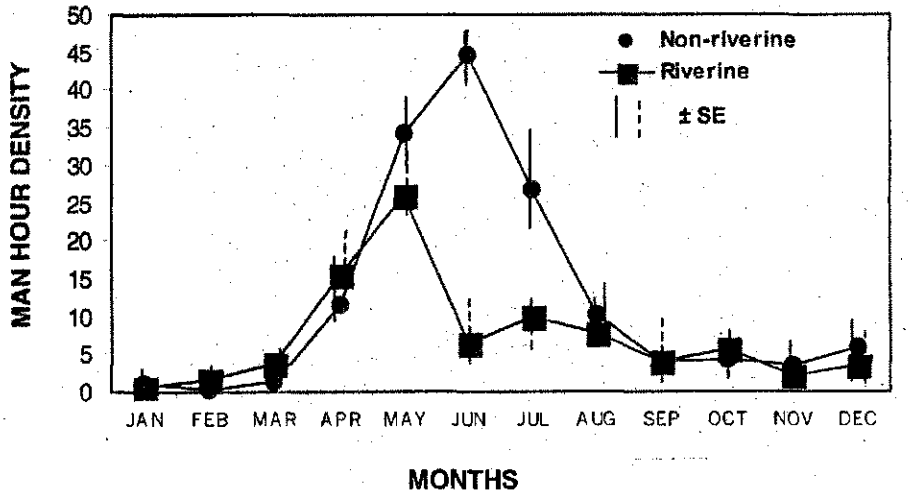


*Fig. 3:* Man hour density of *An. stephensi* in riverine and non-riverine zones

Species-wise data on total catch were also tested by analysis of variance to test the significance of preponderance of *Culex* and malaria vectors. Results showed that the estimates of *Culex* population and species-wise anophelines were not significant in both the areas.

Table 2 shows the results of blood meal analysis. It was found that anthropophilic index (AI) of *An. culicifacies* and *An. stephensi* in the riverine area was 3.7 and 0.45 per cent respectively and in the non-riverine area it was 2.7 and 1.4 per cent respectively. The differ-

**Table 1. Summary of ANOVA results: Day time resting preference of mosquito species based on total catch**

| Area/Species | Mean catch | | | ANOVA estimate | ANOVA (Sources) | | | Errors |
|---|---|---|---|---|---|---|---|---|
| | CS | HD | CS and HD | | Between structure (CS and HD) | Between five species | Species x Type of structure | |
| *Riverine* | | | | | | | | |
| *An. culicifacies* | 0.71 | 0.61 | 0.66 | SS | 42.457 | 127963.799 | 548.96 | 25567.654 |
| *An. stephensi* | 5.71 | 5.58 | 5.65 | DF | 1 | 4 | 4 | 30 |
| *An. annularis* | 0.28 | 0.28 | 0.28 | F | 0.05 | 37.537 | 0.161 | |
| *An. subpictus* | 38.17 | 44.2 | 41.18 | P | 0.8249 | 2.837 E-11 | 0.9564 | |
| *Culex* | 156.18 | 140.08 | 148.13 | | | | | |
| *Non-riverine* | | | | | | | | |
| *An. culicifacies* | 1.92 | 1.56 | 1.74 | SS | 844.561 | 99455.039 | 3776.712 | 23401.196 |
| *An. stephensi* | 14.62 | 13.03 | 13.82 | DF | 1 | 4 | 4 | 30 |
| *An. annularis* | 0.38 | 0.27 | 0.32 | F | 1.083 | 31.875 | 1.21 | |
| *An. subpictus* | 49.85 | 49.84 | 49.85 | P | 0.3064 | 2.072 E-10 | 0.327 | |
| *Culex* | 108.78 | 156.83 | 132.8 | | | | | |

CS — Cattlesheds; HD — Human dwellings; DF— Degree of freedom; SS — Sum of squares; F — Variance ratio; P — Probability; E — Exponent.

**Table 2. Human blood index (HBI) of vector species in riverine and non-riverine areas**

| Resting sites | Riverine | | | | Non-riverine | | | |
|---|---|---|---|---|---|---|---|---|
| | *An. stephensi* | | *An. culicifacies* | | *An. stephensi* | | *An. culicifacies* | |
| | No. tested | HBI | No. tested | HBI | No. tested | HBI | No. tested | HBI |
| Human dwelling | 262 | 0.76 (2) | 27 | 7.4 (2) | 315 | 2.53 (8) | 70 | 2.85 (2) |
| Cattleshed | 405 | 0.24 (1) | 27 | 0.0 (0) | 537 | 0.74 (4) | 78 | 2.56 (2) |
| Total | 667 | 0.45 (3) | 54 | 3.7 (2) | 852 | 1.40 (12) | 148 | 2.70 (4) |

Figures in parentheses are observed number.

ence between the AI's of these vector species in two areas was not statistically significant (p = 0.655 and 0.104).

In the riverine area, a total of 197 *An. culicifacies* and 287 *An. stephensi* were dissected. The sporozoite rates (SR) were 0.5 and zero per cent respectively. The gland positivity of *An. culicifacies* was found only in Mukundpur locality. In non-reverie area, 186 *An. culicifacies* were dissected and the SR was 0.53 per cent. The results of vector incrimination by IRMA technique are given in Table 3. A total of 765 *An. stephensi* from non-riverine area were assayed, four were found positive for circumsporozoite (CS) antigen of *P. vivax* and two for that of *P. falciparum*. The gross sporozoite rate was 0.58 per cent and for *Pf* it was 0.26 per cent. A total of 59 *An. culicifacies* and 560 *An. stephensi* were assayed from the riverine area by IRMA and one specimen of *An. culicifacies* was found positive for CS antigen of *P. vivax*, thus giving a sporozoite rate of 1.69 per cent. Three specimens of *An. stephensi* were found positive for *Pv* CS antigen with a sporozoite rate of 0.53 per cent. Similarly, 35 specimens of *An. annularis* from both the areas were assayed and none was found positive.

**Table 3. Sporozoite rate of *An. culicifacies* and *An. stephensi* using the IRMA technique**

| Locality | *An. stephensi* | | | | *An. culicifacies* | | | |
|---|---|---|---|---|---|---|---|---|
| | No. assayed | Sporozoite rate | | | No. assayed | Sporozoite rate | | |
| | | *Pv* | *Pf* | Total | | *Pv* | *Pf* | Total |
| *Riverine* | | | | | | | | |
| Mukundpur | 196 | 0.51 | 0 | 0.51 | 44 | 2.27 | 0 | 2.27 |
| Kanchanpuri | 99 | 1.01 | 0 | 1.01 | 3 | 0 | 0 | 0 |
| Gazipur | 224 | 0.44 | 0 | 0.44 | 8 | 0 | 0 | 0 |
| M. Khadar | 41 | 0 | 0 | 0 | 4 | 0 | 0 | 0 |
| Total | 560 | 0.53 | 0 | 0.53 | 59 | 1.69 | 0 | 1.69 |
| *Non-riverine* | | | | | | | | |
| Haiderpur | 173 | 0.00 | 0 | 0.00 | 15 | 0 | 0 | 0 |
| Rithala | 463 | 0.86 | 0.43 | 1.3 | 17 | 0 | 0 | 0 |
| Neelwal | 27 | 0 | 0 | 0 | 3 | 0 | 0 | 0 |
| Jatkhor | 102 | 0 | 0 | 0 | 60 | 0 | 0 | 0 |
| Total | 765 | 0.52 | 0.26 | 0.78 | 95 | 0 | 0 | 0 |
| Total of two areas | 1325 | 0.52 | 0.15 | 0.67 | 154 | 0.64 | 0 | 0.64 |

To distinguish *An. stephensi* races on the basis of average ridge number in eggs of 21 iso female lines from riverine and 75 from non-riverine areas were examined. The results showed that in riverine and non-riverine areas the type form was 61.9 and 26.6 per cent, intermediate was 23.8 and 29.33 per cent and the variety *mysorensis* was 11.3 and 44 per cent respectively. In riverine areas, a maximum of 75 per cent type form was found in Kanchanpuri locality and the remaining 25 per cent was intermediate. In the non-riverine area type form was found only in Haiderpur and Rithala localities and the maximum of 51 per cent was in Rithala locality.

For the prevalence of *An. culicifacies* sibling species, a total number of 34 and 40 specimens from riverine and non-riverine areas respectively were examined in the months of May to July (pre-monsoon) for ovarian polytene chromosomes and 98.7 per cent specimens were of species A. In the month of October (post-monsoon), 35 and 13 specimens were examined in riverine and non-riverine areas respectively. Percentage of species B was 11.4 and 15.3 respectively which suggests its correlation with breeding habitats after rains.

Table 4 gives the results of insecticide susceptibility tests carried out in wild caught adults using

**Table 4. Insecticide susceptibility results**

| Locality (area) | Species | Insecticide | Total no. exposed | Per cent mortality after 24 hours |
|---|---|---|---|---|
| Mukundpur (Riverine) | *An. culicifacies* | DDT[(4 per cent) 1 h] | 15 | 53.3 |
| | | DL [(4 per cent) 1 h] | 15 | 46.6 |
| | | Malathion [(5 per cent) 1 h] | 15 | 100.0 |
| | | Deltamethrin [(0.025 per cent) 1 h] | 15 | 100.0 |
| Rithala (Non-riverine) | *An. culicifacies* | DDT [(4 per cent) 1 h] | 10 | 90 |
| | | DL [(4 per cent) 1 h] | 10 | 40 |
| | | Malathion [(5 per cent) 1 h] | 10 | 100.0 |
| | | Deltamethrin [(0.025 per cent) 1 h] | 10 | 100.0 |
| Madanpur (Riverine) | *An. stephensi* | DDT [(4 per cent) 1 h] | 15 | 86.6 |
| | | DL [(4 per cent) 1 h] | 15 | 66.6 |
| | | Malathion [(5 per cent) 1 h] | 15 | 93.3 |
| | | Deltamethrin [(0.025 per cent) 1 h] | 15 | 100.0 |
| Jatkhor (Non-riverine) | *An. stephensi* | DDT[(4 per cent) 1 h] | 15 | 46.6 |
| | | DL [(4 per cent) 1 h] | 15 | 66.6 |
| | | Malathion [(5 per cent) 1 h] | 15 | 66.6 |
| | | Deltamethrin [(0.025 per cent) 1 h] | 15 | 100.0 |
| Rithala (Non-riverine) | *An. stephensi* | DDT[(4 per cent) 1 h] | 45 | 22.2 |
| | | DL [(4 per cent) 1 h] | 45 | 33.3 |
| | | Malathion [(5 per cent) 1 h] | 30 | 43.3 |
| | | Deltamethrin [(0.025 per cent) 1 h] | 15 | 100.0 |

**Table 5. Positive percentage of anopheline and adult emergence (habitat-wise)**

| Breeding habitat | No. searched | Per cent (+)ve rate | Per cent anophelines emerged from samples | | |
|---|---|---|---|---|---|
| | | | Total emerged | *An. culicifacies* | *An. stephensi* |
| *Riverine area* | | | | | |
| Pond | 327 | 25.0 | 221 | 1.35 | 20.05 |
| Ditch | 350 | 30.5 | 326 | 0.92 | 27.76 |
| Tank | 123 | 9.7 | 103 | 0 | 66.0 |
| Drain | 532 | 5.3 | 94 | 23.4 | 19.14 |
| OHT | 118 | 0.85 | 0 | 0 | 0 |
| Pits | 502 | 31.6 | 394 | 2.03 | 28.68 |
| Waste water | 610 | 15.4 | 209 | 0 | 24.4 |
| Pool | 292 | 75.3 | 165 | 1.21 | 2.42 |
| Lake | 51 | 19.6 | 270 | 10.0 | 63.33 |
| River water | 21 | 14.28 | 193 | 17.97 | 26.6 |
| Well | 39 | 7.7 | 4 | 0 | 75.0 |
| *Non-riverine area* | | | | | |
| Pond | 230 | 30.0 | 249 | 6.82 | 31.07 |
| Ditch | 401 | 39.9 | 404 | 2.97 | 50.24 |
| Tank | 456 | 17.3 | 346 | 1.15 | 43.35 |
| Drain | 876 | 10.9 | 295 | 4.40 | 31.86 |
| OHT | 47 | 4.25 | 0 | 0 | 0 |
| Pits | 674 | 35.75 | 586 | 0.85 | 10.92 |
| Waste water | 519 | 27.16 | 180 | 1.66 | 29.44 |
| Pool | 39 | 32.7 | 41 | 4.87 | 19.51 |
| Well | 70 | 1.42 | 0 | 0 | 0 |

discriminatory doses of commonly used insecticides. *An. culicifacies* in both the areas were 100 per cent susceptible to malathion and deltamethrin. In non-riverine area 90 per cent mortality against DDT was observed as against 53.3 per cent in riverine area. The results of susceptibility status of *An. stephensi* when exposed to four per cent DDT and 0.4 per cent dieldrin in riverine and non-riverine areas it was varing between 22 and 87 per cent mortality after 24 hours.

