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INDIAN JOURNAL OF MALARIOLOGY

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CONTENTS

Volume 24
Number 1
June 1987

Short Notes

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Indian Journal of Malariology
Vol. 24, June 1987, pp. 1–7.

Progress Towards a Malaria Vaccine

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INTRODUCTION

Rationale for the development of a malaria vaccine

Major advances were made against malaria in the 1950s and 1960s as a result of the widespread use of insecticides and inexpensive as well as effective antimalaria drugs. However, the global malaria situation has deteriorated since then. This can be attributed to two major causes. These are (1) the failure or inability to continue with effective mosquito control measures in the face of rising costs and the development of insecticide resistance in mosquitoes and (2) the increasing incidence of drug resistance in the parasites. Other factors such as increased migration of people have also contributed to the increase in malaria transmission. The World Health Organization estimates that there are currently 210–220 million cases of malaria every year worldwide of which 85% are caused by *Plasmodium falciparum*, the most dangerous of the malarial parasites (WHO, 1983). *P. falciparum* is responsible for about one million deaths annually, particularly in Africa, among infants and young children. Of great concern is the appearance of strains of *P. falciparum* that are resistant to the most widely used and potent anti-malarial drug, chloroquine. Chloroquine resistant *P. falciparum* was first observed in Thailand in 1959 and in 1960 in Colombia. Since then resistant strains of parasites have spread to large areas of South America, East Asia and East Africa and is now a problem also for the Indian subcontinent. Resistance of *P. falciparum* to the alternative drug combination sulphadoxine/pyrimethamine has also been reported since the mid 1970s. Consequently there are now multiple resistant strains of *P. falciparum*, particularly in Thailand, that have to be treated with a combination of quinine and tetracycline as a last resort. Although a new blood schizonticide, mefloquine has recently been introduced, in general, the development of new drugs to combat malaria has been slow.

The drawbacks faced in controlling mosquitoes with insecticides and in drug treatment of malaria have been some of the reasons that have led to a consideration of an immunological approach to the control of malaria. Encouragement for this venture comes from the considerable success of vaccines against many bacterial and viral diseases. However, the development of a successful vaccine depends on the presence of protective antigens that are shared between different strains of a given malaria parasite. Hopefully antigens that are shared between different *Plasmodium* species can also be identified. Should extensive antigenic variation, as is observed with African trypano-

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somes, be a feature of *Plasmodia*, then the usefulness of vaccines is likely to be severely limited. While antigenic variation does occur in some *Plasmodia* proteins, the extent of this phenomenon among many potentially useful antigens has been shown to be limited.

THE TARGETS FOR A VACCINE

The sporozoites are the forms of the malarial parasite that are introduced into man by the bite of an infected mosquito. Destruction of the sporozoites by immune attack, in contrast to the later stages, may prevent the development of any of the symptoms of disease. The sporozoites are therefore an obvious target for vaccination. It has been known for some time that vaccination with sporozoites, inactivated by irradiation, is at least partially effective in preventing malaria in animal models and in man (Cochrane *et al.*, 1980). Several investigators have also demonstrated that immunization with the asexual blood stages of the parasite is effective in preventing malaria (Siddiqui, 1977; Reese *et al.*, 1978). In this case an initial infection is established before an immune response, mounted by the host, destroys the parasites. Vaccination against the sexual blood stages, the gametocytes, does not result in the prevention of disease. It was believed at first that the ability of the parasites to infect mosquitoes can be suppressed, by vaccination with gametocytes and that this can then effectively block the transmission of malaria (Mendis and Targett, 1979). More recent data suggests that under certain conditions antigametocyte antibodies enhance transmission (Mendis, 1986). This result questions the value of attempts to block transmission by immunization against gametocytes. In comparison to the mosquito and erythrocytic stages of the parasite, much less is known about the exo-erythrocytic stages in the liver. It is possible that, in the future, antigens may be identified that may be useful in protecting the liver against these stages.

Many laboratories have concentrated on investigating one of the different stages of the malarial parasite with a view of developing a vaccine. However, it is unlikely that immunization with antigen from any one stage of the parasite is likely to be effective in preventing disease. This is because the majority of the possibly protective antigens identified, at this time, are stage specific. This then means that there is unlikely to be a great deal of cross-immunity between the different stages. For example, the development of malaria in an individual vaccinated with the sporozoite stage antigens would only require that a single sporozoite escape the host immune response and reach the liver, in order to initiate the disease process. Furthermore, an immune response against many different antigens is likely to be more effective than that against a single antigen of the parasite. Hence an effective vaccine against malaria will depend on the production of a "cocktail" vaccine directed against the many different antigens located on different stages of the parasite.

A given form of the malarial parasite contains thousands of different proteins and other macromolecules which are potentially antigenic in man. However, in the search for a vaccine, the surface antigens of the parasite are likely to be of particular relevance (Ramasamy, 1981). This is because antibodies and immune effector cells interact with the living parasite only through antigenic molecules located on the outside of the parasite. The outer membrane of the parasite also contains the molecules that are involved in binding to target cells (erythrocytes and hepatic cells) and in metabolite transport. Antibodies binding to membrane structures may therefore interfere with the entry of parasites into cells and disrupt other vital membrane functions. For these reasons many laboratories have concentrated in characterizing surface antigens on the different parasite stages. However, some intracellular parasite antigens that are externalised at some stage of parasite development may also

be of relevance for vaccine development. Monoclonal antibodies against molecules located in intracellular organelles known as rhoptries have been shown to inhibit parasite invasion *in vitro* (Schofield *et al.*, 1986). This probably correlates with a role for rhoptry contents during the invasion of red cells by the parasites (Bannister *et al.*, 1982). At least two groups of antigens have been located in the rhoptries (Schofield *et al.*, 1986; Campbell *et al.*, 1984) and these as well as other intracellular molecules that function during invasion, such as the glycophorin binding protein (Perkins, 1984) may be useful vaccine candidates.

THE NATURE OF A POTENTIAL VACCINE

The development of an *in vitro* culture technique for growing *P. falciparum* asexual blood stages (Trager and Jensen, 1976) has enabled sufficient quantities of parasites to be obtained for a variety of immunological and biochemical investigations. This method is too expensive and not easily adapted to the production of enough parasite material for a mass vaccination program. Neither sporozoites nor gametocytes can be obtained in sufficient numbers for a similar purpose.

Since many of the tentative protective antigens that have been characterized so far are proteins, recombinant DNA technology is a useful method for the production of large amounts of antigens. Application of this technology has resulted in the commercial production of medically useful proteins such as human insulin and gamma interferon. The parasite protein, or parts of it containing the protective epitopes, can be synthesised in bacteria, yeast or even eukaryotic cell lines in industrial quantities by this process. An alternative rDNA approach is to insert the parasite genes into a viral vector such as *Vaccinia* and then use the virus to deliver the vaccine. Replication of the virus results in the production of parasite proteins which then stimulate a host immune response (Smith *et al.*, 1986). The *Vaccinia* insert can be engineered in such a way as to result in the expression of parasite proteins on the surface of the infected host cell, a feature which enhances the immune response (Langford *et al.*, 1986).

Recently, it has been demonstrated that antibodies directed against small synthetic peptides (a few amino acids long) are capable of binding to the complete protein containing that particular amino acid sequence (Shinnick *et al.*, 1983). This discovery has greatly simplified our approaches towards producing a vaccine. Instead of the need to clone the gene for a complete protein into bacteria and then look for the synthesis of relevant proteins, it is now only necessary to synthesize small peptides that correspond to the more immunodominant regions of particular proteins. Sufficient peptides for vaccination can be produced by chemical synthesis alone.

Considerable attention has been focussed on protein antigens of the parasite since these are relatively easy to characterize. More recently the carbohydrate moieties of blood stage parasite glycoproteins have been shown to play a role in binding human antibodies (Ramasamy and Reese, 1985; 1986). Greater emphasis is likely to be placed in determining the role of carbohydrates in parasite immunogenicity in the future.

A synthetic vaccine based on defined molecules is likely to have numerous advantages over vaccines based on parasite extracts or attenuated parasites. Immunization with the latter can give rise to undesirable side effects such as immunosuppression, inflammation and toxic reactions. Synthetic vaccines can be designed to avoid such effects and this would make them more acceptable for human use.

CHARACTERIZATION OF ANTIGENS

Major advances in characterizing putative protective antigens have been largely confined to the sporozoite and asexual blood stages.

Sporozoites

The sporozoite is covered on its almost entire outer surface by a single major protein, the circumsporozoite (CS) protein. The CS protein elicits a strong antibody response when animals are vaccinated with irradiated sporozoites. Monoclonal antibodies against the CS protein of the murine malarial parasite *P. berghei* have been shown to inhibit sporozoite invasion of cultured liver cells (Leland *et al.*, 1984). The immunogenicity of the CS protein has been shown to be largely determined by a short, repeating epitope in many *Plasmodium* species. The gene for the CS proteins of *P. falciparum* has recently been cloned into *E. coli* in two laboratories in the United States (Dame *et al.*, 1984; Enea *et al.*, 1984). DNA sequence analysis of the cloned genes revealed that a large portion of the CS protein is composed of 41 four amino acid repeats. Thirty seven of the repeats are identical (Asparagine-Alanine-Asparagine-Proline) while four have an alternative related sequence (Asparagine-Valine-Aspartic acid-Proline). Monoclonal antibodies against the 58,000 m.wt. CS protein of *P. falciparum* abolish the infectivity of sporozoites after antibody treatment indicating the importance of the CS protein in the immune response against sporozoites (Zavala *et al.*, 1985). Also relevant to the possible production of a vaccine based on the CS protein are the observations that the repeating epitope is common to *P. falciparum* strains from Asia, America, and Africa (Enea *et al.*, 1984). In addition to the repeated epitopes present on the CS protein, DNA sequence analysis reveals that on either side of the repeat there are regions that are common to different *Plasmodium* species. Such regions may also be useful for the production of a potential vaccine (Vergara *et al.*, 1985). Recently the structure of the CS protein of *P. vivax* has been determined (Arnot *et al.*, 1985). The immunodominant epitope on this protein is made up of a repeating block of nine amino acids.

Asexual blood stages

A large number of proteins against which antibodies are produced in infected humans and immunized monkeys have been identified in asexual blood stage parasites (Newbold, 1984). Some of these are largely present in a particular blood stage form while others are present throughout, i.e., from the ring stage parasites to released merozoites. Among these proteins are many that have been shown to be present on the surfaces of merozoites or the parasitized erythrocytes. These surface proteins are particularly important for the purposes of inducing protective immunity. While some biochemical parameters such as molecular weight, isoelectric point and the presence or absence of carbohydrates, have been used to characterize the proteins, the amino acid sequence of many of these proteins is not known. Using recombinant DNA technology to clone the genes for blood stage antigen into bacteria, and sequencing the DNA, the amino acid sequence of some *P. falciparum* blood stage proteins have now been deduced (Coppel *et al.*, 1983; 1984; Holder *et al.*, 1985) including that of a major glycoprotein present on the surface of merozoites (Holder *et al.*, 1985). This antigen, when used to immunise *Saimiri* (squirrel) monkeys conferred significant protective immunity against *P. falciparum* infection (Hall *et al.*, 1984). The determination of the structure of other merozoite surface proteins is now in progress in several laboratories. The presence of repeats of a short amino acid sequence is now recognized as a feature of many *Plasmodia* proteins. The first parasite protein to be sequenced was a heat stable (S) antigen that is secreted by blood stage parasites and contains a tandem repeat of eleven amino acids (Coppel *et al.*, 1983). The S antigen however, shows considerable antigenic heterogeneity in that, antigens from different isolates of *P. falciparum* do not cross-react immunologically. The S antigen is therefore not of value as a vaccine. More recently the gene for a protein

present on the surface of ring stage parasitized erythrocytes (RESA) has been cloned and sequenced. This reveals that the protein is composed of repeated eight, four and three amino acid sequences that are highly conserved between different isolates of the parasite (Coppel *et al.*, 1984). The exposed location of RESA on the surface of infected erythrocytes renders it vulnerable to immune attack. The importance of RESA as a candidate malaria vaccine was recently shown by the ability of parts of the RESA molecule synthesized in bacteria to protect *Aotus* (owl) monkeys against falciparum malaria (Collins *et al.*, 1986). Another protein of interest, present on the surface of erythrocytes infected with *P. falciparum* is the cytoadherence protein (Leech *et al.*, 1984). This molecule mediates the adhesion of late stage parasites to the capillary endothelium, a process that helps *P. falciparum* to avoid the lymphoid organs. Although the cytoadherence protein shows considerable antigenic variation, the affinity for endothelium indicates the presence of a conserved region in the molecule that might be a target for immunity.