Table 5 gives the data on anopheline breeding habitats. The immature stages were found in a variety of aquatic habitats, ranging from large water bodies such as ponds to small collections

**Table 6. Results of active surveillance in riverine and non-riverine areas (1989 to 1991)**

| Month* | Riverine | | | | | | | | | | | | SPR | SfR | Cases/ 000 | *Pf*/ 000 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gazipur | | | Kanchanpuri | | | Mukundpur | | | Madanpur Khadar | | | | | | |
| | BS | *Pv* | *Pf* | BS | *Pv* | *Pf* | BS | *Pv* | *Pf* | BS | *Pv* | *Pf* | | | | |
| Aug | 49 | 0 | 0 | 27 | 0 | 0 | 36 | 3 | 0 | 16 | 0 | 0 | 2.34 | 0 | 0.11 | 0 |
| Sep | 94 | 1 | 0 | 28 | 4 | 0 | 15 | 2 | 0 | 72 | 0 | 0 | 3.34 | 0 | 0.27 | 0 |
| Oct | 22 | 0 | 0 | 65 | 1 | 0 | 60 | 9 | 0 | 57 | 0 | 0 | 4.9 | 0 | 0.39 | 0 |
| Nov | 28 | 0 | 0 | 65 | 0 | 0 | 43 | 1 | 1 | 31 | 0 | 0 | 1.2 | 0.6 | 0.07 | 0.03 |
| Dec | 12 | 0 | 0 | 42 | 1 | 0 | 20 | 1 | 0 | 17 | 1 | 0 | 3.29 | 0 | 0.11 | 0 |
| Jan | 25 | 0 | 0 | 17 | 0 | 0 | 7 | 0 | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 0 |
| Feb | 15 | 0 | 0 | 27 | 1 | 0 | 14 | 1 | 0 | 4 | 0 | 0 | 3.33 | 0 | 0.07 | 0 |
| Mar | 18 | 0 | 0 | 8 | 1 | 0 | 16 | 0 | 0 | 19 | 0 | 0 | 1.96 | 0 | 0.03 | 0 |
| Apr | 18 | 0 | 0 | 16 | 1 | 0 | 6 | 0 | 0 | 3 | 0 | 0 | 2.32 | 0 | 0.03 | 0 |
| May | 16 | 1 | 0 | 29 | 0 | 0 | 17 | 1 | 0 | 5 | 0 | 0 | 2.98 | 0 | 0.07 | 0 |
| Jun | 15 | 1 | 0 | 15 | 0 | 0 | 11 | 1 | 0 | 11 | 0 | 0 | 3.84 | 0 | 0.07 | 0 |
| Jul | 13 | 2 | 0 | 45 | 0 | 0 | 12 | 0 | 0 | 4 | 0 | 0 | 2.7 | 0 | 0.07 | 0 |
| Total | 325 | 5 | 0 | 384 | 9 | 0 | 257 | 19 | 1 | 246 | 1 | 0 | 2.88 | 0.08 | 1.36 | 0.03 |
| | Non-riverine | | | | | | | | | | | | | | | |
| | Rithala | | | Haiderpur | | | Neelwal | | | Jatkhor | | | | | | |
| | BS | *Pv* | *Pf* | BS | *Pv* | *Pf* | BS | *Pv* | *Pf* | BS | *Pv* | *Pf* | | | | |
| Aug | 73 | 6 | 3 | 20 | 1 | 0 | 2 | 0 | 0 | 9 | 0 | 0 | 9.6 | 2.88 | 0.47 | 0.14 |
| Sep | 36 | 12 | 0 | 36 | 7 | 1 | 5 | 0 | 0 | 1 | 0 | 0 | 25.64 | 1.28 | 0.95 | 0.04 |
| Oct | 77 | 7 | 0 | 33 | 3 | 1 | 7 | 0 | 0 | 32 | 0 | 0 | 7.38 | 0.67 | 0.52 | 0.04 |
| Nov | 71 | 6 | 18 | 50 | 4 | 2 | 11 | 0 | 0 | 20 | 0 | 0 | 19.7 | 13.15 | 1.43 | 0.95 |
| Dec | 56 | 1 | 20 | 11 | 2 | 1 | 5 | 0 | 0 | 8 | 0 | 0 | 30 | 26.25 | 1.14 | 1 |
| Jan | 17 | 1 | 3 | 16 | 0 | 0 | 10 | 0 | 0 | 3 | 0 | 0 | 8.69 | 6.52 | 0.19 | 0.14 |
| Feb | 36 | 5 | 4 | 19 | 1 | 0 | 1 | 0 | 0 | 8 | 0 | 0 | 15.5 | 6.25 | 0.47 | 0.19 |
| Mar | 21 | 6 | 0 | 27 | 1 | 0 | 11 | 0 | 0 | 13 | 0 | 0 | 9.7 | 0 | 0.33 | 0 |
| Apr | 18 | 4 | 0 | 30 | 0 | 0 | 2 | 0 | 0 | 5 | 0 | 0 | 7.27 | 0 | 0.19 | 0 |
| May | 26 | 5 | 0 | 11 | 1 | 0 | 8 | 0 | 0 | 8 | 0 | 0 | 11.3 | 0 | 0.28 | 0 |
| Jun | 13 | 3 | 0 | 16 | 2 | 0 | 4 | 0 | 0 | 7 | 0 | 0 | 12.5 | 0 | 0.23 | 0 |
| Jul | 34 | 2 | 0 | 16 | 1 | 0 | 9 | 0 | 0 | 8 | 0 | 0 | 4.47 | 0 | 0.14 | 0 |
| Total | 478 | 58 | 48 | 285 | 23 | 5 | 75 | 0 | 0 | 122 | 0 | 0 | 13.95 | 5.52 | 6.41 | 2.53 |

*Months include two years data starting from August 1989 to July 1991; BS — Blood slide examined/collected; *Pv — Plasmodium vivax; Pf — Plasmodium falciparum.*

of water as found in pits. The intensity of breeding was higher in the non-riverine areas. Table 5 also shows the vector species that emerged from the immatures collected from various water habitats searched to identify at adult stage during study period. In the riverine area *An. culicifacies* adult emergence was not found among the larvae collected from waste water, wells and tanks. However, *An. stephensi* were emerged from the larvae collected from all the breeding places searched. In both the areas breeding of *An. culicifacies* and *An. stephensi* was found mainly in clear water, except in some polluted, blocked cemented drains with grass growth in the non-riverine area where *An. stephensi* breeding was observed.

The results of fortnightly active surveillance in eight localities of two areas are presented in Table 6. In the riverine area, out of 1,212 blood smears collected, 35 were positive for *Pv* and *Pf* giving a SPR of 2.89 per cent. Relative prevalence of *Pv* and *Pf* was 97.14 and 2.85 per cent respectively, and the slide falciparum rate (SfR) was 0.08 per cent. In the non-riverine area 960 blood smears were collected, of which 134 were positive giving a SPR of 13.95 per cent and a SfR of 5.52 per cent. Relative prevalence of *Pv* and *Pf* was 60.45 and 39.55 per cent respectively. Two years pooled data of malaria cases in two areas showed that maximum cases occurred during July to November.

## DISCUSSION

The densities of adult anopheline mosquitoes were estimated by two techniques, hand collection and total catch. The percentage of *An. stephensi* (15.68 per cent) collected by both the techniques was more than *An. culicifacies* (3.16 per cent) in both the riverine and non-riverine areas. Earlier Bhatia *et al.*[21] during a survey in villages near Delhi, showed nine PMH density of *An. culicifacies* and 4.2 of *An. stephensi* by both the techniques in four years period. Pattanayak *et al.*[7] also found a higher density of *An. culicifacies* (PMH 3.4) than *An. stephensi* (PMH 0.7) in a search lasting 356 hours. However, in the present study the density of *An. stephensi* was much higher than that of *An. culicifacies*. The low density of *An. culicifacies* in the riverine area was due to shifting of preferred larval habitat from the river bed pools to large lakes/channels and pumping reservoirs. This may be due to extensive pollution of the river area caused by sewage and sullage out pourings by 17 major storm water drains. Breeding of *An. culicifacies* was maximum in the northern part of the riverine area where water pollution was at minimal level. In the non-riverine area *An. culicifacies* breeding was not observed in the western Yamuna canal which at present is cement lined and allows fast flow of water. Earlier this canal used to be the main source of *An. culicifacies* breeding.[22]

Seasonal prevalence of *An. culicifacies* density showed two patterns. In the riverine area the peak density was observed in the months of April and October, while in the non-riverine area it was in May and August. Such variations in the dynamics of *An. culicifacies* were also reported earlier.[21,23-25] Species-wise seasonal prevalence of sibling species A and B of *An. culicifacies* showed that species A was predominant in pre-monsoon and in post-monsoon. However, spe-

cies B increased after the onset of monsoon. Earlier studies carried out by Subbarao *et al.*[19] established the role of species A in the transmission of malaria and indicated the higher percentage of species B in riverine villages after post-monsoon which varied between 10 and 60 per cent. In this study, there was not much variation in the proportion of species B in riverine and non-riverine areas which may be due to extensive pollution observed in riverbed pools and lesser number examined. *An. stephensi* was found throughout the year in both the study areas. Mean man hour densities and peaks of *An. stephensi* showed wide variation over months in both the areas and thus it could be considered that *An. stephensi* density was regulated by seasonal factors. Dry summer conditions were found favourable for *An. stephensi* as also reported earlier.[21,26]

To sum up it can be said that urbanization has brought in an ecological succession of vectors. The major vector of malaria in Delhi is *An. stephensi*. All empty lands/marshy swamps, ponds and river water which are contaminated with open sullage/sewage pourings, do not support *An. culicifacies* breeding. Influence of *An. culicifacies* is restricted to northern localities of the riverine area where water is not polluted with sewage pouring compared to southern part of the riverine area.

Day-time resting preferences of *An. culicifacies* and *An. stephensi* in human dwellings and cattlesheds in both the areas did not differ significantly when subjected to ANOVA. Variations in the densities between the two areas were observed. Such variation could be due to ecological factors. Though the two areas are in close proximity to each other, they are ecologically and physiogeographically distinct from one another. The sub-soil water level is very high (0.6 to 1 m) in riverine area and receives 600 mm annual rainfall and soil is coarse to fine loamy. Non-riverine area is characterized by patches on saline and soil type is fine loamy and sand loam and normal annual rainfall is 300 to 500 mm and sub-soil water level is 3 to 10 m. Thus, it is not the proximity and spatial continuity, but the ecosystem, which has influence on the prevalence and densities of *An. culicifacies* and *An. stephensi*.

The differences in AI of *An. culicifacies* and *An. stephensi* in riverine and non-riverine areas were not significant and indicated that *An. stephensi* is more zoophilic than *An. culicifacies*. Afridi *et al.*[27] found the AI rate in Delhi to be 1.8 and 1.4 per cent for *An. culicifacies* and *An. stephensi* respectively. Though the AI of *An. culicifacies* was high compared to that of *An. stephensi,* if density is taken into consideration along with AI the *An. stephensi* number which fed on the human population was greater in the non-riverine area.

The overall sporozoite rate of *An. culicifacies* and *An. stephensi* was 0.7 per cent indicating malaria transmission in the study areas. Previous studies on incrimination of *An. culicifacies* in Delhi carried out in areas adjoining the River Yamuna and in south Delhi showed that *An. culicifacies* has a role in malaria transmission in these areas but no infected specimens were detected in the non-riverine area of northwest Delhi.[28] Our study showed that *An. culicifacies*

has a role in malaria transmission in the non-riverine area also. On the basis of these results it was found that in the riverine area *An. culicifacies* played a greater role in malaria transmission in only the northern part of the area where water pollution is at minimal level while *An. stephensi* played almost the similar role in both the areas. The results also indicated that localities with a high percentage of type forms had high sporozoite rates indicating the active role of type forms in malaria transmission.

Insecticide susceptibility test showed that *An. culicifacies* and *An. stephensi* in both the areas were 100 per cent susceptible to deltamethrin. In non-riverine area mortality of *An. culicifacies* (90 per cent) was compared to riverine area where mortality was 53.3 per cent. Susceptibility status of *An. stephensi* also varied from locality to locality in both the areas which could be due to different selection pressure and needs more investigations.

The study revealed that *P. vivax* and *P. falciparum* infections are endemic in Delhi with a relative ratio of 68 : 32. No *P. malariae* cases were found. In non-riverine and riverine areas, the cases per 1000 was 6.41 and 1.36 respectively indicating that the non-riverine area was more malarious than the riverine. A variation in malaria prevalence rate within the area was observed. *P. vivax* cases were found throughout the year, while *P. falciparum* cases were observed from August to February. Earlier Hodgson[22] reported 65 per cent *P. vivax* and 35 per cent *P. falciparum*. Senior-White[4] found 62 per cent *P. vivax* and 38 per cent *P. falciparum*.[22] Choudhury[29] showed *P. vivax* 71.9 per cent and *P. falciparum* 26.5 and 1.4 per cent mixed infection. Sharma *et al.*[30] reported 83.68 per cent *P. vivax*, 16.09 per cent *P. falciparum* and 0.38 per cent mixed infection. Thus the prevalence of *Pv* has been dominating over *Pf* since 1912. It could, therefore, be inferred that the ecosystem of Delhi is more in favour of *P. vivax* endemicity than *P. falciparum* but disruption in the ecosystem at times may result in a transient spurt in *Pf* cases.[31]

In historic prospective the riverine area was more malarious than non-riverine areas. In contrast to that now a major part of the riverine area (except northern part) is less malarious and other non-riverine areas of Delhi are becoming more malarious. The changes are attributed mainly due to ecological changes, pollution of the Yamuna river and rapid developmental activities which contribute to the anopheline vector breeding habitats and malaria transmission. To conclude, it can be said that both the areas are no longer receptive to *An. culicifacies* and the main vector is *An. stephensi* and accordingly a control strategy be planned based on situational analysis of malaria proneness and vectors.

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# *Plasmodium falciparum* Dihydrofolate Reductase Val-16 and Thr-108 Mutation Associated with *in vivo* Resistance to Antifolate Drug: A Case Study

SUKLA BISWAS[a]

Due to increasing trend in chloroquine resistance, the antifolate (sulpha-pyrimethamine combination) drugs are gaining more importance in the treatment of uncomplicated falciparum malaria. The efficacy of sulpha-pyrimethamine combinations in the treatment is compromised by the development of resistance in parasite. The occurrence of mutations at active sites in *Plasmodium falciparum* gene sequences coding for dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) confer resistance to pyrimethamine and sulphadoxine. This study presents the characterization of a *P. falciparum* sample from a patient who did not respond to standard doses of a pyrimethamine/sulpha regimen. Although parasitaemia fell rapidly, the infection had not resolved six days later as because the response to treatment selected resistant sub-population. The *in vitro* drug sensitivity assays demonstrated resistance to pyrimethamine, sulphadoxine and cycloguanil; while polymerase chain reaction (PCR) and restriction digest based methods indicated that at known drug resistant loci the isolate had a genotype of DHFR Val-16 and Thr-108 previously only associated with cycloguanil resistance. As per the published reports this type of paired mutations in natural isolates are rare. It is of considerable interest to carry out studies on alleles of this gene in relation to resistance at epidemiological level.

**Keywords:** Dihydrofolate reductase, Drug resistance, Malaria, *P. falciparum*, Point mutation

## INTRODUCTION

Chloroquine resistant *Plasmodium falciparum* cases started emerging and spreading since 1960 in malaria endemic regions of the globe. It created an urgent need to look for the alternative drugs to treat mainly those patients who show therapeutic failure with chloroquine (CQ). The antifolates became the drugs of choice as a second line of treatment for CQ resistant

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*P. falciparum* cases. The antifolates include dihydrofolate reductase (DHFR) inhibitors such as pyrimethamine, cycloguanil (the active metabolite of proguanil), trimethoprim and dihydropteroate synthetase (DHPS) inhibitors such as sulfonamides and dapsone.[1] The combination of these two inhibitors provide effective synergistic action against *P. falciparum*.[2] However, resistance to this second line of drugs has also been reported from many parts of the world including southeast Asian countries, mainly Thailand, where sulpha-pyrimethamine (SP) combination had been used intensely during early 1970s resulting in RIII level of resistance. Later on SP became ineffective in those areas.[3] Studies have shown that differential resistance to the antifolate drugs arises from the point mutations in the active sites of the DHFR and DHPS enzymes.[4-8] However, there is a lack of information regarding the molecular basis of antifolate drug resistant parasites in India. In CQ resistant areas of India, SP combination drugs are used for the treatment of uncomplicated *P. falciparum* malaria. Resistance to SP has been reported mainly from northeastern, eastern, central and southern regions of India.[9]

In the present study, I report a case of *P. falciparum* malaria patient who did not respond to the recommended doses of CQ and metakelfin (a combination of sulphamethoxypyrazine-pyrimethamine). This patient came as a traveller to the northeastern state (Manipur), then to the capital city of India (Delhi) from the area near Thailand-Burma border, where resistance to SP combination has been reported. The blood samples collected from this patient on Day 0 (before SP treatment), Day 2 (48 hours after SP treatment) and Day 6 (five days after SP treatment) were tested *in vitro* for antimalarial drug sensitivity and for DHFR gene mutations.

## MATERIALS AND METHODS

The peripheral blood smear of the patient was found positive with ring stage at a density of 75000/µl blood on Day 0. After 48 hours — on Day 2 parasite density was 500/µl and on Day 6 peripheral smear was found positive with ring forms at a density of 3000/µl. After obtaining informed consent, about 0.5–1 ml uncoagulated blood was collected aseptically. The parasite isolate, named as FNEM, was tested *in vitro* for its susceptibility to chloroquine (CQ), pyrimethamine (PM), cycloguanil (CG), sulphadoxine (SD) following the methods published elsewhere.[1,2,10] For testing against antifolates, washed erythrocyte pellet was suspended in conditioned media (with low *p*-aminobenzoic acid and low folate) enriched with 10% human AB+ serum (obtained from blood donor belonging to malaria non-endemic area). Culture was done at 5% haematocrit in 96-well tissue culture plates predosed with various dilutions of CQ (0.2–12.8 µM), PM (0.001–2 µM), CG (0.004–4 µM), SD (2–500 µM). The culture was done by candle jar technique for 44 hours.[11] Smears were stained and examined under microscope. Schizonts having eight or more nuclei were counted per 200 asexual parasites and the minimum inhibitory concentration (MIC) was determined.

Samples collected at three phases were tested for the known polymorphic residues of DHFR at the codon sites 16 and 108 to assess the

molecular basis of the differential response to SP treatment. Parasite genomic DNA was extracted following the published method.[12] The DHFR gene amplification, mutation-specific restriction analysis were done to detect the mutations at codon sites 16 and 108 following published primers and methodologies.[13,14] The known controls of both wild (3D7) and mutant (HB3 and FCR3) along with three known *P. falciparum* isolates (one drug sensitive and two drug resistant) and one negative blood sample were also taken for the assay for comparison.

## RESULTS

The *in vitro* drug sensitivity results are shown in Table 1. The isolate showed higher MIC values in case of CQ and the antifolates when compared with the data of sensitive (K-S: FJB D9) and resistant (K-R: FSH14) strains. The MIC values on Day 0 for CQ, PM, CG and SD were 6.8, 0.92, 0.96 and 300 µmol/l and on Day 6 were 6.6, 0.95, 1.2 and 320 µmol/l respectively. The drug sensitivity assay for Day 2 isolate could not be done since parasitaemia was very low.

**Table 2. Amino acid residues at critical positions of DHFR domain**

| Parasite | | Positions | |
|---|---|---|---|
| | | 16 | 108 |
| FNEM | Day 0 | *Val* | *Thr* |
| | Day 2 | *Val* | *Thr* |
| | Day 6 | *Val* | *Thr* |
| 3D7 (w) | | Ala | Ser |
| HB3 (m) | | Ala | *Asn* |
| FCR3 (m) | | *Val* | *Thr* |

Mutant genotypes are indicated in italics letters; w: Wild; m: Mutant;FNEM: The clinical isolate obtained from traveller on three different days; 3D7: African strain; HB3: Honduras strain; FCR3: The Gambia strain.

Amino acid residues of the codons in the DHFR domain of the clinical isolate before and after treatment are shown in Table 2. Analysis of mutations at codons 16 and 108 were done by amplification of the DHFR gene followed by digestion with restriction enzymes Alu I, Bsr I, ScrF I and Nla III. Digestion with Alu I, Bsr I and ScrF I produced two bands of 386 and

**Table 1. *In vitro* susceptibility to various antimalarial drugs (Drug susceptibility: Minimum inhibitory concentration ± S.D. in µM)**

| Parasite | | CQ | PM | CG | SD |
|---|---|---|---|---|---|
| FNEM | Day 0 | 6.8 ±0.3 | 0.92±0.04 | 0.96±0.07 | 300 ± 6 |
| | Day 6 | 6.6±0.4 | 0.95±0.08 | 1.20±0.18 | 320 ±24.1 |
| K-S | | 0.56±0.04 | 0.003±0.001 | 0.005±0.001 | 25.6 ± 1.6 |
| K-R | | 7.0±0.2 | 1.0±0.04 | 1.25±0.15 | 375 ±6.25 |

K-S: Drug sensitive clone of Indian *P. falciparum* isolate (FJB D9), obtained from a patient responded to CQ and SP; K-R: Drug resistant clone of Indian *P. falciparum* isolate (FSH14), obtained from a patient showed treatment failure to CQ and SP; FNEM: The clinical isolate obtained from the traveller; CQ: Chloroquine; PM: Pyrimethamine; CG: Cycloguanil; SD: Sulphadoxine.