CELLULAR IMMUNITY

Early work in a murine malaria model demonstrated the importance of cellular (as opposed to antibody mediated) responses in protective immunity against sporozoites (Chen *et al.*, 1977). This has been confirmed more recently by the demonstration of the importance of T cell derived gamma interferon in generating protective immunity (Ferreira *et al.*, 1986). There is increasing evidence for similar soluble factors functioning in the immune response to the human malaria parasites (Troye-Blomberg *et al.*, 1985). It has therefore become important to identify sites on parasite antigens that are recognised by T cells. The sites recognised by T cells can be different from those recognised by the B cell antigen receptor, and for an effective immune response, may have to be incorporated into a synthetic vaccine. The recent suggestion that T cell sites tend to be amphipathic (Delisi and Berzovsky, 1985) may simplify the determination of such sites. There appears to be a considerable degree of histocompatibility antigen restriction in the recognition of malaria antigens by T cells and this will have to be taken into account during the development of a synthetic vaccine (Good *et al.*, 1986). It is also clear that T cell epitopes included in a vaccine will have to stimulate T helper cells rather than T suppressor cells.

ADJUVANTS AND CARRIERS

In many of the early reports of immunization with crude extracts of parasite and the recent successful trial with RESA (Collins *et al.*, 1986), complete Freunds Adjuvant (CFA) was used to augment the immune response to antigens. The need for using adjuvants with synthetic vaccines based on purified molecules is clearly recognised. However, CFA is not acceptable for human use and more suitable adjuvants such as muramyl dipeptide (MDP) have to be tested for their efficacy in synthetic malaria vaccines. The use of small synthetic peptides as vaccines also necessitates the use of larger proteins as covalently linked 'carrier' molecules. Potential carriers include parasite molecules that contain T helper cell stimulating epitopes. Carriers that have already been used with *P. falciparum* antigens are diphtheria and tetanus toxoids. The development of suitable carrier molecules for synthetic peptides that can be safely used in man, is an area that requires further investigation.

FUTURE PROSPECTS

Since the amino acid sequences of some protective antigens of *P. falciparum* have already been determined, many of these will undergo monkey and human trials in the near future. Other candidate antigens are also likely to emerge soon from several laboratories. Efforts will be made to identify protective regions of the

molecules as reported recently for RESA (Collins *et al.*, 1986) and then use synthetic peptides or recombinant proteins corresponding to such regions for immunizations. Adjuvants suitable for human use will also be tested at the same time. While very little cross-reaction between the protective antigens of *P. falciparum* and the antigen of other human malarial parasites has been reported till date, there is likely to be some homology in the structure of these antigens. DNA probes derived from *P. falciparum* may therefore be potentially useful for identifying genes for homologous protective antigens in parasites such as *P. vivax*, *P. malariae* and *P. ovale*. In addition, DNA libraries and monoclonal antibodies against *P. vivax* are already available in several laboratories. These are also being utilised to characterise protective antigens. We can therefore, at the present time, look forward with considerable optimism to the development of a malaria vaccine in the near future.

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Seasonal Prevalence of Sibling Species A and B of the Taxon *Anopheles culicifacies* in Villages around Delhi

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Two riverine villages, Basantpur, Faridabad district (Haryana) and Arthala, Ghaziabad district (Uttar Pradesh) and a non-riverine village, Mandora, Sonepat district (Haryana) were surveyed for two years to examine the seasonal variations in the prevalence of sibling species A and B of *Anopheles culicifacies*. Species A was predominant almost throughout the year. The proportion of species B increased after the onset of monsoons i.e., July onwards and the maximum observed was between September–October. The proportion of species B in Mandora varied between 5–10% while in riverine villages it varied between 10–60%. In Basantpur in September–October the proportion of species B exceeded species A while in Arthala it was between 25–35%. The high proportion of species B observed in Basantpur was correlated with extensive breeding observed in riverbed pools in post-monsoon months. Several species A specimens were found positive for sporozoites in Arthala and Basantpur thus establishing the role of species A in the transmission of malaria.

INTRODUCTION

Anopheles culicifacies is a major vector of malaria in rural areas of the Indian sub-continent. This taxon is a complex of three sibling species, species A, B and C (Green and Miles, 1980; Vasantha *et al.*, 1982; 1983; Subbarao *et al.*, 1983).

Preliminary surveys carried out in northern India revealed that species A and B are sympatric (Subbarao *et al.*, 1984). Further, it was observed that the sibling species composition varied in riverine and non-riverine villages (Subbarao *et al.*, 1980). A study on the prevalence of sibling species A and B of *A. culicifacies* was initiated in riverine and non-riverine villages around Delhi. The villages were surveyed for two consecutive years and the results are reported in this paper.

MATERIAL AND METHODS

Three ecologically different villages viz., Basantpur and Mandora, Faridabad and Sonepat districts respectively in Haryana and Arthala (Ghaziabad district) in Uttar Pradesh were selected for studies on species A and species B from June 1981 to December 1983.

Description of the study sites

Arthala is situated about 2 kms from Hindon river and 35 kms south of Malaria Research Centre. A brewery is situated in the vicinity of the village. This is not a typical rural village as most of the houses are of cement. People of this village

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are either labourers in the factory or farmers. The human and cattle population is about 10,000 and 1200 respectively.

Basantpur is a typical riverine village situated on the banks of the Yamuna and located approximately 30 kms southwest of the Malaria Research Centre. Both temporary and permanent houses are common in the village. It is inhabited by about 500 people and the cattle population is roughly three times that of human population. The main occupation of the people is agriculture. During the rains people migrate to other villages due to floods.

Mandora is a typical rural village with both kutcha and pukka houses and a canal runs 3–4 kms away from the village. The village is 40 kms northwest of Malaria Research Centre. Agriculture is the main occupation of the villagers. The human and cattle population of the village is about 6000 and 2000 respectively.

Methods of mosquito collection

In each village, few human dwellings, mixed dwellings and cattlesheds were selected for mosquito collection. Fortnightly visits were made to each village and resting adult anopheline mosquitoes were collected with the help of suction tube and flash light. In each catching station 15 minutes were spent by 4–5 insect collectors. From the total anophelines collected, *A. culicifacies* were identified and man hour densities were calculated.

In all the three villages mosquito collections were started in June 1981, but man hour densities were determined from October 1981. Semi-gravid females were used to study the polytene chromosomes to determine the proportion of species A and species B in the field.

Occasionally larvae collected from the breeding sites were brought to the laboratory and reared. *A. culicifacies* adults that emerged from these immatures were also examined cytologically for sibling species identification.

Chromosome preparation and species identification

A. culicifacies collected from the field were brought to the laboratory. Ovaries from the semi-gravid females were removed and fixed in modified Carnoy's fixative (1:3 glacial acetic acid and methanol) and were stored in the refrigerator till required. Polytene chromosomes were prepared according to the method described by Green and Hunt (1980). The adults that emerged from the larvae collected in the field were processed similarly for chromosome preparations. Polytene chromosomes were observed under bright field illumination with a Leitz Dialux 20 microscope.

The diagnostic inversion genotypes of species A and species B are X $+^{a}$ $+^{b}$ 2 $+^{g^1}$ $+^{h^1}$ and Xab $2g^1$ $+^{h^1}$ respectively (Subbarao *et al.*, 1983). It may be mentioned here that when this study was initiated, inversions on chromosomes 2 were not identified and only the X-chromosome inversions a and b were used for the identification of the sibling species. From the later part of 1982, chromosome 2 inversions were also used in the identification.

Vector incrimination studies

To ascertain the role of sibling species in the transmission of malaria, ovaries from the semi-gravid females were used for species identification and the same mosquitoes were examined for the presence of sporozoites in the salivary glands.

RESULTS AND DISCUSSION

Anopheline species prevalent in the study villages were *A. annularis*, *A. culicifacies*, *A. stephensi* and *A. subpictus*. The densities of *A. culicifacies* were relatively high from May to November in

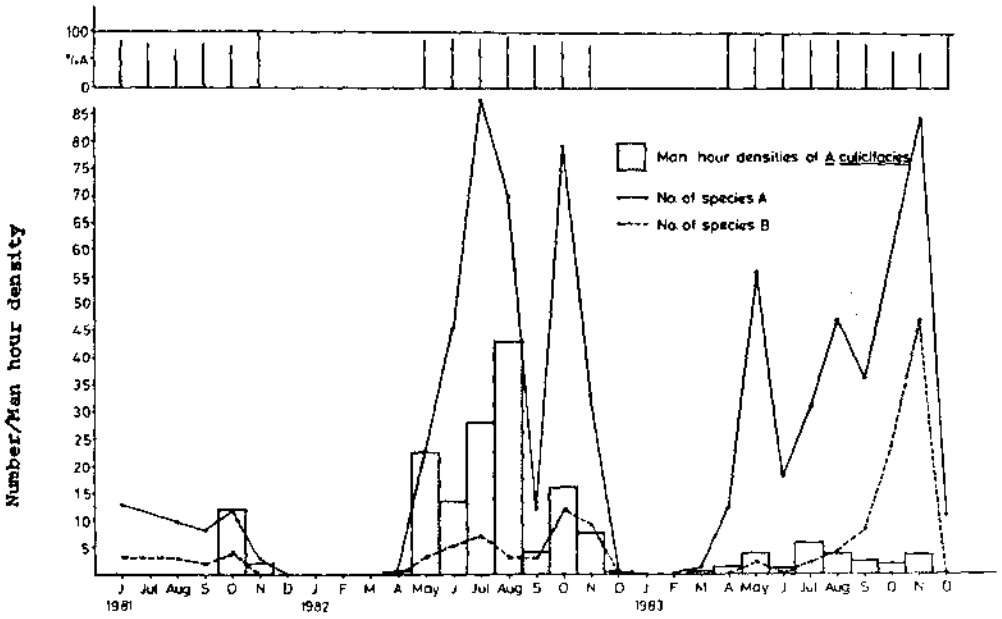

Fig. 1: Seasonal variations in the prevalence of species A and B of *A. culicifacies* in riverine village, Arthala.