322 bp. The Nla III digestion of the wild type (Ala-16) produced three fragments of 568, 93 and 47 bp, whereas mutant type (Val-16) produced two fragments of 568 and 140 bp. The isolate collected on three different days showed paired mutation of Thr-108 and Val-16 having ScrF I and Nla III restriction sites when compared to the banding patterns of mutant clone FCR3. The restriction pattern after digestion with respective enzymes in test sample (lane 5), reference control (C1:3D7; C2:HB3; C3:FCR3), known parasite isolates (lanes 1, 3, 4) and negative sample (lane 2) are shown in Figs. 1-4.

## DISCUSSION

Of the reported DHFR mutations in a number of geographical isolates from southeast Asia, Africa and South America involve single point mutation in Ser to Asn-108. Mutations at Asn to Ile-51 and Cys to Arg-59 in association with Asn-108 found to be involved with greater resistant pyrimethamine strains. Isolates refractory to both pyrimethamine and cycloguanil possess mutations at Cys to Arg-59, Ser to Asn-108 and Ile to Leu-164.[12,15,16] *P. falciparum* strains showing cycloguanil resistance involves paired mutations at Ala to Val-16 and Ser to Thr-108. This type of parasites with paired mutations also show slight reduction in sensitivity to pyrimethamine.

In CQ resistant areas of India antifolates are being given as alternative treatment. The use of proguanil as prophylactic has been recommended by WHO in CQ resistant areas. Due to the wide use of the antifolates for prophylaxis and therapy, appearance of drug resistant parasites in population is not surprising. The study subject was a non-responder to SP, the peripheral smear was found positive after 48 hours (Day 2) and also within a week (Day 6) after consumption of full doses of the drug. The isolate also showed reduced susceptibility to the antifolates by *in vitro* micro test. From the available data the molecular basis of *in vitro* resistance of *P. falciparum* to the antifolates is understood, but the determinants of treatment failure with SP still remain unknown.[17] Antifolate resistant parasites generally select for the variants Asn-108 alone or in combination with Ile-51 or Arg-59 or Leu-164 in DHFR. The paired mutations of Val-16 and Thr-108 as an indicator of high cycloguanil and low pyrimethamine resistance are not very common. The molecular analysis of 208 isolates from diversified regions as south Asia, middle east, Africa and South America did not show presence of this type of mutant form.[8,16] Of 50 isolates from Vietnam, only one showed paired mutation of Thr-108 and Val-16.[18] The molecular analysis of *P. falciparum* isolates collected from various geographic locations in India and Thailand has not shown any genotype containing paired mutation at codons 16 and 108.[19] This clinical specimen has shown reduced sensitivity *in vitro* to both pyrimethamine and cycloguanil, which is correlated with the mutation at positions 16 and 108. It is not yet known whether both of these drugs share the same or different binding sites in DHFR.[17] Though the SP use is becoming common in some places of India due to non-responsiveness to CQ, yet there is not a high selection pressure from drug treatment. Because of the polyclonality of the natural isolates the resistant clones may spread among

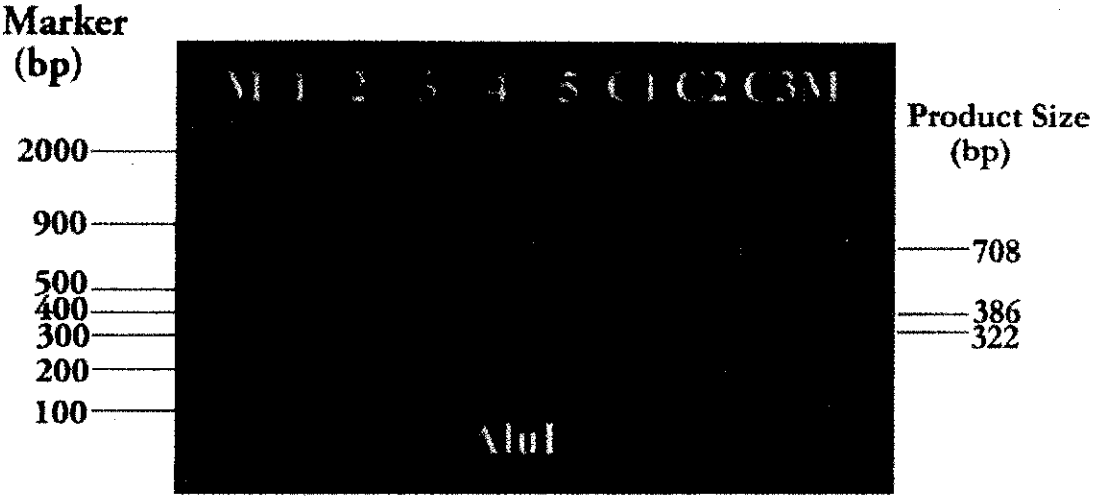


*Fig. 1:* Restriction digestion pattern of *Pf*-DHFR gene (708bp) at codon 108 by restriction enzymes Alu I in three *Pf* isolates in lanes 1, 3, 4 and for FNEM in lane 5. Lane 2 for parasite negative sample. Presence of two cleaved products of 386bp and 322bp in lane 1 shows the wild type Ser-108 as obtained in 3D7 (lane C1). Single band of 708bp in lanes 3, 4, 5, C2 and C3 demonstrate undigested product. DNA size marker of 100bp was loaded in lane M


*Fig. 2:* Restriction digestion pattern of *Pf*-DHFR gene (708bp) at codon 108 by restriction enzymes Bsr I in three *Pf* isolates in lanes 1, 3, 4 and for FNEM in lane 5. Lane 2 for parasite negative sample. Presence of two cleaved products of 386bp and 322bp in lanes 3,4 shows the mutant type Asn-108 as obtained in HB3 (lane C2). Single band of 708bp in lanes 1, 5, C1 and C3 demonstrate undigested product. DNA size marker of 100bp was loaded in lane M

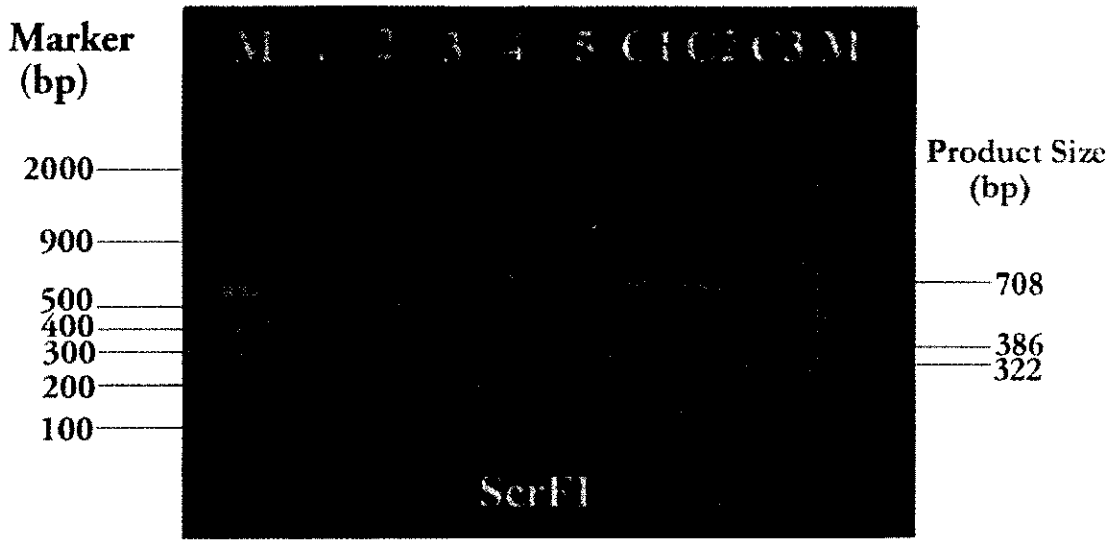


*Fig. 3:* Restriction digestion pattern of *Pf*-DHFR gene (708bp) at codon 108 by restriction enzymes ScrF I in 3 *Pf* isolates in lanes 1, 3, 4 and for FNEM in lane 5. Lane 2 for parasite negative sample. Presence of two cleaved products of 386bp and 322bp in lane 5 shows the mutant type Thr-108 as obtained in FCR3 (lane C3). Single band of 708bp in lanes 1, 3, 4, C1 and C2 demonstrate undigested product. DNA size marker of 100 bp was loaded in lane M

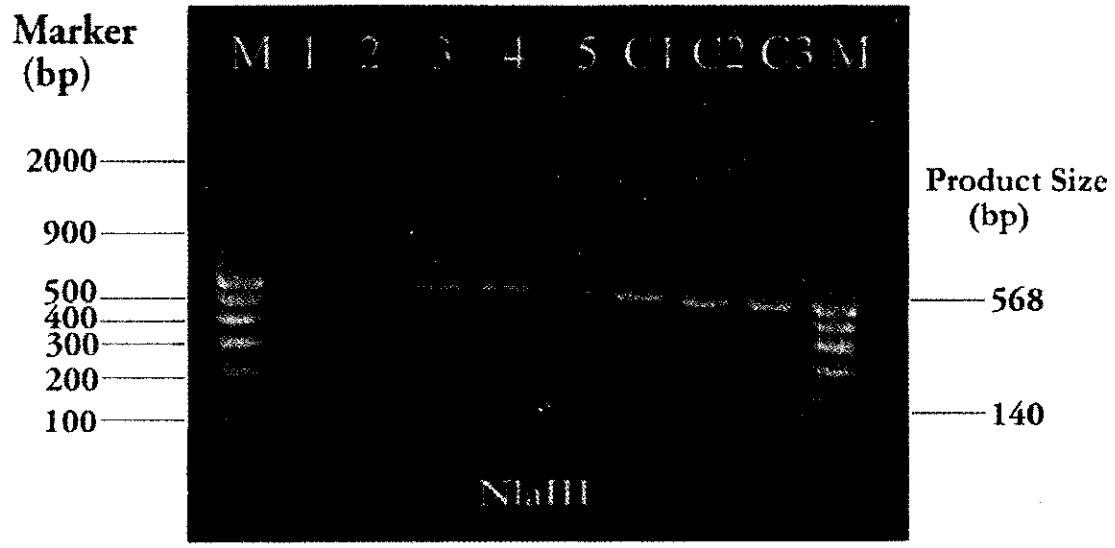


*Fig. 4:* Restriction digestion pattern of *Pf*-DHFR gene (708bp) at codon 16 by restriction enzymes Nla III in three *Pf* isolates in lanes 1, 3, 4 and for FNEM in lane 5. Lane 2 for parasite negative sample. Presence of two bands of 568bp and 140bp in lane 5 shows the mutant type Val-16 as obtained in FCR3 (lane C3). Presence of thick band of 568bp, two faint bands of 93bp and 47bp in lanes 1, 3, 4, C1 and C2 demonstrate wild type Ala-16. DNA size marker of 100 bp was loaded in lane M

patients even in the absence of selection pressure of the drug. The molecular methods and their application in surveillance programme to detect drug resistant mutants may offer an additional tool in epidemiological surveys.