Table 1. Man hour densities in different collection sites in the two study villages*, Arthala and Basantpur

1982	Arthala			Basantpur		
	Cattlesheds	Mixed dwellings	Human dwellings	Cattlesheds	Mixed dwellings	Human dwellings
January	—	0	—	—	1.75	—
February	0	0.06	—	0.1	1.5	0.37
March	0	0	0	0.85	0	0.43
April	2.25	—	0	2.5	0.44	1.4
May	20.96	6.68	1.6	18.75	1.45	—
June	6.33	9.0	5.5	—	2.31	—
July	24.14	4.0	6.72	0.32	0.28	—
August	45.75	10.5	12.12	1.33	3.08	—
September	10.0	1.6	0.79	3.43	5.5	0.06
October	43.46	13.29	4.57	35.0	25.54	2.73
November	18.43	5.77	2.06	24.78	12.0	13.68
December	3.0	1.5	0.5	4.55	10.8	2.6

* Mandora is not included because *A. culicifacies* almost totally disappeared after malathion spray in March 1982. Data of 1981 and 1983 are not included because only in 1981 sibling species identification was done and in 1983 data was found to be same as in 1982.

all three study villages (Figs. 1, 2 and 3). In winter months in the two riverine villages Arthala and Basantpur, *A. culicifacies* was almost absent while in the non-riverine village, Mandora, the densities remained more than 5 per man hour. Of the three types of collection sites viz.,

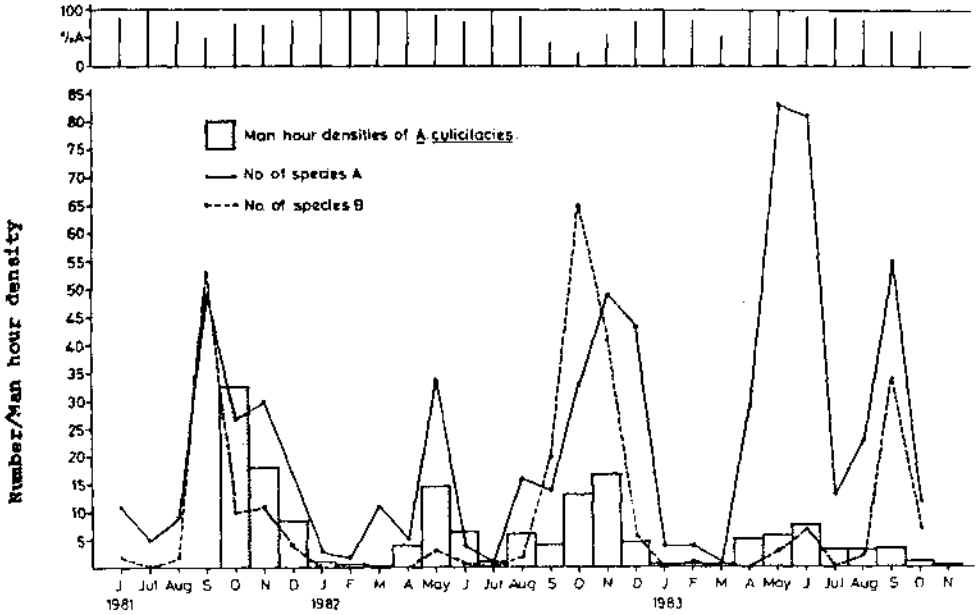

Fig. 2: Seasonal variations in the prevalence of species A and B of *A. culicifacies* in riverine village, Basantpur.

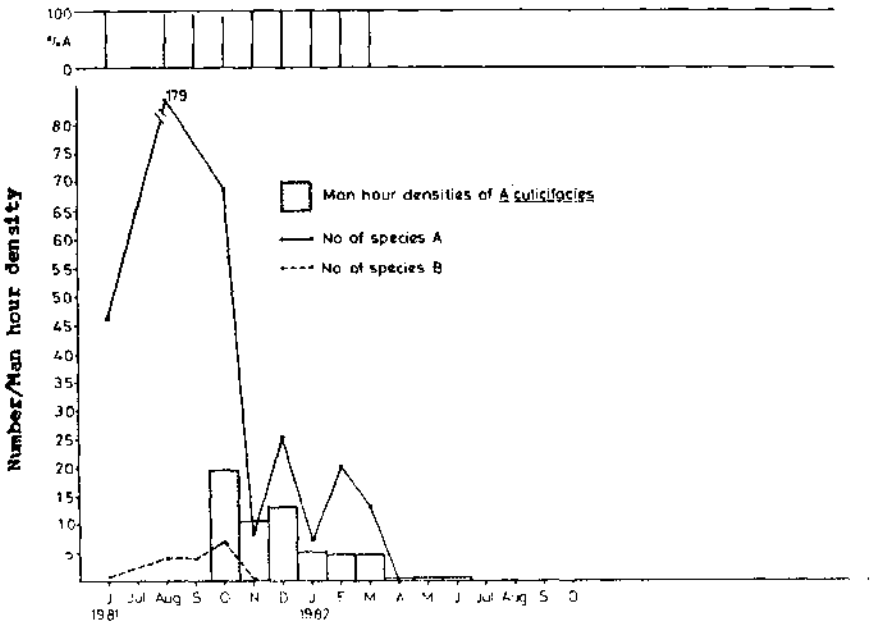

Fig. 3: Seasonal variations in the prevalence of species A and B of *A. culicifacies* in non-riverine village, Mandora.

cattlesheds, human dwellings and mixed dwellings, densities of *A. culicifacies* were found to be relatively higher in cattlesheds than the same in the other two structures (Table 1).

In 1982 HCH was sprayed in August in Arthala and in May-June in Basantpur which resulted in a sudden decline in the *A. culicifacies* population in the following months but in later months the population reached normal densities. In Mandora 3 rounds of malathion were sprayed in 1982 and 1983 which resulted in the total absence of *A. culicifacies* in our collections (Subbarao, 1984). In 1983 natural densities of *A. culicifacies* were very low in both Arthala and Basantpur.

In all the three villages, the two sibling species A and B were found sympatric. The relative proportions of species A and B varied with the seasons but roughly remained the same during the years even though the densities of *A. culicifacies* varied (Figs. 1, 2 and 3). Though the proportion of species B varied in the three types of catching stations no specific pattern was observed (Table 2). Thus, it appears that the two sibling species do not have any specific preference for resting habitats.

In all three villages species A was the predominant species almost throughout the year. The proportion of species B increased after the onset of monsoons (i.e., from July onwards) and reached a maximum in September-October. The increase was more conspicuous in the two riverine villages Arthala and Basantpur (Figs. 1 and 2). In Basantpur, the proportion of species B even exceeded species A while in Arthala maximum observed was about 35%. In Mandora which is not a riverine village, the proportion of species B never exceeded 10% (Fig. 3).

In all types of breeding sites viz., ponds, rainwater pools, riverbed pools and riverbanks, species A and B were found. However, the three study villages differed in having different types of breeding sites.

In Mandora *A. culicifacies* breeding was observed in ponds and was mostly restricted to a big pond in the centre of the village. In Arthala, a riverine village, breeding was found only in rainwater pools and small ponds. Because of the embankment no riverbed pools were formed, while in Basantpur a typical riverine situation exists and breeding was observed on the riverbank and in the riverbed and rainwater pools. With the recession of floods after monsoon there was an increase in the number of riverbed pools providing extensive breeding grounds for the mosquitoes.

As mentioned, in post-monsoon months the proportion of species B increased and it was significantly high in Basantpur village. This suggests that there may be a correlation between breeding in riverbed pools and the proportion of species B observed in Basantpur. Indeed it was found that in the larval surveys done, although not regularly, sibling species composition in the breeding sites corresponded with the adults.

Suguna *et al.* (1983) also observed an increase in species B proportion in post-monsoon months in villages situated on the banks of Thenpenniar river in Tamil Nadu. In these villages species B predominated from September to December and was 95% in October. Similar seasonal variations in the prevalence of *A. gambiae* and *A. arabiensis*, two sibling species of the *Anopheles gambiae* complex were reported (White and Rosen, 1973; Joshi *et al.*, 1975). Data of sporozoite positive *A. culicifacies* identified for sibling species is given in Table 3. Though the dissections were not extensive, data clearly indicates the role species A plays in the transmission of malaria in Arthala and Basantpur. In Mandora sporozoite positive mosquitoes were not identified for sibling species. Studies are in progress to identify the sporozoite species.

It may be mentioned that in Mandora, malaria incidence was always high. In 1981 the slide positivity rate in September-October was about

Table 2. Species B in different collection sites in Basantpur

Study period* 1982	Overall %	% in Cattlesheds	% in Mixed dwellings	% in Human dwellings
September	63.1	50	80	**
October	67	78	52	68
November	44.7	35.2	40	53

* As the proportion of species B was high in these months.
** Only two gravid *A. culicifacies* were found which could not be identified.

Table 3. Incrimination of sibling species of the *Anopheles culicifacies* complex in the two study villages

Village	Period	No. dissected	No. positive for sporozoites	Cytological identification	Proportions of	
					Sp. A	Sp. B
Arthala	August, 1982	211	8	4 species A 4 unsuitable	94.8	5.2
	September-October, 1983	249	8	5 species A 3 unsuitable	77.8	22.2
Basantpur	September-October, 1983	243	1	species A	60.1	39.9

80% and several *A. culicifacies* were found positive for sporozoites (MRC Annual Report, 1981). As species A is predominant in this village, it can be concluded that species A might be transmitting malaria. Arthala also experiences high malaria incidence and in 1982 the slide positivity rate was about 50% in August-September (MRC Annual Report, 1982). However, in Basantpur malaria cases were rare.

In northern India, *Plasmodium vivax* is found predominant in pre-monsoon months while *P. falciparum* in post monsoon months (Sharma *et al.*, 1983). The appearance of peak densities of species B at the time when *P. falciparum* is prevalent raises the question of whether it is a mere coincidence or there is a relationship between the two. Studies are in progress to identify the *Plasmodium* species of sporozoite with the help of labelled antibodies against sporozoite antigen.

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Antiparasitic Agents. Part 7—Antimalarial activity of Alkyl 5(6)-(4-Aminophenyl) sulphonobenzimidazole-2-carbamates

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The antimalarial activity of alkyl 5(6)—(4-aminophenyl) sulphonobenzimidazole-2-carbamates (*6*, *7*) in experimental animals is reported. At an intraperitoneal dose of 3 mg/kg ×4 days, compounds *6* and *7* showed 100% elimination of ***Plasmodium berghei*** infection in mice for 25 days. When tested against ***Plasmodium knowlesi*** in rhesus monkeys, both the compounds delayed the patency but did not exhibit curative effects upto an oral dose of 20 mg/kg × 7 days.

INTRODUCTION

During our efforts to develop ideal antiparasitic agents, the synthesis of a wide class of 2,5(6)-disubstituted benzimidazoles and related compounds were carried out in this laboratory (Sharma and Abuzar, 1983; Abuzar and Sharma, 1985; Abuzar *et al.*, 1985). Some of the compounds of this series showed close structural resemblance with 4,4-diaminodiphenylsulphone (DDS, *1*), a potent antimalarial (Fig. 1). This prompted us to evaluate the antimalarial efficacy of alkyl 5(6)-substituted benzimidazole-2-carbamates of the type *2–7* when methyl and ethyl 5(6)-(4-aminophenyl) sulphonobenzimidazole-2-carbamates (*6* and *7*) were found to exhibit marked activity against *Plasmodium berghei* in mice and *Plasmodium knowlesi* in rhesus monkeys which is reported in this communication.

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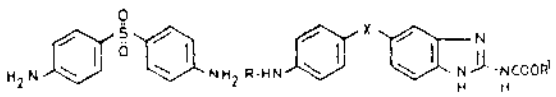

1 (DDS)

2 X = S, R = $COCH_3$, R' = CH_3
3 X = S, R = $COCH_3$, R' = C_2H_5
4 X = SO_2, R = $COCH_3$, R' = CH_3
5 X = SO_2, R = $COCH_3$, R' = C_2H_5
6 X = SO_2, R = H, R' = CH_3
7 X = SO_2, R = H, R' = C_2H_5

Fig. 1: Structure of DDS.

MATERIAL AND METHODS

Chemistry

Alkyl 5(6)-(4-acetylaminophenyl)thiobenzimidazole-2-carbamates (*2, 3*) (Abuzar *et al.*, 1985) were oxidised with potassium permanganate in 80% acetic acid at room temperature (5–6 hr) to yield alkyl 5(6)-(4-acetylaminophenyl) sulphonobenzimidazole-2-carbamates *4* (yield 83%, m.p. 270°C) and *5* (yield 78%, m.p. 275°C) respectively. Hydrolysis of *4* and *5* with 10% aqueous HCl gave the desired alkyl 5(6)-(4-aminophenyl) sulphonobenzimidazole-2-carbamates *6* (yield 79%, m.p. 246°C) and *7* (yield 85%, m.p. 250°C).

Primary screening

Mice (18 ± 2gm) of either sex were inoculated intraperitoneally with 1 million RBCs parasitized with *P. berghei* on day zero and were used for testing. The test compounds were given orally or intraperitoneally once a day for 4 days, 4 hours after introducing the infective inoculum to the animals. In each experiment 3–5 animals were used per group and 3–5 animals were kept as controls. The parasite counts were made from tail blood films on alternate days 5, 7 upto 25 days and compared with the parasite counts of control, untreated and infective animals (Peters, 1965).

Secondary screening

Rhesus monkeys (*Macaca mulatta*, 4–6 kg) of either sex were used in this study. The animals were maintained on standard animal feed supplemented with soaked gram, green vegetables, apples, bananas and carrots in well ventilated rooms (35°C, photoperiod 12 hours) of the primate house of this Institute. Before infecting the animals, they were kept under quarantine and conditioning period of 15 days during which they were tested for tuberculosis by X-rays.

Counted number of erythrocytes (1×10^4 to 1×10^7) parasitized with *P. knowlesi* (W_1 strain) were injected in monkeys through cephaneous veins. Thin and thick smears were prepared and stained with Giemsa. Parasitaemia was recorded per 10^4 erythrocytes (Singh and Dutta, 1981).

RESULTS AND DISCUSSION

Compounds *6* and *7* were administered intraperitoneally to mice infected with *P. berghei* at an initial dose of 10 mg/kg given for 4 days. On day 5, post-treatment, both the compounds caused 100% elimination of parasitaemia which persisted upto the total observation period of 25 days. A similar spectrum of activity was observed when *6* and *7* were given at the dosages of 5 and 3 mg/kg × 4 days. However, at a dose of 1 mg/kg, 100% reduction of parasite count could be observed only upto day 7 and thereafter the parasite count started increasing gradually (Table 1).

Table 1. Antimalarial activity of compounds *6* and *7* against *P. berghei* in Mice

Compd. No.	Dose, i.p., mg/kg × 4 days	% Reduction of parasitaemia on days				
		5	7	9	11	25
6	10	100	100	100	100	100
	5	100	100	100	100	100
	3	100	100	100	100	100
	1	100	100	94.9	91.5	74.6
7	10	100	100	100	100	100
	5	100	100	100	100	100
	3	100	100	100	100	100
	1	100	100	92.5	91.0	65.0
DDS	10	100	100	100	100	100
	5	100	100	100	100	100
	3	100	100	100	100	100
	1	100	100	100	100	100

Compounds *6* and *7* were also evaluated against the secondary screen *P. knowlesi* in rhesus monkeys. Both the compounds were administered

orally at a dose of 10 mg/kg × 7 days when *6* was found to be inactive while compound *7* was able to suppress the parasitaemia initially but there was recrudescence after 3 days of completion of treatment. When the dose was increased to 20 mg/kg, compound *6* exhibited slight suppression of parasitaemia and recrudescence was observed. Compound *7*, on the other hand, cleared the parasitaemia by *5th* day of the treatment but again there was recrudescence 7 days after completion of the treatment and both the monkeys developed high parasitaemia.

The antimalarial screening results of *6* and *7*, summarised in Table 1, indicate that incorporation of one of the amino functions of DDS into benzimidazole heterocycle retains the antimalarial activity though it is not superior to that of the parent drug. It is also noteworthy that presence of an alkoxycarbonylamino group at 2—position of the benzimidazole ring is essential for biological activity, a structural requirement which is complementary to the alkyl benzimidazole-2-carbamates showing antiparasitic activity (Sharma and Abuzar, 1983). Incorporation of both the amino groups of DDS into benzimidazole rings leading to the formation of 2,2'-dicarbalkoxyamino-5, 5'-dibenzimidazolysulphones (Abuzar *et al.*, 1986) showed no antimalarial activity which indicates that the presence of 4-aminophenylsulphonyl residue is essential for antimalarial activity in the analogues of DDS (Sweeny and Strube, 1979).

The fact that compounds *6* and *7* exhibit curative effect against *P. berghei* in mice and suppressive effects against *P. knowlesi* in rhesus monkeys, undoubtedly points out that there is ample scope to synthesize more analogues of DDS using more versatile antiparasitic pharmacophores at one of its phenyl rings. This will not only help in evolving more meaningful SAR in this class of compounds but will also increase the probability to discover potent antiparasitic agents in general and an antimalarial agent in particular.

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Survey of the Anopheline fauna of the Western Ghats in Tamil Nadu, India

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Thirty one anopheline species were collected in hill ranges of the Western Ghats, between August 1985 and October 1986. *Anopheles (Anopheles) crawfordi* and *Anopheles (Cellia) dirus* were recorded for the first time in Tamil Nadu. Very low densities of the malaria vector *Anopheles (Cellia) fluviatilis* were recorded (0.9% of total collection).

INTRODUCTION

The foothills of the Western Ghats were once hyperendemic for malaria. These hill ranges have an extremely rich flora and fauna. The earliest surveys of anophelines were carried out in the early decades of this century, and culminated in the publication of a monograph on the Indian anophelines by Christophers in 1933. Subsequently, studies were carried out by Covell and Harbhagwan (1939) and Vedamanikkam (1949; 1952) in the Wynaad area, Russell and Jacob (1942) in the Nilgiris, Measham and Chowdhuri (1934) in the Anaimalais, Ramachandra Rao (1945) and Jaswant Singh and Jacob (1944) in North Kanara District, and Brooke Worth (1953) in Hassan District. The number of species recorded in this area by these workers was 30. In all cases *Anopheles (Cellia) fluviatilis* was incriminated as the vector of malaria. Later Wattal *et al.* (1962) and Kalra and Wattal (1962) re-examined the specimens from early surveys in the collection of National Institute of Communicable Diseases, and added *Anopheles (Anopheles) barbumbrosus* and *An (Cel) elegans* to the list of species.

Indoor residual spraying with DDT during early years of the National Malaria Control and Eradication Programmes brought about a spectacular decrease in the incidence of foothill malaria in Tamil Nadu. Although elsewhere in the state there was a resurgence of malaria during the late sixties and early seventies (Roy *et al.*, 1979), this did not occur in the foothills, and these areas are now reportedly silent. Over the past four decades the ecology of Western Ghats has also been greatly altered. There has been extensive deforestation due to the construction of dams, roads, industries and rehabilitation camps, and the natural forests or sholas, have been replaced over large areas by plantations. No systematic surveys of the anopheline fauna have been carried out in the post DDT era, and the present study was undertaken to update knowledge of distribution and prevalence of vector species, and to assess their disease potential for the future.

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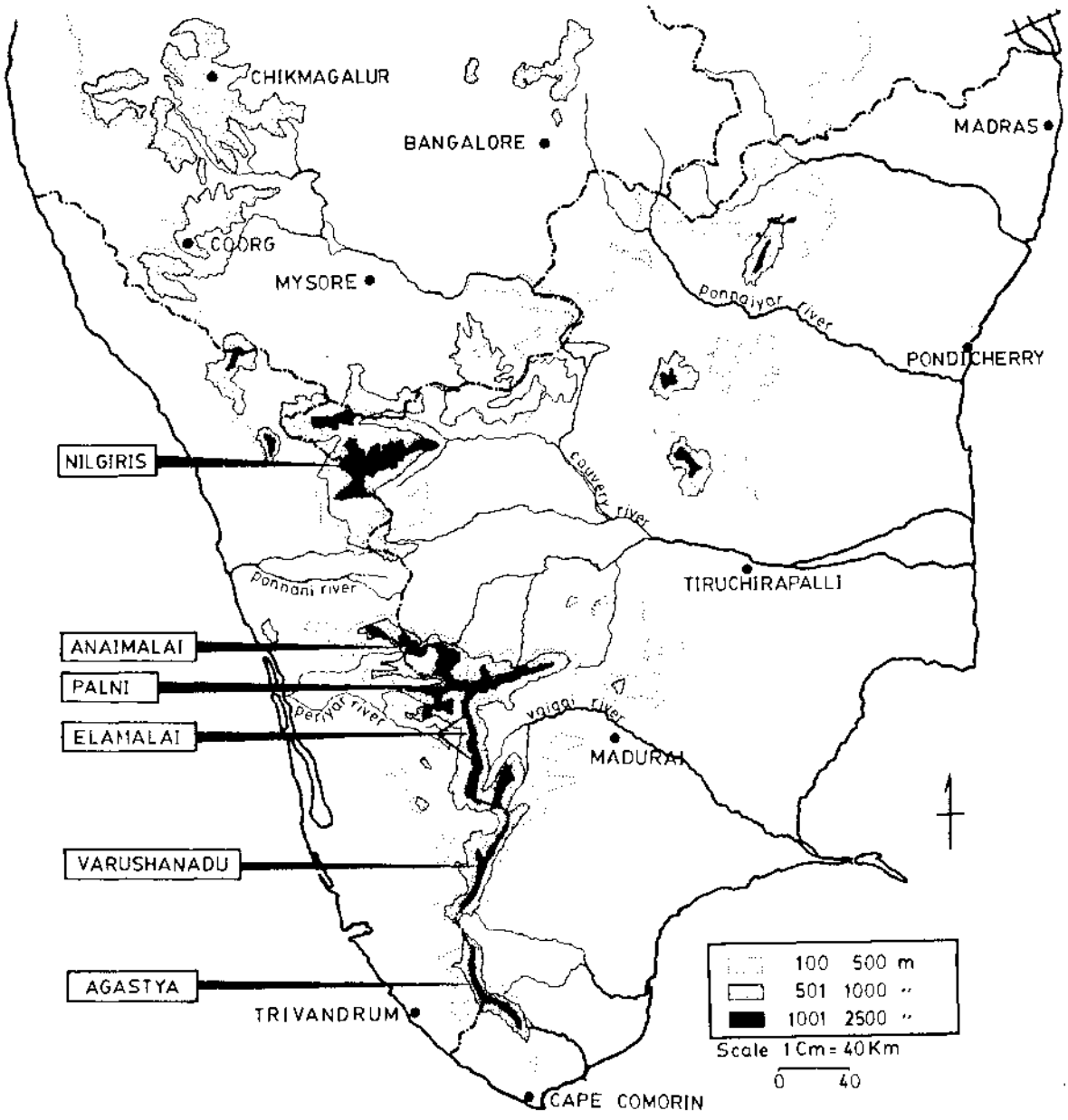

Fig. 1: Localities surveyed in Western Ghats.

MATERIAL AND METHODS

The hill ranges which constitute the Western Ghats occupy the western and southern portion of the Indian peninsula plateau. They lie between 8°11'N to 20°02'N and 73°35'E to 77°27'E and rise to a height of 2600 m in some places. There is considerable variation in annual rainfall but the whole area receives good rains from both monsoons, with no more than four dry months. The southwest monsoon (July-September) is more effective on the western slopes while the northeast monsoon (October-December) has a strong influence on the eastern slopes. The vegetation consists of tropical thorny and mixed deciduous forest, with only a few patches of subtropical evergreen forest (Shola) remaining. Afforestation plantations of teak (*Tectona grandis*), *Eucalyptus* sp., wattle, babul (*Acacia* sp.) and *Pinus* sp. are observed on the plateaux. Extensive tea and coffee plantations and orchards are seen.

Altogether thirteen survey tours (273 man days) were carried out between August 1985 to October 1986 to six hill ranges viz. Agastya, Varushanadu, Elumalai, Palni, Anaimalai and Nilgiri (Fig. 1). Since the studies were confined to Tamil Nadu, it is mainly the eastern slopes of the Western Ghats which have been surveyed, except in the Palni and Nilgiri hills where the collections were also made on western slopes. Studies were repeated at least once in a different season for each hill range, except Anaimalai and Palni. Four tours were made to the Nilgiri hills.