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# Serological Appraisal of Malaria Status in Tribal Area of Orissa, India

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A cross-sectional seroepidemiological study conducted in 173 tribal residents (including all age groups) from three villages of Sundergarh district of Orissa using ELISA as a tool revealed high levels of antibody titres against both AR1 and *Pf* antigens. The mean ±S.D. ELISA O.D. obtained for AR1 were 0.73±0.2, 1.037±0.196, 1.05±0.42 and for *Pf*, 0.70±0.2, 1.0±0.28, 0.94±0.4 respectively for Badramoli, Jharbeda and Boneikela population. Parasitological results showed high incidence of malaria in < 5 years age group in Badramoli and Boneikela villages when compared to other age groups. *An. fluviatilis* was found to be the principle malaria vector as found in the HBI results. High ELISA O.D. and high equivalent transmission index (ETI) indicate a high malaria transmission in the area. Seroepidemiological studies could be used for effective surveillance and stratification of endemicity in a given area which can help in executing intervention strategy for malaria control.

**Keywords:** Annual parasite index, Enzyme-linked immunosorbent assay, Equivalent transmission index, Malaria, Tribals

## INTRODUCTION

With the increasing development of resistance to antimalarial drugs in *Plasmodiumm falciparum* strains, resurgence of malaria in India has emerged as a major public health problem. In states like Orissa, particularly the tribal area with its unique geographical and socioeconomic conditions and recurrence of natural calamities malaria situation has always been very grim. In fact 30 per cent of *P. falciparum* cases and 48 per cent of all malaria deaths reported in India are from Orissa alone.[1,2] The uninterrupted transmission in tribal areas of Orissa is mainly because, the tribal population generally resides in forest area, which is inaccessible and hence

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proper medication and organized control measures can't be carried out effectively. The study of malariometric indicators and their association with potential risk factors is very important to understand the malariogenic situation. In an endemic environment individuals develop certain degree of protective immunity against the malaria parasite. Seroreactive antimalarial antibodies directed against merozoite surface antigen (MSA-1) and *Pf*155/RESA (vaccine candidate) have been demonstrated in these communities.[3-5] Assessment of immunological status of the host is an important and essential criterion to differentiate the non-immune community from the immune community, thus providing a reliable ground for determining the degree of endemicity and immune profile of the area. In the present study, antimalarial antibodies directed against *P. falciparum* antigen and synthetic peptide antigen AR1 (*Pf*155/RESA) have been determined in the tribal population of Sundergarh district during August–November 1997 using the enzyme linked immunosorbent assay (ELISA). The R1 antigen represents an epitope in the carboxyl terminal immunodominant repeat region of the ring infected erythrocyte surface antigen (RESA/*Pf*155) of *P. falciparum*. The results of the study are presented in this paper.

## MATERIALS AND METHODS

### Study area

Sundergarh district, which is most seriously and predominantly affected by falciparum malaria, is situated in the Garhjat hills of eastern deccan plateau with an altitude range of 200-900 m above mean sea level. Thirty-six per cent of the area is covered with forests and is inhabited predominantly by tribals (62 per cent of tribal population). The three study villages namely, Badramoli, Jharbeda under Bisra PHC and Boneikela under Gurundia PHC (all tribal villages) were selected on the basis of high prevalence of malaria.[6]

### Sampling

A total of 173 finger prick blood samples were collected randomly from a cross-sectional population with their full consent during August–November 1997. All the age groups including children were included in the study. Blood samples were collected on Whatman paper No. 3 and stored at –20°C till used. Thick and thin blood smears were also prepared on glass slides from the same person. The slides were stained with JSB stain and examined under oil immersion compound microscope. Prevalence data and malariometric indices for the study villages were collected from the District Malaria Officer, Sundergarh for the period 1995–1997 to know the endemicity of malaria in the region.

### Synthetic peptide AR1 antigen

It is a nonapeptide (EENVEHDA-CYS) and is custom synthesized commercially from M/s. Cambridge Research Biochemicals, U.K. It is an octapeptide epitope form 3' repeat region of the ring infected erythrocyte surface antigen (RESA) with a CYS at the c' terminal end.

### *Pf* antigen

Soluble extract of trophozoite- schizont enriched fraction through percoll gradient was used for ELISA as reported earlier.[3]

**Antimalarial IgG antibody detection:** Each blood sample (50 µl) on filter paper was eluted with 1 ml phosphate buffer saline (pH 7.4) at ± 4°C overnight. Enzyme linked immunosorbent assay (ELISA) was carried out according to published procedure.[7]

Using linear regression analysis of ELISA O.D. value and known annual parasite index (API) of a given area a formula was constructed to calculate ETI = 270.55 x ELISA O.D.+7.40 (using microsoft programme) where 270.55 and 7.4 are constant numbers.

**Mosquito blood meal analysis**

Adult mosquito of vector species like *An. culicifacies* and *An. fluviatilis* were collected from the study villages in the morning during 0600-0800 hours from human dwellings and cattlesheds using suction tube. The stomach blood of mosquito was smeared on Whatman filter paper No. 1 and the samples were assayed by microdot ELISA technique.[8]

**RESULTS**

Results of malaria microscopy of 173 persons from three malaria endemic villages of Sundergarh district (Orissa) are given in Table 1. The slide positivity rate (SPR) in all the three villages was found to be highest in the age group of 0–5 years but was showing declining trend in the higher age groups. The parasitological data for the study villages collected from the State Health Department for the years 1995, 96 and 97 revealed that Badramoli and Jharbeda had past history of low malaria transmission, Boneikela village had the high prevalence of malaria compared to the other two villages. The comparison of parasitological values in different age groups revealed that children popula-

**Table 1. Age related parasite prevalences in three villages of Sundergarh district during August-November 1997**

| Village | Age group (yrs) | Average age Mean ± S.D. | No. examined | SPR |
|---|---|---|---|---|
| Badramoli | 0–5 | 2.29± 1.35 | 16 | 37.5 |
| | 6–19 | – | – | – |
| | >20 | 34±10.92 | 13 | 13.7 |
| Jharbeda | 0–5 | 2.66± 1.86 | 19 | – |
| | 6–19 | 14.25± 1.70 | 18 | 22 |
| | >20 | 57.06±10.45 | 27 | 13.5 |
| Boneikela | 0–5 | 4.16± 1.57 | 11 | 18 |
| | 6–19 | 12.11± 4.8 | 26 | 11.5 |
| | >20 | 35.0 ±10.66 | 33 | 3 |

SPR — Slide positivity rate.

tion had the highest SPR compared to other age groups indicating their vulnerability to malaria infections.

Results of the ELISA tests performed on filter paper blood samples for the detection of anti AR1 and anti *Pf*IgG levels are shown in Table 2. Majority of the study population was found to contain high levels of IgG antibodies. Jharbeda village population showed high seropositivity followed by Boneikela and Badramoli. ETI which has been determined from known mean ELISA O.D. and its corresponding API from low, moderate and high endemic areas were compared. Regression equation has been developed by using low, moderate and high transmission status. ETI gives more reliable information on malaria transmission, where high ELISA O.D. and high ETI indicate a high malaria transmission area. Sero-reactivity such as ELISA O.D. and ETI all lead to a conclusion that these three villages had high malaria transmission throughout the year.

Two malaria vector species *An. culicifacies* and *An. fluviatilis* which are widely prevalent in the study area were assayed for host identification from mosquito blood meal and the results are shown in Table 3. A clear distinction of human blood index (HBI) between the two species in-

**Table 2. Seroreactivity against AR1 peptide and *Pf* antigen in the population residing in three villages of Sundergarh district during 1997 transmission season**

| Village | ELISA O.D. | | % Seropositivity | | API | ETI |
|---|---|---|---|---|---|---|
| | AR1 Mean±S.D. | *Pf* Mean± S.D. | R1 | *Pf* | | |
| Badramoli | 0.73±0.20 | 0.70±0.20 | 87.5 | 80.15 | 23.5 | 184.82 |
| Jharbeda | 1.037±0.196 | 1.0±0.28 | 97.8 | 96.6 | 13.2 | 266.76 |
| Boneikela | 1.055± 0.42 | 0.94±0.40 | 92.3 | 84.3 | 47.2 | 283.24 |

API—Annual parasite index; ETI—Equivalent transmission index.

**Table 3. Mosquito blood meal host identification in vectors collected from the study villages in Sundergarh district, Orissa**

| Village | Species | No. tested | Host | | |
|---|---|---|---|---|---|
| | | | Human | Cattle | HBI |
| Badramoli | *An. culicifacies* | 42 | 0 | 42 | 0 |
| | *An. fluviatilis* | 5 | 5 | 0 | 1 |
| Jharbeda | *An. culicifacies* | 96 | 0 | 96 | 0 |
| Boneikela | *An. culicifacies* | 16 | 1 | 15 | 0.062 |
| | *An. fluviatilis* | 16 | 16 | 0 | 1 |

HBI— Human blood index.

dicate that *An. fluviatilis* is the major vector in this area although it exhibits varied role in the malaria transmission under different ecological conditions.

## DISCUSSION

The epidemiology of malaria is the product of a complex interactions between host, vector and parasite that are specific to each location in which malaria occurs.[9] In places such as Orissa and particularly tribal area of Orissa where transmission is intense and malaria is hyperendemic, prevalence of malaria parasitaemia decreases with age. Results from the present study show that Badramoli village had history of low malaria transmission but during sample collection most children were found parasite positive. They had lower AR1 ELISA O.D. compared to SPR. The reason of low antibody titre may be first time exposure of small children to the malaria parasite and subsequent exposure may enhance the antibody titre.[10] The high API during 1997 indicated high malaria transmission during the year. Jharbeda village showed a decline in SPR with increase of age. The ELISA O.D. was high but the API was lower which indicates higher immunity in the population making them less infective. Seroepidemiological information including API and ELISA O.D. can be expressed in a better way using ETI. It has been found that ETI gives more reliable information which explains high incidence rate and high ELISA O.D. The study population in the village Boneikela had the highest API, ELISA O.D. and human blood index which explains that Boneikela was the worst affected malaria endemic village compared to rest. API which is widely accepted as a malariometric index to measure malaria transmission may not be dependable in areas where active surveillance is difficult due to inaccessibility and socio-cultural practices of the population.

Results of the present study showing high levels of antimalarial antibodies of IgG type directed against AR1 and *Pf* antigens is a clear indication of highly endemic status of these tribal villages of Sundergarh district of Orissa. Observation of high levels of IgG isotype in tribals is in fact an indication of sufficient exposure to malariogenic conditions with active process of transmission. Presence of high levels of IgG type of antimalarial antibodies in young children (0-5 years) is an indication of fresh transmission. Serological technique such as ELISA is a suitable and sensitive technique to measure the endemicity of malaria in a given community. Similar approach had been used to measure endemicity in other countries also.[11] In the present study ELISA using *Pf* crude antigen and AR1 synthetic peptide antigen was found to be useful technique to evaluate the endemicity in India. Although, purified and enriched parasite (*Pf*) preparation gives reasonable reproducibility and correlate well with endemicity, it is not feasible to obtain such antigens in large quantities to carry out epidemiological studies at large-scale. These problems, however, can be successfully solved by using synthetic peptide as these offers several advantages like stringent quality control, long shelf life, stability at ambient temperature and large-scale production. Such antigens can be used widely in endemic areas. Studies using synthetic peptide antigen have given satisfactory results so far.[12] It could, therefore, be recom-

mended for wide use and considered as a promising and dependable candidate in serology of malaria to evaluate endemicity.[13-14]

Finally, it may be appropriate to comment that high levels of antimalarial antibodies in endemic situation is an indication of the ongoing process of active transmission. Immune responses in the host, which provides some degree of protection and help in preventing clinical malaria. But development of sterile immunity is rare. By screening the population through seroepidemiological surveillance tests, endemic status could be predicted well in advance so that execution of control strategies be taken successfully.