Altitude was measured by a portable altimeter and recorded. Immatures were collected from representative habitats, such as streams, rivers, pools, tree holes etc., and reared to the adult stage. Identification was based mainly on adult characters; however the larval and pupal chaetotaxy was also examined whenever necessary. For this purpose samples of larvae were reared individually to obtain associated material. Generic and subgeneric abbreviations used follow Reinert (1982).

RESULTS AND DISCUSSION

Thirty one species of anophelines were collected, 11 belonging to subgenus *Anopheles* and 20 to subgenus *Cellia* (Table 1). The Nilgiri hills had the richest anopheline fauna, which became apparent on the first tour. For this reason the area was visited once in each season, so that comparisons could be made with the earlier studies of Russell and Jacob (1942). *An (Cel) maculatus* was numerically the most abundant species, and was found everywhere except in the Agastya hills in the far south. Other ubiquitous species were *An (Ano) aitkenii* and *An (Ano) elegans* which were represented in six and five respectively of the hill ranges visited. As shown in Fig.2 most of the species were found up to 1250 m above sea level, with the maximum number occurring between 300 m and 1000 m. Above 2000 m only four species were encountered, of which *An (Ano) gigas* and *An (Ano) lindesayi*, which are well known high altitude species, were abundant.

Habitat distribution of various species is illustrated in Fig.3. Jungle streams were the most favourable habitat, from which 22 species were collected. Pools and springs were also productive. Paddy fields were encountered only in Nilgiris, where they contributed 14 species. Six species were reared from larvae collected in tree holes. These were *An (Ano) aitkenii*, *An (Ano) annandalei*, *An (Ano) sintoni*, *An (Cel) elegans*, *An (Cel) culicifacies* and *An (Cel) dirus*. Of these, the last two species were found only in one hole each, and these were somewhat atypical, being open, wide (25-50 cm diam.) holes near the base or in fallen tree trunks.

Notes on a few individual species follow :

(1) *Anopheles (Anopheles) crawfordi* Reid 1953, is a member of the *An. hyrcanus* group which is

Table 1. Anopheles species collected as immatures in different ranges of the Western Ghats

SPECIES / Hill ranges / Mandays	Agastya	Varushanadu	Elamalai	Palni	Anaimalai	Nilgiri	Total
	35	28	35	20	20	135	273
1. *Anopheles (Anopheles) aitkenii* James, 1903	12	326	10	60	52	26	486
2. *Anopheles (Anopheles) annandalei* Prashad, 1918	0	0	0	0	0	2	2
3. *Anopheles (Anopheles) barbirostris* Van der Wulp, 1884	0	99	4	0	24	38	165
4. *Anopheles (Anopheles) barbumbrosus* Strickland and Chowdhury, 1927	0	0	0	1	0	73	74
5. *Anopheles (Anopheles) crawfordi* Reid, 1953	0	0	0	0	0	5	5
6. *Anopheles (Anopheles) gigas* Giles, 1901	0	0	6	94	25	28	153
7. *Anopheles (Anopheles) insulaeflorum* (Swell and Swell), 1919 (1920)	0	0	0	0	4	0	4
8. *Anopheles (Anopheles) lindesayi* Giles, 1900	0	0	0	131	1	0	132
9. *Anopheles (Anopheles) nigerrimus* Giles, 1900	0	0	0	0	0	2	2
10. *Anopheles (Anopheles) peditaeniatus* (Leicester), 1908	0	0	0	0	0	38	38
11. *Anopheles (Anopheles) sintoni* Puri 1929	3	0	0	0	0	0	3
12. *Anopheles (Cellia) aconitus* Doenitz, 1902	0	0	0	0	0	16	16
13. *Anopheles (Cellia) annularis* Van der Wulp, 1884	0	0	0	0	0	1	1
14. *Anopheles (Cellia) culicifacies* Giles, 1901	0	24	18	0	0	218	260
15. *Anopheles (Cellia) dirus* Peyton and Harrison, 1979	0	0	0	0	0	72	72
16. *Anopheles (Cellia) elegans* (James), 1903	11	4	27	39	0	38	119
17. *Anopheles (Cellia) fluviatilis* James, 1902	0	8	3	0	1	24	36
18. *Anopheles (Cellia) jamesii* Theobald, 1901	8	0	2	0	0	114	124
19. *Anopheles (Cellia) jeyporiensis* James, 1902	0	0	0	1	0	209	210
20. *Anopheles (Cellia) karwari* (James), 1902	0	0	0	0	2	7	9
21. *Anopheles (Cellia) maculatus* Theobald, 1901	0	64	109	2	126	536	837
22. *Anopheles (Cellia) majidi* Young and Majid, 1928	0	0	0	0	0	89	89
23. *Anopheles (Cellia) moghulensis* Christophers, 1924	0	61	1	1	0	8	71
24. *Anopheles (Cellia) pallidus* Theobald, 1901	0	0	0	0	0	1	1
25. *Anopheles (Cellia) splendidus* Koidzumi, 1920	0	0	0	0	0	177	177
26. *Anopheles (Cellia) subpictus* Grassi, 1899	12	3	16	0	0	15	46
27. *Anopheles (Cellia) tessellatus* Theobald, 1901	0	0	0	0	0	68	68
28. *Anopheles (Cellia) theobaldi* Giles, 1901	0	470	1	0	0	0	471
29. *Anopheles (Cellia) vagus* Doenitz, 1902	10	0	0	0	0	25	35
30. *Anopheles (Cellia) varuna* Iyengar, 1924	0	32	86	0	0	14	132
31. *Anopheles (Cellia)* sp. (undet)	0	9	0	0	0	2	11
TOTAL	56	1100	283	329	235	1846	3849

known to occur in Assam (Harrison and Scanlon, 1975). Its range is now extended to southern India for the first time. Five specimens were obtained, as determined on adult characters as well as larval and pupal chaetotaxy. This species is reported to be confined to areas of high rainfall in Thailand (Harrison and Scanlon, *loc. cit.*) and it is of interest that it was collected from Bospara, Gudalur Taluk (Nilgiri District) a high rainfall pocket under the influence of the southwest monsoon, in the western part of Nilgiris, contiguous with Wynaad in Kerala. Other members

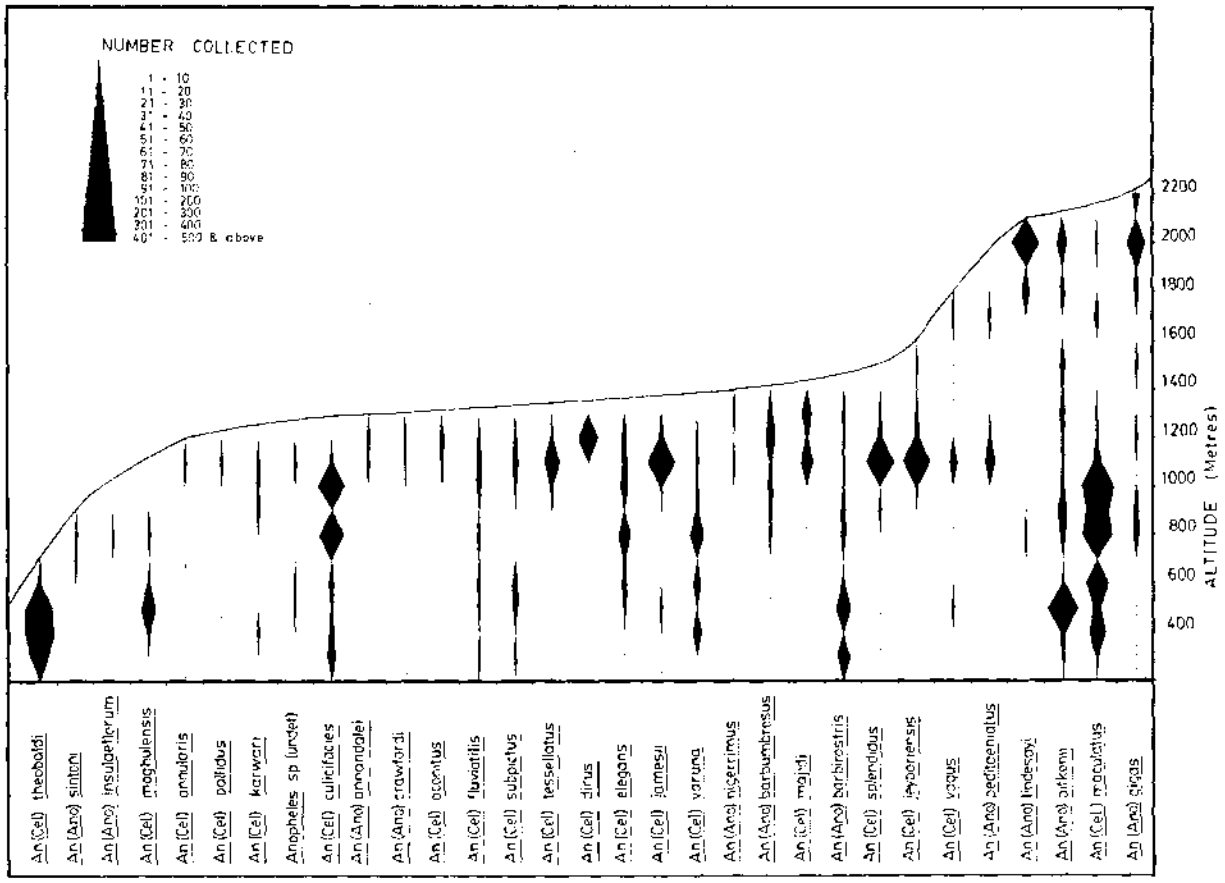

Fig. 2: Distribution of species of *Anopheles* at different altitudes in the Western Ghats.

of the *hyrcanus* group found in small numbers in several localities were *An (Ano) peditaeniatus* and *An (Ano) nigerrimus*.

(2) *Anopheles (Cellia) dirus* Peyton and Harrison, 1979, and *Anopheles (Cellia) elegans* (James), 1903. These species belong to the *An leucosphyrus* group. *An dirus* is now known to be a complex of sibling species, of which the form in the northeastern states is species A, and that in the Western Ghats is species E (E.L. Peyton, personal communication). This species has been recorded in North Kanara (Kalra and Wattal, 1962) and in the Sagar-Shimoga forest of Karnataka (H.R. Bhat, personal communication). In the present survey it was confined to Bospara, Gudalur (Nilgiri District), the same high rainfall area mentioned earlier, where it was found breeding in an elephant foot print in association with *An elegans* on one occasion. Elsewhere, in the eastern part of the Nilgiris and other parts of the ghats which receive rainfall chiefly under the influence of the northeast monsoon, *An elegans* alone was found.