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# Dynamics of Malaria Transmission under Changing Ecological Scenario in and around Nanak Matta Dam, Uttaranchal, India

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To understand the transmission dynamics of malaria in three different ecotypes, namely watershed (forest), seepage (Nanak Matta Dam) and plain (non-forest, non-dam) areas of Nainital and Udham Singh Nagar districts of Uttaranchal, entomological and parasitological investigations were carried out from July 1996 to June 1997. In the three ecotypes, average per man hour densities of adult vector species in human dwellings and cattlesheds recorded were high for *Anopheles culicifacies* from April to September and October to March for *An. fluviatilis*. Prevalence of both *An. culicifacies* and *An. fluviatilis* was higher in the forest area as compared to other two areas. Observations on gonotrophic condition revealed endophilic tendency of both vector species. Higher number of both vector species were found in outdoor than indoor during night human bait collections. Out of 864 specimens of *An. fluviatilis* dissected, one showed natural infection of sporozoites in salivary glands in the month of November from the forest area only. Sibling species study of *An. fluviatilis* revealed the presence of species S for the first time in the forest area. Parasitological investigations also depicted high incidence of malaria in the forest area as compared to other two areas. Overall results from the study indicated active malaria transmission in the forest area.

**Keywords:** *An. culicifacies, An. fluviatilis,* Malaria transmission, Sibling species

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## INTRODUCTION

Foothill Himalayas (terai) in District Nainital of Uttaranchal were reported to be hyperendemic. The area earned the notoriety of death trap of malaria and was considered unfit for human habitation and was getting depopulated because of high morbidity and mortality. Earlier studies indicated prevalence of four anopheline species — *An. minimus, An. fluviatilis, An. culicifacies* and *An. varuna,* where *An. minimus* was incriminated as the chief vector of malaria with a sporozoite rate of 2.5 per cent in eastern terai.[1] In *An. fluviatilis* gut infections were recorded in two out of 1871 dissected, accordingly *An. fluviatilis* was considered of no significance as a malaria vector in western terai.[2]

During 1947, with the influx of displaced persons from Pakistan, terai area was brought under colonization scheme in a mass-scale for reclamation of land for resettlement with the large-scale deforestation and DDT spray in the area, marshes got drained and wild animals retreated into deeper forest. Consequently, *An. fluviatilis* showed increased anthropophilic nature as high as 47 per cent.[3] Due to increased man mosquito contact, *An. fluviatilis* replaced *An. minimus* as the chief vector,[4,5] latter was, however, got dislodged from the area due to DDT spray.[6] Ecological pressure continued in the terrain system under intensive cultivation practices and soon it was found that malaria incidence declined considerably. However, almost four decades after an outbreak occurred in the terai where *An. culicifacies* and *An. fluviatilis* were incriminated as vectors of malaria,[7] but *An. fluviatilis* was later considered as a secondary vector due to low anthropophily and resting behaviour.[8]

Under these changing ecological scenario, it was decided to undertake longitudinal studies in and around Nanak Matta Dam (terai) area, Uttaranchal to define the role of vector species. The study lasted one year from July 1996 to June 1997 and the present paper records the observations made on this important subject.

## MATERIALS AND METHODS

### Study area

Two villages each from three ecologically different areas like watershed (forest), seepage (Nanak Matta Dam) and plain (non-forest, non-dam) were selected for the study. Ecological characteristics of each ecotype are briefly described below.

**Watershed area (Forest):** Haspur and Jolashal villages (pop. 537) of Mota Haldu PHC in Nainital district are situated in the deep forest area, cross erased by monsoon streams and rivulets. All houses are *kachha,* made up of wood and mud. Some houses have outdoor two tier open structures locally known as *machan* for shelter. Mostly the houses are scattered in small groups known as "*Khattas*". Ethnic proportion of the population was 54 per cent hill residents, 23.7 per cent muslims (Gujjars) and 22.3 per cent Tharu tribals. Census survey revealed cattle to human ratio as 2.7 : 1.

**Seepage area (Nanak Matta Dam):** The dam area villages include Bengali colony and

Kishanpur of Nanak Matta PHC of District Udham Singh Nagar. The population of these villages was 1162. The earthern dam is seepage prone which creates swampy/marshy conditions in the surrounding areas. The ratio of cattle to human population was 1 : 3.1.

**Plain area (Non-forest, non-dam):** The plain area villages are Jhankat and Khempur of Nanak Matta PHC of District Udham Singh Nagar. The population of these villages was 849. The villages have compact houses made-up of bricks, mud and cement. Sub-soil water level is very high and Artesian well water is used for agriculture and drinking purpose. Cattle to human ratio was 1 : 1.8. The land is fertile and the socio-economic status of inhabitants is better than that of other two ecotypes.

Following detailed studies were carried out in above ecotypes.

**Entomological study:** Per man hour density, resting behaviour, abdominal condition, night biting, whole night resting collections and sporozoite rate of *An. culicifacies* and *An. fluviatilis* were recorded as per WHO standard techniques.[9] Sibling species composition and blood meal analysis of *An. culicifacies* and *An. fluviatilis* from indoor resting collections were carried out as per techniques described by Subbarao *et al.*[10]

**Parasitological study:** Blood smears from febrile patients were examined microscopically for malaria parasite. Presumptive treatment of 600 mg chloroquine base to adults and proportionate doses to children were given as per NAMP schedule.

**Meteorological data:** Rainfall, average temperature and relative humidity data from July 1996 to June 1997 were collected from G.B. Pant Agriculture and Technology University, Pantnagar, Uttaranchal.

## RESULTS

### Entomological observations

**Man hour density (MHD):** Data on per man hour density of *An. culicifacies* and *An. fluviatilis* for different seasons in three areas revealed that in the forest area the average per man hour density of *An. culicifacies* ranged from 2.5 to 113.8 and of *An. fluviatilis* 13.3 to 82.2, in the dam area it varied from 1.0 to 24.7 for *An. culicifacies* and 0.0 to 31.3 for *An. fluviatilis,* whereas in the plain area the vector density varied from 0.4 to 14.2 for *An. culicifacies* and 0.7 to 30.9 for *An. fluviatiliş*. High density of *An. culicifacies* was recorded in pre-monsoon and monsoon months (April to September) and of *An. fluviatilis* during post-monsoon months (October to March) in all the three areas.

Prevalence of both vector species was highest in the forest area. Overall the prevalence pattern of both vector species had inverse relationship in all three areas in cattlesheds and human dwellings (Fig. 1).

**Gonotrophic conditions:** The per cent proportion of unfed (UF), fullfed (FF), semigravid (SG) and gravid (G) condition of vector species were recorded. In the forest area the above proportion was 4.2, 39.0, 30.5 and 26.3 of *An. culicifacies* and 3.6, 34.7, 30.2 and 31.5 of

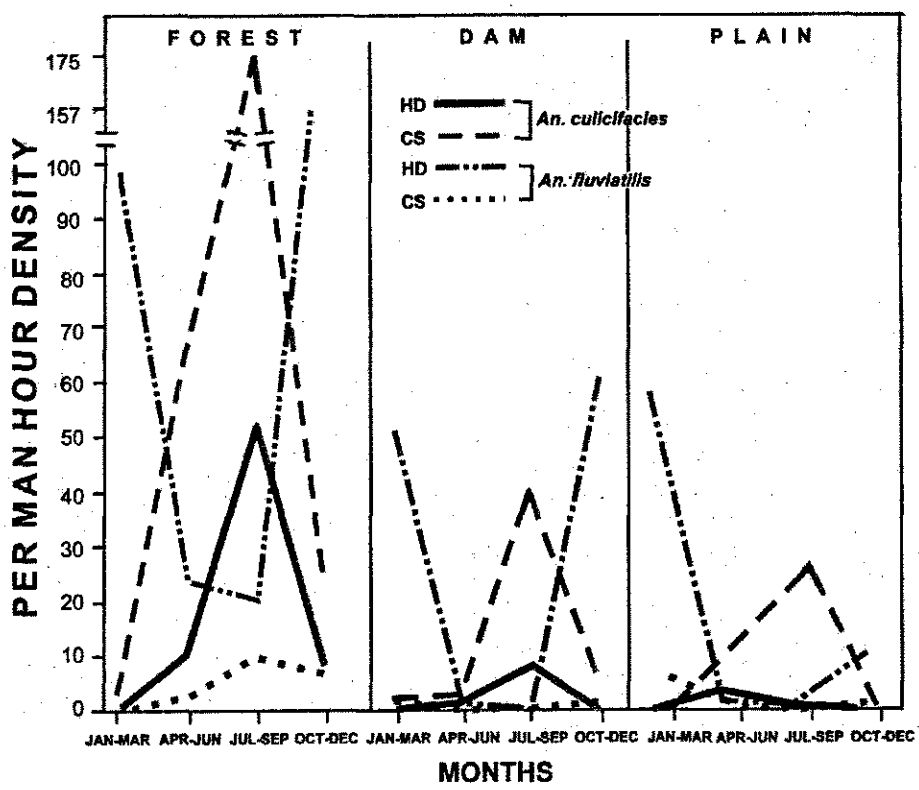


*Fig. 1:* Prevalence pattern of *An. culicifacies* and *An. fluviatilis* in cattlesheds and human dwellings from different ecotypes (July 1996 to June 1997)

*An. fluviatilis,* respectively. In the dam area the UF, FF, SG and G proportion was 6.3, 39.6, 35.1 and 19.0 of *An. culicifacies* and 7.4, 30.8, 36.7 and 25.1 of *An. fluviatilis* while in the plain area this proportion was 13.2, 36.8, 32.1 and 17.9 of *An. culicifacies* and 11.2, 23.8, 36.0 and 29.0 of *An. fluviatilis,* respectively. From the results of gonotrophic conditions it is concluded that both *An. culicifacies* and *An. fluviatilis* showed endophilic tendency.

**Night bait collections:** The results of night human bait collections are shown in Table 1. In the forest area *An. culicifacies* biting was high during third and fourth quarters (zero to 0600 hours) of night and biting rate per night per bait in indoor and outdoor was 0.5 and 1.4 respectively whereas *An. fluviatilis* biting was recorded whole night with a peak in second quarter (2100 to 2400 hours) of night and biting rate per bait per night in indoor and outdoor was 1.1 and 3.0, respectively. In the plain area *An. culicifacies* biting rate per night per bait was zero in indoor and 0.4 in outdoor, whereas no biting of both vector species were recorded in the dam area. The average biting rate was 0.95

**Table1. Results of whole night indoor and outdoor human biting collections of vector anophelines (July to September 1996 and May to June 1997)**

| Vector species | Study area | Biting hour collections | | | | | | | | | | Biting rate/ night/ bait | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | I quarter | | II quarter | | III quarter | | IV quarter | | Total | | | |
| | | ID | OD | ID | OD | ID | OD | ID | OD | ID | OD | ID | OD |
| *An. culicifacies* | Forest | – | 3 | – | 4 | 3 | 4 | 3 | 6 | 6 | 17 | 0.5 | 1.4 |
| | Dam | – | – | – | – | – | – | – | – | – | – | – | – |
| | Plain | – | 2 | – | – | – | – | – | 1 | – | 3 | – | 0.4 |
| *An. fluviatilis* | Forest | 1 | 9 | 5 | 12 | 5 | 6 | 2 | 10 | 13 | 36 | 1.1 | 3.0 |
| | Dam | – | – | – | – | – | – | – | – | – | – | – | – |
| | Plain | – | – | – | – | – | – | – | – | – | – | – | – |

ID — Indoor; OD — Outdoor; Total night collections in each quarter : 12 in forest, nine in dam and eight in plain areas; Total baits : 12 each in indoor and outdoor in forest area, nine each in indoor and outdoor in dam area, and eight each in indoor and outdoor in plain area.

and 2.05 of *An. culicifacies* and *An. fluviatilis* in the forest area, respectively and 0.2 of *An. culicifacies* in the plain area.