(3) *Anopheles (Cellia)* sp. undetermined: Five

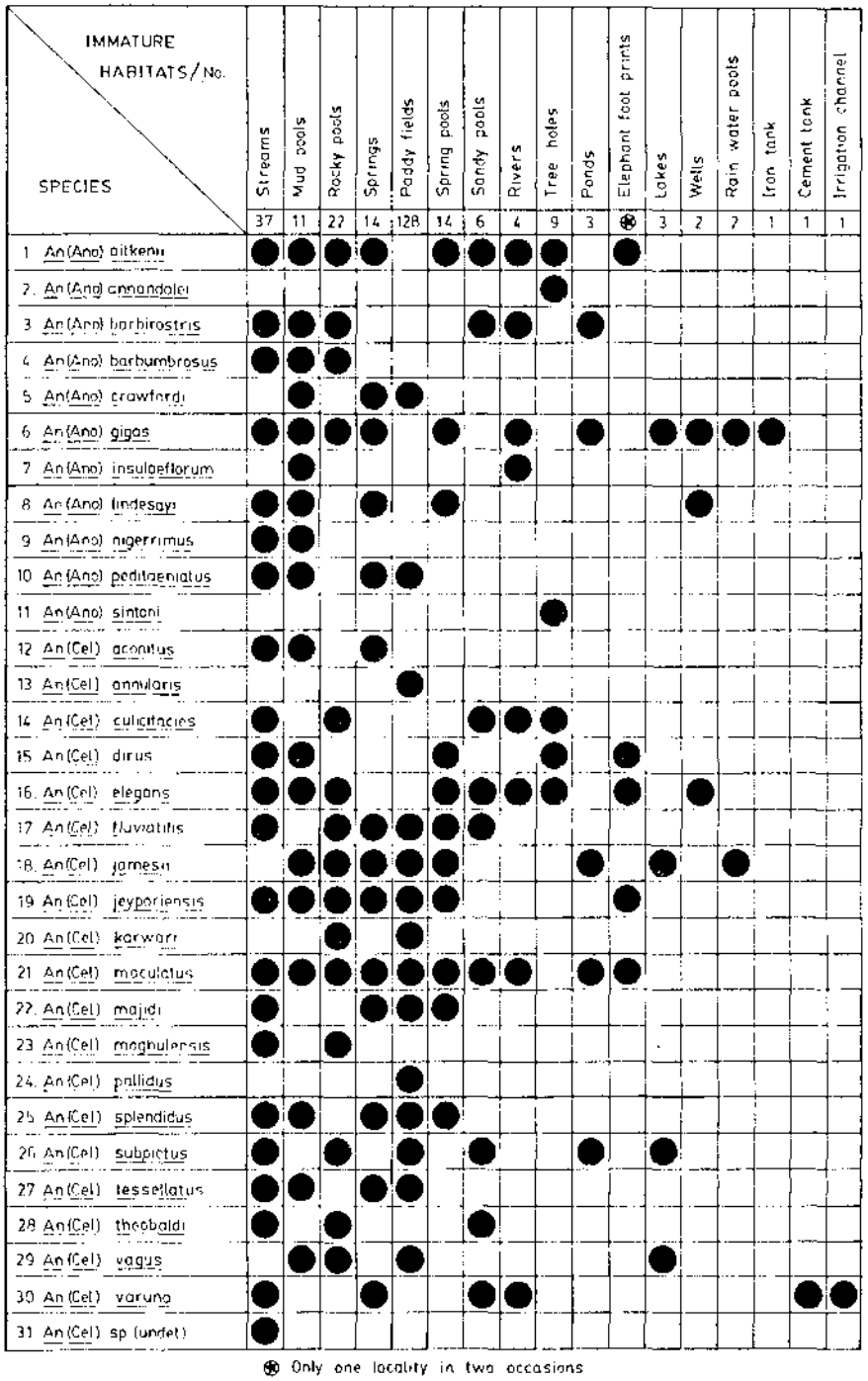

Fig. 3: Summary of the immature habitats of the species of *Anopheles* in the Western Ghats.

males and six females were reared from larvae collected in streams in two localities in the Varushanadu and Nilgiri Hills. These specimens closely resemble *An maculatus*, except that the hind tarsomeres are dark. They differ from *An stephensi* in lacking the white speckling on the palpi which is characteristic of the latter species. In the absence of larval material, which would be diagnostic, the male terminalia were examined. Christophers (1933) has described presence of an accessory hair between the harpago and club in *An stephensi*, which is absent in *An maculatus*. Our specimens were similar to the latter species. These specimens may represent a variant of *An maculatus*, which is known to be a highly variable species (Christophers, *loc. cit.*), and they were in fact collected in association with this species. Efforts are continuing to obtain associated immature material.

(4) *Anopheles (Cellia) fluviatilis* James, 1902 was abundant in the pre DDT era and was the major malaria vector. Russell and Jacob (1942) found that the species constituted 11.92 per cent of all anophelines collected in the Nilgiris throughout the year. Covell and Harbhagwan (1939) in Wynaad and Ramachandra Rao (1945) in North Kanara obtained corresponding values of 8.8% and 5.0% respectively. By contrast, only 36 specimens of *An fluviatilis* could be obtained in the whole of this study, 0.9% of the total anophelines collected. Russell and Jacob (*loc. cit.*) found that February and September were peak breeding months in the western and eastern parts of the Nilgiris respectively. They could collect an average of 11.0 to 20.0 larvae per 15 minutes collection period at the peak season. In the present study only eleven specimens could be collected during tours to both areas in both February and September. Immediately after two years of extensive use of DDT in Wynaad, Vedamanikkam (1949) recorded a sharp decline in the immature population, which led the same author in the year 1952 to comment that *An fluviatilis* was fast becoming extinct in the area. While not extinct, it may be concluded that now *An fluviatilis* does not have a high potential as a vector in this area because the population density is so low. However, *An culicifacies* may be a greater potential hazard. In the U.P. Terai, where there has been extensive deforestation for agricultural settlement, *An fluviatilis* has been replaced as the major malaria vector by *An culicifacies* (Sharma *et al.*, 1984). In Orissa also, where deforestation has been extensive, the latter species has been found sporozoite positive in the interior where it was not previously implicated in malaria transmission (Nagpal and Sharma, 1986; R.T. Collins, personal communication). Vigilance is essential to prevent this trend from repeating itself in the Western Ghats' ecosystem.

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Three new Electrophoretic Allelomorphs of Glucose-6-phosphate dehydrogenase

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Three new G-6-PD variants, one fast moving with 110% and two slow moving variants with 90% and 50% electrophoretic mobilities were observed. Enzyme activity studies showed that the enzyme with 110% mobility was labile and this enzyme was designated as G-6-PD "Garhwal". Pedigree analysis of variants G-6-PD 50% mobility in two families of Buksa tribe indicated its X-linked inheritance and that it is an allelomorph of the normal B-type prevailing in the population. This variant is designated as G-6-PD "Haldwani" and the 90% mobility variant as G-6-PD "Delhi".

INTRODUCTION

A number of Glucose-6-Phosphate dehydrogenase (EC 1.1.1.49) variants have been reported from different ethnic and racial groups. The two common electrophoretic forms are G-6-PD A and B. Phenotype B is the most common type with 100% electrophoretic mobility. Phenotype A has a faster mobility of 110% and is found in about 30% of the negroid population. A number of individuals were found to be deficient for this enzyme. Frequency of deficient individuals in certain populations has been shown to be related to the endemicity of falciparum malaria and also to drug induced hemolysis (WHO, 1967). In the surveys conducted on metropolitan Delhi population and Buksa tribe of Haldwani to study correlation of the genetic markers to malaria endemicity, three new electrophoretic variants of G-6-PD enzyme were observed which are reported in this paper.

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MATERIAL AND METHODS

Blood was collected into a heparinized vial by pricking a finger tip and processed for hemolysate (WHO, 1967). Electrophoretic phenotypes were identified on 7.5% horizontal polyacrylamide slab and starch gel using Tris-EDTA-Boric acid (TEB) pH 8.8 buffer (WHO, 1967). Samples with variant enzyme along with normal samples were also tested on 7.5% polyacrylamide horizontal slab gel electrophoresis using 0.1 M phosphate buffer (pH 7.1) according to Joshi *et al.* (1985). Quantitative estimation of enzyme activity was done (WHO, 1967) using Beckman Model DU 2 spectrophotometer. Thick and thin smears from all subjects were examined for the presence of malaria parasite.

RESULTS AND DISCUSSION

A total of 626 samples from metropolitan Delhi population and 635 samples from Buksa tribe of

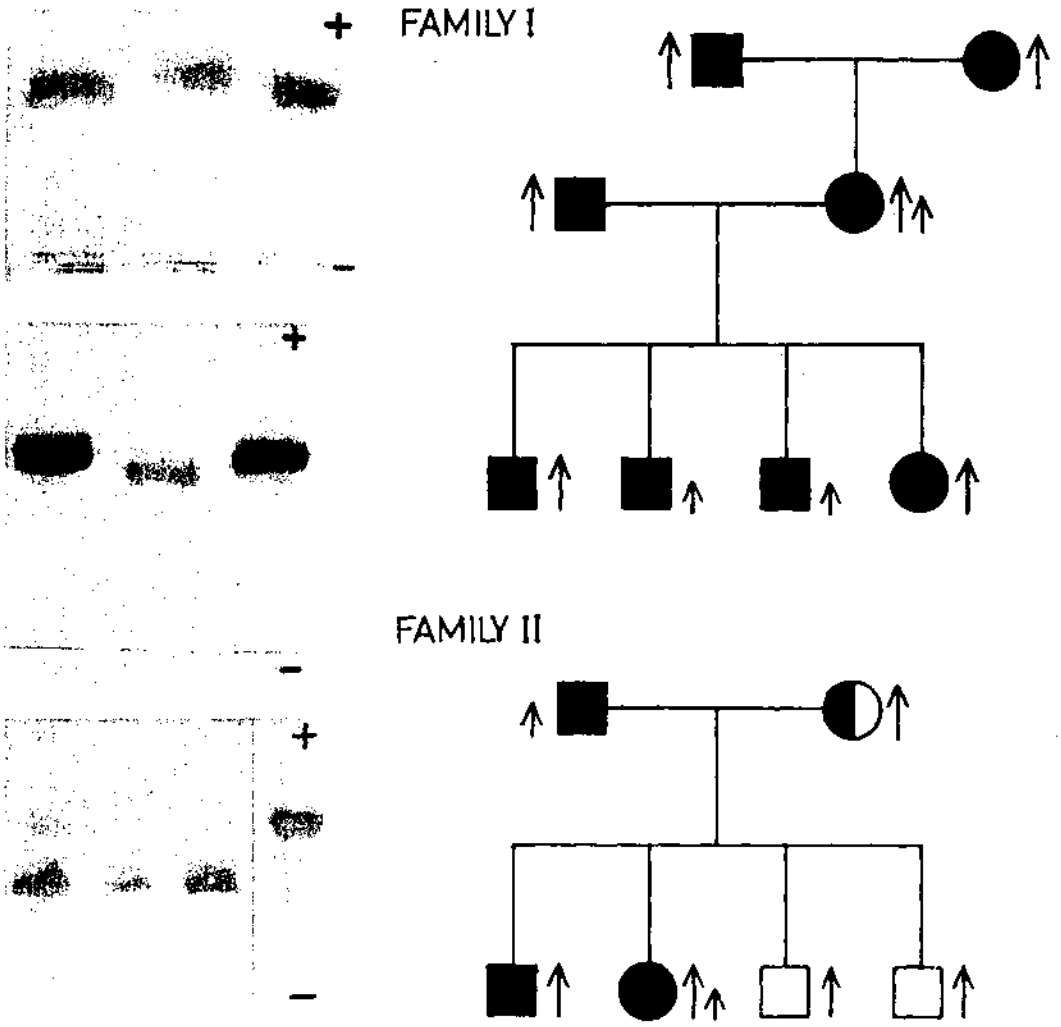

Fig. 1: Electrophoretogram showing 1 & 3 normal G-6-PD B phenotype and 2, fast moving G-6-PD "Garhwal".

Fig. 2: Electrophoretogram showing 1 & 3, G-6-PD B phenotype and 2, slow moving G-6-PD "Delhi".

Fig. 3: Electrophoretogram showing 1, heterozygote for slow moving G-6-PD "Haldwani" and normal B phenotype (female); 2 & 3, "Haldwani" type (male); 4, normal B phenotype (male).

Fig. 4: Pedigree analysis data showing X-linked inheritance of slow variant of G-6-PD in Buksa tribe of Haldwani ■ Non-deficient, □ Deficient, ↑ 100% Electrophoretic mobility, Type B, ↑ 50% Electrophoretic mobility, "Haldwani" type and ↑↑ Heterozygote for B and "Haldwani" type.

U.P., Terai, Haldwani, Distt. Nainital were analyzed. Except for three samples from Delhi and eight samples from Buksa tribe, all the other samples exhibited G-6-PD B phenotype on electrophoresis. Of the three samples in Delhi population, one exhibited 90% mobility and two exhibited 110% mobilities as compared to the common B phenotype which is considered to be of 100% mobility (Fig. 1). In all the eight samples of Buksa population enzyme band with 50% mobility was observed. All the samples with variant enzymes were from male subjects except for one in Buksa population which was from a female subject. Samples with slow and fast moving bands were confirmed on two buffers and two media as suggested by WHO (1967).

The fast moving variant of Delhi population resembled the common A phenotype in having 110% mobility and this electromorph was lighter in colour as compared to normal B type when electrophoresed after storage for 3-4 days. Enzyme activity in the red blood cells of one faster moving sample was 10.52 I.U. and of the 28 normal B type samples ranged between 9.34 and 15.48 I.U. On the eighth day after storage at −10°C only 44% activity was retained in the faster moving sample as compared to 70% in the normal B type sample, confirming the labile nature of the variant enzyme observed during electrophoresis. Thus, though the fast moving samples resembled A phenotype in their mobility (110%) they appeared to be different variants because of their labile nature. Because the A phenotype was reported to be stable (WHO, 1967), the fast moving variant is considered to be a new variant and designated as G-6-PD "Garhwal". Both the subjects exhibiting this variant are from Garhwal, a high altitude region of Uttar Pradesh, but are presently living in Delhi. Of the 626 samples, 82 were from high altitude region. A sample with 90% mobility was of a local resident and exhibited normal enzyme activity on the electrophoretogram (Fig. 2). Due to non-availability of the sample for further analysis enzyme activity tests could not be performed. This new variant is tentatively designated as G-6-PD "Delhi".

Of the eight variant samples of Buksas seven were male subjects with a slow moving band of 50% electrophoretic mobility, while the eighth sample was that of a female heterozygous for normal B type (100%) and slow (50%) bands (Fig. 3). On both the buffer systems i.e., TEB (pH 8.8) and phosphate buffer (pH 7.1), the Rf value of the observed slow band was 12 and that of common B type band was 24. The variant phenotype was observed in 5 families residing in 5 different villages out of 16 villages surveyed.

Data available on 2 families indicated X-linked inheritance of the slow variant and that it is an allelomorph of the normal B type prevailing in the Buksa population (Fig. 4). As all the villages were located at Gadarpur PHC of Haldwani, the variant is designated as G-6-PD "Haldwani". The three variant enzymes identified in this study are determined by 3 isoalleles at the G-6-PD locus. It may be mentioned that the variant samples were found both in malaria positive and negative groups.

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Genetic Markers in Malaria Patients of Delhi

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A total of 355 subjects affected with malaria and 305 negative controls from metropolitan Delhi were investigated with regard to ABO blood group, Haptoglobin, Haemoglobin and Glucose-6-phosphate dehydrogenase markers. Except for the Hp system no other marker studied has shown significant difference between the two groups with regard to the distribution pattern of polymorphic forms. A significant increase in the incidence of functional ahaptoglobinemia (Hp 0 -0) was observed in the malaria positive group. G-6-PD deficiency was detected in two individuals.

INTRODUCTION

Malaria is still a serious public health problem in many parts of the world. The hypothesis that all individuals might not be equally susceptible to malarial infection was proposed by Haldane in 1949 (cited from Siniscalco *et al.*, 1973). The factors responsible for such individual variations need to be identified and such an identification would help in understanding the existing differences in the incidence of malaria in different populations. Relationship between some human red cell genetic markers and malaria has already been shown in African populations viz., the selective advantage of G-6-PD deficiency and sickle cell haemoglobin against *P. falciparum* (Luzzatto *et al.*, 1983) and Duffy negative antigen against *P. vivax* infection (Miller *et al.*, 1975). A few reports on the distribution of genetic markers and malaria in Indian populations are available (Gupta and Raichowdhuri, 1980; Vasantha *et al.*, 1982) but no conclusive correlations have been drawn. Therefore, surveys of genetic markers were undertaken in Indian populations with the objective to correlate the frequencies of genetic markers to malaria endemicity or incidence. In recent years, a high incidence of *P. falciparum* and *P. vivax* malaria was reported from some areas in Delhi (Choudhury, 1983). Four genetic markers viz., ABO, Haptoglobin (Hp), Haemoglobin (Hb) and Glucose-6-phosphate dehydrogenase (G-6-PD) were selected to study the polymorphism and positive correlation of some of the polymorphic forms of these markers with malaria endemicity. Results of the survey carried out in Delhi are reported in this paper.

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MATERIAL AND METHODS

The study was carried out on patients with fever who came for blood test to the malaria clinic run by the National Malaria Eradication Programme, Delhi and the non-fever cases from the staff of Malaria Research Centre, Delhi to serve as base line data. Relevant history was taken

from every patient. Blood samples were collected during the years 1983 and 1984.

Blood samples collected from the NMEP clinic and the MRC included subjects from various socio-economic groups representative of metropolitan population. About 0.3 to 0.4 ml of blood was collected by pricking a finger in heparinized vials from 660 subjects. Simultaneously a thick and thin blood smear was also made. Methods used for the collection, storage and analysis of samples were same as described by Joshi *et al.*, (1985; 1987a).Blood smears were stained by JSB and examined for malaria parasites under bright field Leitz Dialux 20 microscope at a magnification of 1000x.

Most of the samples collected (approximately 90%) were from adults. The sex ratio in the sample was 4.5 males to one female. These proportions did not vary between the malarious and non-malarious groups. Most of the subjects were in the age group ranging between 10–60 years. Subjects above 14 years were categorized as adults. It was difficult to persuade women and children among the patients to give blood. Because of poor representation of women and children in the sample, no subdivision viz., males, females, children, adult etc. was done for distribution frequency analysis.

RESULTS

In a total of 660 samples collected, 540 were from febrile and 120 from afebrile subjects. Of the 540 fever cases, 355 were positive for malaria infection and 185 were negative. The 120 afebrile subjects from MRC were also negative for malaria infection. *Plasmodium vivax* was predominant, being found in 259 cases and *P. falciparum* in 91 cases, while 5 cases were found with mixed infection of *P. vivax* and *P. falciparum*.

Table 1. Distribution of ABO and Haptoglobin Polymorphs

Groups	Non-malarious			Malarious		
Polymorphic Forms	Afebrile	Febrile	Total	*P. vivax*	*P. falciparum*	Total
ABO						
A	16	50	66	73	23	96
	(21.9)	(29.2)	(27.3)	(28.9)	(25.6)	(28.4)
	25	53	78	87	31	118
	(34.3)	(31.0)	(36.0)	(34.4)	(37.8)	(34.9)
AB	8	22	30	27	3	30
	(10.9)	(12.9)	(7.0)	(10.7)	(3.7)	(8.9)
O	24	46	70	66	28	94
	(32.9)	(26.9)	(28.7)	(26.0)	(32.9)	(27.8)
Total	73	171	244	253	85	338
Haptoglobin						
1–1	4	3	7	13	2	15
	(4.9)	(1.7)	(2.7)	(6.4)	(2.8)	(5.5)
2–1	28	46	74	61	23	84
	(34.2)	(26.3)	(28.8)	(29.9)	(32.4)	(30.5)
2–2	47	119	166	95	27	122
	(57.3)	(68.0)	(64.6)	(46.6)	(38.0)	(44.4)
0—0	3	7	10	35	19	54
	(3.6)	(4.0)	(3.9)	(17.1)	(26.8)	(19.6)
Total	82	175	257	204	71	275

Figures in parentheses are % frequencies.

As there was no significant difference in the distribution pattern of genetic markers studied between the two groups viz., the afebrile and febrile negative for malaria infection, these two groups were pooled for further analysis and served as the control group (Table 1). It may be noted that due to unavoidable circumstances all the samples could not be analyzed for all the systems and therefore, the number of samples tested vary for each enzyme system.

ABO Polymorphism

Two hundred and forty four samples from the non-malarious group and 338 samples from malaria positive group were tested for ABO grouping by standard tube test. Observed numbers and per cent frequency of phenotypes are given in Table 1. Chi-square test revealed that the distribution of the phenotypes did not differ significantly between the two groups ($\chi^2_3 = 2.06$, $p > 0.50$). No significant difference was found in the distribution of ABO polymorphs among *P. vivax* and *P. falciparum* cases ($\chi^2_3 = 4.85$, $p > 0.10$, Table 2).

Haptoglobin

Two hundred and fifty seven samples in control group and 275 samples in malaria positive group were analyzed for haptoglobin polymorphism and the data is given in Table 1. Chi-square value ($\chi^2_3 = 49.76$, $p < 0.01$) indicated that the frequencies of polymorphic forms in control and malaria positive group differed significantly. A significant increase in the frequency of Hp 0–0 (19.6%) was observed in the malaria positive group in comparison with the control group (3.9%) and vice versa for Hp 2–2 frequencies. This suggests that the disease may be responsible for the high Hp 0-0 (functional ahaptoglobinemia) incidence. These findings are supported by Trape *et al.* (1985) who reported that malaria causes ahaptoglobinemia and thus is responsible for a high frequency of Hp 0–0 in Africa. Functional ahaptoglobinemic individuals are those with no detectable Hp phenotypes on electrophoresis. However, functional ahaptoglobinemic patients were not examined during convalescence to establish the direct correlation between Hp 0–0 and malaria. Such a study would also indicate the time after which detectable haptoglobins are seen again in the blood.

The malaria positive group comprised of 204 *P. vivax* and 71 *P. falciparum* cases. A slightly higher incidence of Hp 0-0 i.e., 26.8% was observed among *P. falciparum* cases as compared to 17.1% among *P. vivax* cases. No statistically significant difference was observed between the two malaria positive groups with regard to the distribution of haptoglobin polymorphs ($\chi^2_3 = 4.67$, $p > 0.10$, Table 2).

Haemoglobins

The samples examined for haemoglobin variations by electrophoresis included 219 controls, 180 *P. vivax*, 68 *P. falciparum* and 3 mixed infections of *P. vivax* and *P. falciparum*. A single

Table 2: Comparative chi-square test values

Groups tested	Afebrile vs Febrile	Control vs Malarious	Control vs *P. vivax*	Control vs *P. falciparum*	*P. vivax* vs *P. falciparum*
System					
ABO	2.13	2.06	0.94	5.56	4.85
Hp	4.36	49.76*	30.58*	39.81*	4.67

Degree of freedom = 3
*$P < 0.001$.

sample from *P. vivax* infected group showed Hb AS (sickle cell trait) phenotype while the remaining samples were of normal HbA type.

G-6-PD

A total of 281 samples from the control and 345 from malaria positive groups were analysed for G-6-PD deficiency by flourescent spot test and electrophoresis. Two of the samples tested were found to be deficient and the rest were normal. Both the deficient subjects were among the controls, one each in febrile and afebrile groups comprising 187 males and 94 females. The deficient sample showed faint bands on electrophoresis as compared to others at the G-6-PD 'B' position i.e., they were of Gd(–), B phenotype. The normal samples were found to be the common Gd(+), B phenotype except for three samples which showed electrophoretic variations. Details of these three samples are presented in another communication (Joshi *et al.*, 1987a). In the present study both the groups, malarious and non-malarious, were found to be homogenous with regard to phenotypic distribution of ABO and Hp polymorph and the allelic frequencies were in Hardy-Weinberg equilibrium

Table 3: Allelic Frequencies of ABO and Hp in malarious and non-malarious groups

Alleles	Frequencies in	
	Malarious	Non-malarious
A	0.205	0.22
B	0.255	0.25
O	0.54	0.54
Chi-square testing for random mating	6.83*	0.652 NS
Hp^1	0.26	0.18
Hp^2	0.74	0.82
Chi-square testing for random mating	0.012 NS	0.134 NS

*$0.10 > P > 0.05$

NS-Not significant

Note: Number of Hp 0-0 were deleted from the total number for calculation of Hp^1 and Hp^2 gene frequencies

(Table 3). The high incidence of B compared to A allele observed in this study is characteristic of Indian populations and the allelic and phenotypic frequencies for A,B,AB and O polymorphs observed in both the groups are found to be within the reported range.