Night human biting collections during winter season could not be carried out due to non-availability of bait, hence whole night indoor resting collections of vector species were made from October to April. In the forest area, *An. culicifacies* and *An. fluviatilis* collection was high during first quarter (1800 to 2100 hours) and fourth quarter (0300 to 0600 hours) of night and the average collection per night was 4.1 and 9.9, respectively. The average collection per night of *An. culicifacies* and *An. fluviatilis* was zero and 2.1 in the dam area whereas in the plain area it was 0.86 and 4.0, respectively.

**Vector incrimination:** A total of 864 *An. fluviatilis* and 151 *An. culicifacies* collected from the forest area were dissected for salivary gland positivity where one specimen of former species was found positive for sporozoite infection in November 1996 but none from the latter species. From the dam and plain areas a total of 80 and 45 *An. fluviatilis* and 17 and 25 *An. culicifacies,* respectively were dissected for salivary gland positivity but none was found infective.

**Sibling species composition and blood meal analysis:** Results of sibling species composition and blood meal analysis of *An. culicifacies* and *An. fluviatilis* are given in Table 2. From the forest area, out of 122 *An. culicifacies* and 239 *An. fluviatilis* analysed, the per cent composition of *An. culicifacies* species B and C was 87.7 and 12.3 and, human blood index (HBI) was 0.009 and zero, respectively, whereas in *An. fluviatilis* the per cent composition of

**Table 2. *An. culicifacies* and *An. fluviatilis* sibling species composition and their blood meal analysis in three different ecotypes (July 1996 to June 1997)**

| Ecotype | Total nos. analysed | *An. culicifacies* per cent sibling species (HBI) | | Total no. analysed | *An. fluviatilis* per cent sibling species (HBI) | |
|---|---|---|---|---|---|---|
| | | B | C | | S | T |
| Forest villages | 122 | 87.7 (0.009) | 12.3 (0.0) | 239 | 1.3 (0.3) | 98.7 (0.004) |
| Seepage area villages (dam site) | 38 | 92.1 (0.0) | 7.9 (0.0) | 14 | 0 | 100 (0.0) |
| Plain area villages | 26 | 92.3 (0.0) | 7.7 (0.0) | 18 | 0 | 100 (0.0) |

HBI — Human blood index; Figures in parentheses indicate HBI.

species S and T was 1.3 and 98.7 and the HBI was 0.3 and 0.004, respectively. In the dam area, out of 38 *An. culicifacies* analysed, the per cent composition of B and C was 92.1 and 7.9; and HBI was zero for both the species whereas all 14 samples of *An. fluviatilis* analysed were species T and the HBI was zero. In the plain area, out of 26 *An. culicifacies* examined the per cent composition of species B and C was 92.3 and 7.7, respectively and the HBI was zero, whereas in *An. fluviatilis,* out of 18 samples analysed all (100 per cent) were species T and the HBI was recorded zero. High percentages of species B in all the areas as compared to C were recorded. However, areawise results revealed comparatively higher proportion of C in the forest area than in other two areas.

## Parasitological observations

The results of active fever surveys conducted in three different ecotypes are given in Table 3. The parasitological indices—slide positivity rate, slide falciparum rate, annual parasite incidence and annual blood examination rate in forest villages were 48.5, 35.7, 245.8 and 50.7, respectively. In the dam and plain areas the SPR was 0.0 and 1.8; the SfR was 0.0 and 0.0; the API was 0.0 and 1.2; and annual blood examination rate (ABER) was 4.8 and 6.4, respectively. Highest parasitological parameters were recorded from the forest area.

Sex-wise analysis of malaria positive cases was made which did not indicate any difference but age group analysis revealed high malaria preva-

**Table 3. Results of active surveillance in three different ecotypes (July 1996 to June 1997)**

| Ecotype | Population | BSE | *Pv* | *Pf* | Total (+)ve | SPR | SfR | API | ABER |
|---|---|---|---|---|---|---|---|---|---|
| Forest | 537 | 272 | 35 | 97 | 132 | 48.5 | 35.7 | 245.8 | 50.7 |
| Dam | 1162 | 56 | 0 | 0 | 0 | 0.0 | 0.0 | 0.0 | 4.8 |
| Plain | 849 | 54 | 1 | 0 | 1 | 1.8 | 0.0 | 1.2 | 6.4 |

lence (60.1 per cent) in young age (5 to 15 years) group in the forest area. The average SfR was recorded highest (49.5 per cent) during October to December months. The entomological results also revealed highest density (157) of *An. fluviatilis* during these months and natural infection of sporozoite in the month of November. Thus, both entomological and parasitological results proved that *An. fluviatilis* was responsible for malaria transmission in the forest area.

Further, the meteorological data like average temperature and relative humidity values were recorded 18.8°C and 79 per cent, respectively which were found suitable for the survival of vector species and transmission of malaria during the month of November in the forest area.

## DISCUSSION

During the present study no human biting of *An. fluviatilis* was observed in the dam and plain areas. Our earlier observation revealed a very low biting behaviour of *An. fluviatilis* (0.08 to 0.33 bites/bait/night) in the dam area of terai.[11] *An. fluviatilis* in terai area had very little preference for human blood and was predominantly zoophagic which is in agreement with the findings reported by Nanda *et al.*[12]

In *An. culicifacies*, sibling species B was reported as a poor malaria vector and species C an efficient vector for malaria transmission,[10,13] whereas in the present study anthropophagy was recorded 0.009 and zero in *An. culicifacies* sibling species B and C, respectively in the forest area.

Species S is highly anthropophagic and an efficient vector of malaria.[12] It is possible that species S was wide spread in terai area of District Nainital but due to deforestation and extensive agricultural practices it is now confined to forest area and playing a role in malaria transmission in forest villages.

High malaria incidence in the forest area may be attributed to high vector densities, suitable ecological niches, low socio-economic status, lack of awareness about protection measures and inaccessibility of area for vector control operations. Moreover, no antimalarial activities were being carried out by the state health authorities in the deep forest area. On the other hand low incidence of malaria in dam and plain areas may be due to low prevalence of vectors, better socio-economic status, more awareness towards health maintenance, easy approach to treatment from primary health centres and spraying of insecticides etc.

From the study, it is concluded that *An. fluviatilis* is playing a key role in transmission of malaria in the forest area as proved by incrimination study. Occurrence of species S of *An. fluviatilis* complex which is an efficient vector, was recorded for the first time from the forest area. Moreover, the relative proportion of species C of *An. culicifacies* complex, an established malaria vector was higher in the forest area than in other two areas. The role of sibling species C needs further elucidation.

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**Short Notes**

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# Prevalence of G-6-PD Deficiency and Sickle-cell Haemoglobin Carriers in Malaria Endemic Tribal Dominated Districts—Mandla and Jabalpur, Madhya Pradesh

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**Keywords:** G-6-PD, Gonds, HbS, Madhya Pradesh, Malaria

Human blood polymorphic systems are important biochemical markers in anthropological surveys especially in relation to disease distribution. Of the several enzyme systems studied in relation to malaria, glucose-6-phosphate dehydrogenase (G-6-PD) deficiency and certain haemoglobins confer a selective advantage to the subjects against malaria infection by *Plasmodium falciparum*.[1] However, the use of certain antimalarials such as primaquine and other 8-amino quinolines increases the oxidant stress in G-6-PD deficient individuals resulting in haemolytic crisis.[2] Similarly, individuals who are homozygous for sickle-cell haemoglobin (HbS) develop sickle-cell crisis under low oxygen conditions.[3] Data on the frequency and distribution of G-6-PD deficiency and other variants, haemoglobin variants and other related genetic markers would be helpful in the administration of proper drugs. Therefore, a study was undertaken on the prevalence of G-6-PD deficiency and other haemoglobinopathies in malaria en-

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**Table 1. Distribution of G-6-PD deficiency and sickle-cell haemoglobin in Gond tribes of Mandla and Jabalpur districts, Madhya Pradesh**

| System | Mandla | | | Jabalpur | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|
| | Obs. Nos. (%) | Exp.% | Gene | Obs. Nos. (%) | Exp.% | Gene | Obs. Nos. (%) | Exp.% | Gene |
| *G-6-PD (Males only)* | | | | | | | | | |
| Deficiency | 6 (7.3) | – | $Gd^-$ 0.073 | 15 (14.85) | – | $Gd^-$ 0.15 | 21 (11.5) | – | $Gd^-$ 0.115 |
| Non-deficiency | 76 (92.7) | – | $Gd^+$ 0.927 | 86 (85.15) | – | $Gd^+$ 0.85 | 162 (88.5) | – | $Gd^+$ 0.885 |
| Heterozygotes (Females) | – | 13.5 | – | – | 25.5 | – | – | 20.4 | – |
| *Hb* | | | | | | | | | |
| AA | 153 (85.96) | 85.4 | $Hb^A$ 0.92 | 131 (74.0) | 73.96 | $Hb^A$ 0.86 | 284 (80.0) | 79.21 | $Hb^A$ 0.89 |
| AS | 23 (12.92) | 14.0 | $Hb^S$ 0.08 | 43 (24.3) | 24.08 | $Hb^S$ 0.14 | 66 (18.59) | 19.58 | $Hb^S$ 0.11 |
| SS | 2 (1.12) | 0.5 | | 3 (1.7) | 2.96 | | 5 (1.41) | 1.2 | |
| $\chi^2$ for random mating | N.S. | | | N.S. | | | N.S. | | |

Obs. — Observed; Exp. — Expected; N.S.—Non-significant.

demic tribal dominated districts—Mandla and Jabalpur, Madhya Pradesh.

Samples were collected from tribal (Gonds) population living in villages of Bizadandi block of Mandla and Kundum block of Jabalpur districts. Kundum and Bizadandi blocks of Madhya Pradesh form a continuous area of hilly terrain which is a part of hard-core malarious belt with high incidence of *P. falciparum* malaria. Area is inhabitated by Gond tribes (80 per cent). Almost all the villages are located either on low hillocks or in shallow valleys near perennial streams. Villages usually comprise of a group of hamlets in which 10 to 20 families live.

Blood samples (0.1–0.2 ml) were collected in heparinized vials (1.5 ml microfuge tube) by pricking a finger and were transported to the laboratory in an ice box within 2–3 hours of collection. Samples were centrifuged and the plasma was removed. The packed cells were washed thrice in 0.9 per cent saline. To the pellet of cells an equal amount of distilled water was added to lyse the cells. Haemolysates were used for G-6-PD and haemoglobin assays.

Haemoglobin (Hb) electromorphs were typed using cellulose acetate membrane and citrate agar electrophoresis (Shandon Scientific Company, U.K.) and stained with Ponceau-S as given by Schmidt and Brosious.[3] Fluorescent spot test was performed for the detection of G-6-PD deficiency.[3] Electrophoretic phenotypes of G-6-PD were identified on 7.5 per cent polyacrylamide horizontal slab gel with phosphate buffer (pH 7) and also by the staining procedure of Mathai *et al.*[4]

A total of 183 samples collected from male subjects (hemizygous for G-6-PD) have been analyzed for G-6-PD activity by fluourescent spot test and also by horizontal polyacrylamide gel electrophoresis. A total of 7.3 per cent males had deficiency of G-6-PD enzyme in Mandla area and 15 per cent in Jabalpur area. No significant difference in the distribution pattern of G-6-PD deficiency was observed between two areas (Table 1). Observed deficiency percentage was also within the range reported for Gond tribes of Sarguja district, M.P. On the basis of calculated gene frequencies (Gd) expected female heterozygote proportion was about 13.5 per cent in Mandla and 25.5 per cent in Jabalpur with a proportion of 20.4 per cent in the total samples (Gond tribes) studied from the area. All the samples tested were of "B" variant, a common variant among the Indian population.

Out of these, 164 samples were analyzed for malaria positivity and comparative chi-square analysis between malaria positive (*P. falciparum* and *P. vivax*) and malaria negative subjects have shown similar distribution pattern in both the groups (Table 2).

For the haemoglobin variants, a total of 355 samples have been analyzed from both male and female subjects (Table 1). Gene frequency of HbS calculated for Mandla and Jabalpur samples was about 0.08 and 0.14 respectively. Distribution of polymorphs of haemoglobin in both the areas was in conformity with Hardy-Weinberg equilibrium (chi-square values were non-significant). However, a significantly higher ($p < 0.02$) frequency of HbS (0.14) was observed in Jabalpur area compared to Mandla

**Table 2. Distribution of G-6-PD deficiency and haemoglobin variants in malaria positive and malaria negative subjects**

| Traits | No. observed | | |
|---|---|---|---|
| | No. (+) ve | | No. (–)ve |
| | *Pf*(+) | *Pv*(+) | |
| *G-6-PD* (Males only) | | | |
| Deficiency | 4 | 1 | 9 |
| Non-deficiency | 43 | 12 | 95 |
| *Hb* | | | |
| AA | 31 | 11 | 154 |
| AS | 7 | 4 | 49 |
| SS | 0 | 1 | 3 |

area (0.08). Observed proportion of HbAS was within the range reported for Gond tribes of Raipur, Ambikapur and Bilaspur districts of Madhya Pradesh.[5] Comparative chi-square analysis between malaria positive (*P. falciparum* and *P. vivax*) and malaria negative groups in pooled data from Mandla and Jabalpur have shown that there is no statistically significant difference in respect of haemoglobin variant distribution between the two groups (Table 2).

Fig. 1 shows the proportion of malaria selective traits in the Gond tribes of the area. Proportion of HbAS heterozygotes observed to be high (19.58 per cent) in the population of Gond tribes. Similarly, the proportion of expected heterozygous females (carriers of G-6-PD deficient gene) is also about 20.4 per cent in the

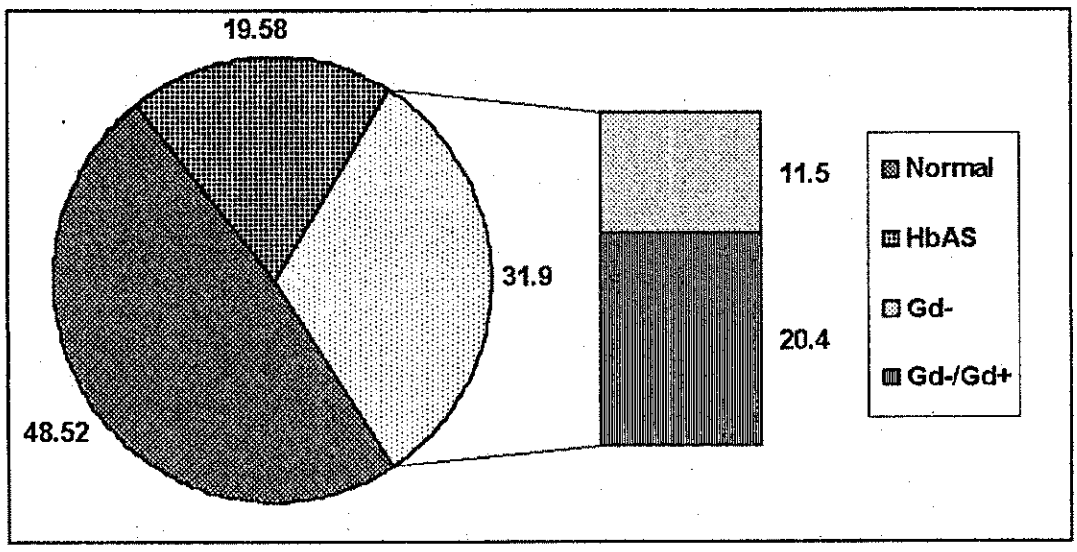


*Fig. 1:* Proportion of sickle-cell haemoglobin and G-6-PD deficiency traits in Gond tribe of study areas

population. This is in addition to observed proportion of hemizygous males (11.5 per cent) in total samples studied. Therefore, giving an overall proportion of 51.48 per cent of these selective traits in the population. In general high incidence of the above genetic disorders have been reported among tribal groups.[6]

A significantly higher incidence of genetic disorders was observed in tribal populations analyzed from Haldwani (malaria endemic) District Nainital, Uttar Pradesh[7] and N.E. states (Joshi *et al.*, unpublished). On the other hand, a low prevalence of G-6-PD deficiency and sickle-cell haemoglobin was reported by us earlier in Delhi,[8] Ghaziabad (U.P.);[9] Faridabad, Sonepat[10] and Gurgaon[11] districts of Haryana populations. Pant *et al.*,[12] have also reported similar low incidence of 1.5 and 1.8 per cent for sickle-cell haemoglobin and G-6-PD deficiency respectively in Muslims of Kheda district, Gujarat.

Red blood cells of subjects with HbAS and $Gd^{-}/Gd^{+}$ are known to limit the parasitaemia or severity of disease by impairing the development of the parasite.[1,2] In the present study also, out of 61 positive slides for malaria parasites, 42 were having scanty or low parasitaemia, while 19 were with moderate parasitaemia. None of the slides had high parasitaemia. All the four G-6-PD deficient subjects diagnosed positive for *P. falciparum* were having low grade parasitaemia. Similarly, out of seven HbAS subjects diagnosed positive for *P. falciparum*, all had low grade parasitaemia, however, one six year old female had enlarged spleen. It can thus be concluded from the observations that Gond tribes of the malaria endemic districts—Mandla and Jabalpur (M.P.) has high prevalence of malaria selective traits–sickle-cell haemoglobin and G-6-PD deficiency. Such high prevalence of these disorders among the population might be having a positive association with the malaria endemicity of the area.

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# Serological Profile following Malaria Outbreak in Mewat Region of Haryana, India

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**Keywords:** AR1 antibody, IgG, Malaria outbreak , *Pf* antigen, Seropositivity

The Mewat area of Gurgaon district in Haryana—a semi arid zone with alluvial plains and scanty vegetation, experienced a malaria outbreak in 1996,[1] which resulted in high morbidity and mortality and reported twenty-three confirmed deaths of *Plasmodium falciparum* (NAMP, Delhi). State Health Department of Haryana had sprayed insecticides and given radical treatment to control malaria at regular intervals as per the schedule. Whether these interventional measures were adequate enough in combating malaria and the impact of these measures on the antimalarial immune status of the population is not clearly known.

Serological studies provide useful additional information which permit the epidemiological evaluation and asseessment of malaria.[2,3] Serological methods are established as additional tools to study the extent and degree of malaria endemicity.[4,5] Malarial antibodies in a population are the result of a cummulative experience of the past several attacks. Therefore, study of immune status of a population is an important parameter for the assessment of active transmission and endemicity of the area.[5] Hence, serological studies were undertaken to know the antimalaria immune status of the population in the months of February and March 1997 and April 2001 in the aftermath of the severe outbreak by conducting cross-sectional surveys.

Mewat region comprises of three community blocks namely Nuh, Punhana and Ferozepur Jhirka (approx. 27° N and 70° E). Four villages namely Ferozepur Namak, Malabh, Salmbha

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and Khedla of CHCs Nuh and Ferozepur Jhirka were selected for the present study. Epidemiological data for the corresponding period was obtained from the District Malaria Office, Gurgaon. A total of 846 finger prick blood samples in the first survey (1997) and 248 in the second survey (2001) from the four villages were collected from a cross-sectional population including all age groups. Blood samples were collected on Whatman filter paper No 3 and stored at –20°C till used. Soluble extract of trophozoite schizont enriched fraction through percoll gradient was used for ELISA as reported earlier,[6] to estimate the anti *Pf* antibody levels. Synthetic Peptide AR1 antigen was procured from M/s. Cambridge Biochemicals, U.K. Antimalarial IgG antibody detection was performed by ELISA as reported earlier by Roy *et al.*[7]

Serological results obtained after one year of the outbreak in cross-sectional surveys conducted in 1997 showed high levels of IgG antibody directed against AR1 and *Pf* crude antigens of malaria parasite in all the four study villages. In Ferozepur Namak the per cent mean seropositivity of AR1 and *Pf* antigens remained high during 1997 and 2001 whereas Salmbha showed elevated levels in 2001 in comparison to that of 1997 but Khedla and Malabh showed slightly low levels in 2001 compared to that of 1997 (Table 1). The results clearly indicate high immune status of the population.

After the outbreak, interventional measures were taken at a large-scale (Source : District Malaria Office) and their impact on the transmission and immune status of the community were measured through serological studies. All

**Table 1. Anti AR1 and anti *Pf* antibody (IgG) levels in the finger prick blood samples collected from residents of CHCs Nuh and Ferozepur Jhirka in the aftermath of malaria outbreak (1997 and 2001)**

| Year | Village | Total no. of filter paper samples | Mean±S.D. ELISA $O.D._{490}$ | | Per cent mean seropositivity | |
|---|---|---|---|---|---|---|
| | | | AR1 | *Pf* | AR1 | *Pf* |
| 1997 | Ferozepur Namak | 211 | 0.96±0.15 | 0.90±0.09 | 98.8 | 98.2 |
| | Salmbha | 195 | 0.66±0.25 | 0.74±0.28 | 82.0 | 82.2 |
| | Malabh | 261 | 0.79±0.20 | 0.95±0.16 | 96.0 | 95.6 |
| | Khedla | 179 | 0.83±0.17 | 0.80±0.15 | 96.4 | 95.8 |
| 2001 | Ferozepur Namak | 47 | 0.65±0.24 | 0.56±0.22 | 98.8 | 98.6 |
| | Salmbha | 83 | 0.01±0.23 | 0.93±0.22 | 96.4 | 94.8 |
| | Malabh | 70 | 0.95±0.26 | 0.75±0.19 | 89.6 | 85.5 |
| | Khedla | 48 | 0.77±0.19 | 0.68±0.18 | 80.0 | 70.0 |

the four villages studied exhibited almost similar level of high antimalarial immune response. This high levels of IgG antibody in individuals probably be due to the elicitation of adequate humoral immune response in the population after one year of the outbreak. High levels of anti AR1 and anti *Pf* IgG antibody after five years of the outbreak in the same village community indicate the persistence of low intensity transmission and parasite exposure in the area. The persistence of high levels of antibodies in the population may be due to the radical and periodic treatments, antimalarial measures, declining transmission and effective intervention measures.

Though there was a gradual decline in the incidence of malaria in this region as found from the epidemiological information provided by the District Malaria Office, Gurgaon, antibodies still remain in a good quantity indicating the high immune status of the population. Serological tools are important to monitor the surveillance and effectiveness of interventional measures. Thus serological tools help in surveillance of malaria situation; to evaluate the immune status of the population; disease transmission pattern; and assessment of efficacy of intervention measures.

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