DISCUSSION

Oliver-Gonzalez and Torregrosa (1944) have reported that malaria like many otherdiseases, share common antigens with the ABO blood groups. No correlation between ABO blood groups and malaria has been observed in African populations (Molineaux and Gramiccia, 1980) and in Dadar and Nagar Haveli tribal population of India by Vasantha *et al.* (1982). However Gupta and Raichowdhuri (1980) reported that malaria parasites share group A antigens and hence are better tolerated by the host's immune system. Athreya and Coriell (1967) postulated that the blood group B may have an advantage in malarious regions.

Indian populations are represented by low Hp^1 and high Hp^2 gene frequencies. The reported incidence of Hp^1 allele for majority of the Indian populations is 0.14 to 0.37 (Baxi, 1977). Gene frequencies calculated for Hp^1 and Hp^2 genes in malaria positive and malaria negative groups fall within the reported range, but with a higher frequency of Hp^1 in malaria group compared to the control group and vice versa for the Hp^2 allele (Table 3). Reported incidence of Hp 0–0 (functional ahaptoglobinemia) in Indian populations varies from 0 to 7 per cent (Seth *et al.*, 1971). Thus the observed incidence of 3.90 per cent Hp 0–0 in non-malarious group falls within the normal range while the 19.6 per cent observed in malaria group is very high. High incidence of Hp 0–0 in the malaria group may be responsible for lowering Hp^2 gene frequency as majority of the Hp 0–0 samples might be of Hp 2–2 types, the commonest polymorphic form. Similar findings have been observed by several workers and there are various hypotheses suggesting that the

majority of Hp 0–0 are Hp 2-2 individuals (Baxi, 1977; Santos *et al.*, 1983; Trape *et al.*, 1985).

High incidence of functional ahaptoglobinemia among malaria positive group can be explained on the basis that during malaria infection depletion of plasma haptoglobin occurs, either due to the formation of Hp-Hb complex with residual haemoglobin liberated at the end of erythrocytic cycle of malaria parasite or due to the circulating antigen antibody complexes causing intravascular haemolysis (Boreham *et al.*, 1981). Welch *et al.* (1978) had shown that G-6-PD deficiency may be responsible for Hp 0–0 cases, which however is not the case in our samples. Trape *et al.* (1985) have also shown that chemoprophylaxis decreases the incidence of ahaptoglobinemia considerably in the populations.

The incidence of HbS in Indian populations varies from zero to 30% and northern Indian populations are generally characterized by a very low incidence of HbS allele (Sharma, 1983). Thus the occurrence of a single case of HbAS in our study samples which are mostly from subjects belonging to northern states is not unexpected. No correlation between malaria positivity and sickle cell allele was observed in this study.

No significant difference was observed between the malaria positive and negative control groups of this study with regard to the frequency of G-6-PD deficiency and electrophoretic variations. Both the deficient subjects were of Punjabi origin. In Punjabi Khatris the G-6-PD deficient allele was reported to be having a frequency of 14% (Ghosh, 1983). Madan *et al.* (1981) have also reported the highest incidence of G-6-PD deficiency (3.75%) among subjects from west Punjab followed by subjects from Delhi (1.32%) in their study conducted on patients visiting a Delhi hospital. The incidence of G-6-PD deficient allele was found to vary from zero to 20% in the Indian subcontinent with a few exceptions (Ghosh, 1983).

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Studies on the Comparative Gametocytaemia of *P. berghei* (NK 65) in male and female *Mastomys natalensis*

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Course of gametocytaemia along with parasitaemia of *P. berghei* (NK 65) infection transmitted through *A. stephensi* has been studied in both sexes of *Mastomys natalensis*. Animals became patent between Day 4 and Day 5 when 1×10^4 sporozoites were inoculated i.v. Parasitaemia increased throughout the course of infection in both sexes of animals. Two peaks of gametocytaemia were discernible in the females, on Day 10 and Day 16 while three peaks in the males, on Day 7, 11 and 20. The ratio of gametocytes to total parasites was maximum during the first peak in both sexes. These observations confirm that the peripheral blood is flooded with waves of gametocytaemia only at certain intervals.

INTRODUCTION

Transmission of malaria depends on the availability of viable male and female gametocytes in the peripheral blood coinciding with the biting habits of suitable vector mosquitoes. As such, it is imperative to have detailed knowledge about the bionomics of the gametocytes indicating when they appear first in circulation and whether their infectivity is correlated with their age and maturity. Earlier workers have observed various characteristics of the gametocytes. Garnham (1931) reported that the gametocytes of *P. falciparum* appear in the blood at regular intervals. Hawking *et al.* (1968) attempted to observe circadian rhythms in infectivity of gametocytes of many malaria parasites. Vanderberg and his coworkers (1968) observed that for establishment of mosquito cycle, infective period of gametocytaemia plays an important role. In a bid to study some of the above aspects of gametocytes it was thought useful to study simultaneously the course of parasitaemia and gametocytaemia of *P. berghei* (NK 65) infection in a susceptible rodent host, *Mastomys natalensis*. This study would help in maintaining the sporogonic cycle in the vector mosquito and in controlling the infection by altering the gametocytaemic periodicity.

MATERIAL AND METHODS

i) Malaria Parasite : NK 65 strain of *P. berghei* received from the Department of Biochemistry, P.G.I. Chandigarh, was used in the experiment.

ii) Vertebrate Host : 8 weeks old male and female *M. natalensis* were used.

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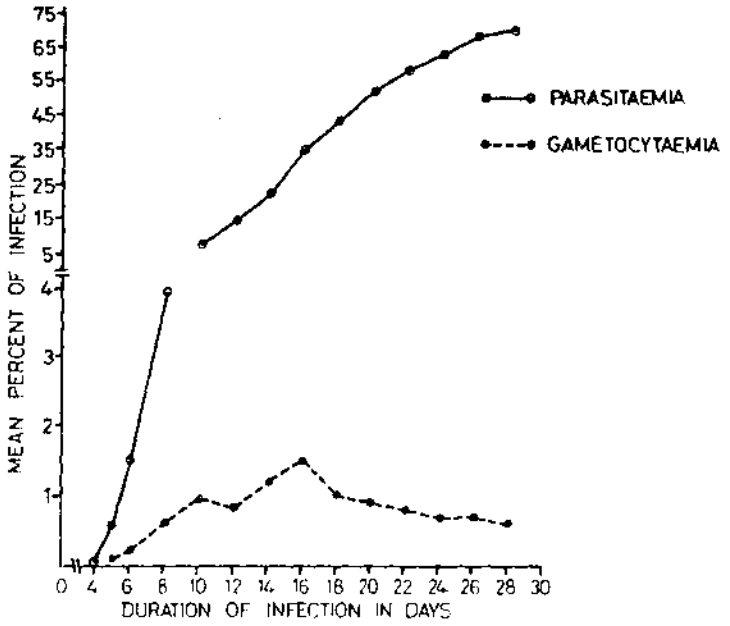

Fig. 1: Graph showing course of parasitaemia and gametocytaemia in female *M. natalensis* infected with *P. berghei* (NK 65).

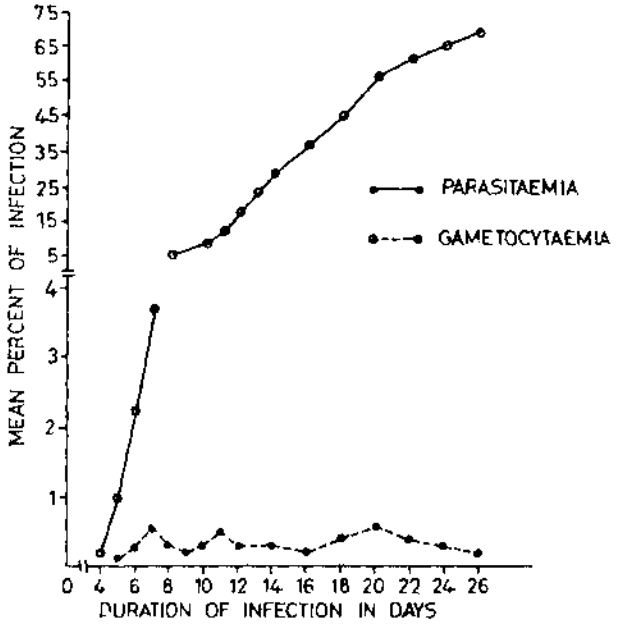

Fig. 2: Graph showing course of parasitaemia and gametocytaemia in male *M. natalensis* infected with *P. berghei* (NK 65).

iii) Sporozoites — Salivary gland sporozoites were isolated and quantitated by using standard method. Each specimen was inoculated with 1×10^4 sporozoites (i.v.).

Total parasitaemia and gametocytaemia were assessed every alternate day from the 4th day post inoculation of sporozoites till death.

RESULTS

Course of parasitaemia in female *M. natalensis*

Table 1 gives in brief the special features observed during the course of *P. berghei* (NK 65) infection in female *M. natalensis*. Five experiments were conducted using 7 animals in each set of experiment. The results were obtained with slight variations. Most of the animals became patent on Day 4 (range: 4–6 days). The survival time was found to be 24.67 ± 0.65 days. The animals died at peak parasitaemia (65–70%).

Fig. 1 depicts the course of parasitaemia in the female *Mastomys*. Parasitaemia increased very slowly till Day 10 ($8.68 \pm 0.44\%$) and afterwards there was a steep rise in the parasitaemia until Day 20 ($52.13 \pm 1.40\%$) and again at a slow rate after Day 20 and reaching the peak on about Day 28 with $70.30 \pm 4.72\%$ parasitaemia when the animals succumbed.

Course of gametocytaemia in female *M. natalensis*

The course of gametocytaemia has been shown in Fig. 1. The gametocytes were detectable in the blood one day after the appearance of trophozoites in the blood i.e., generally Day 5. The number of gametocytes gradually increased (Day 7: $0.37 \pm 0.03\%$, Day 8: $0.63 \pm 0.03\%$, Day 9: $0.77 \pm 0.05\%$) and reached the first peak on Day 10: $0.95 \pm 0.05\%$. Then there was a sudden fall on Day 12: $0.77 \pm 0.07\%$. On Day 14 gametocytaemia again increased to $1.20 \pm 0.09\%$ and reached the second peak on Day 16: $1.48 \pm 0.10\%$ followed by decline from Day 18: $1.09 \pm 0.07\%$ till death on Day 28: $0.65 \pm 0.12\%$.

Course of parasitaemia in male *M. natalensis*

Five experiments were conducted with 8 animals in each experiment. The pre-patent period was observed between 4–6 days. Death occurred at peak parasitaemia ($68.97 \pm 1.07\%$). The survival time observed was 23.63 ± 0.42 days. The course of parasitaemia was similar to that observed in females (Fig. 2).

Course of gametocytaemia in male *M. natalensis*

It is shown in Fig. 2 that in male *M. natalensis* first peak of gametocytaemia was reached on Day 7 ($0.56 \pm 0.03\%$) followed by slight decline on subsequent three days i.e. Day 8, 9 and 10. The second

Table 1. Salient features of the course of *P. berghei* (NK 65) infection in *M. natalensis*

S. No.	Sex	Body wt.(g)	Prepatent period (days) Median/Range	Maximum Parasitaemia(%) $\bar{X} \pm SE\bar{X}$	Survival time (days) $\bar{X} \pm SE\bar{X}$
1.	Female	30–35	4 (4–6)	70.30 ± 4.72	24.67 ± 0.65
2.	Male	30–35	4 (4–6)	68.97 ± 1.03	23.63 ± 0.42

$\bar{X}$: Mean; $SE\bar{X}$: Standard error

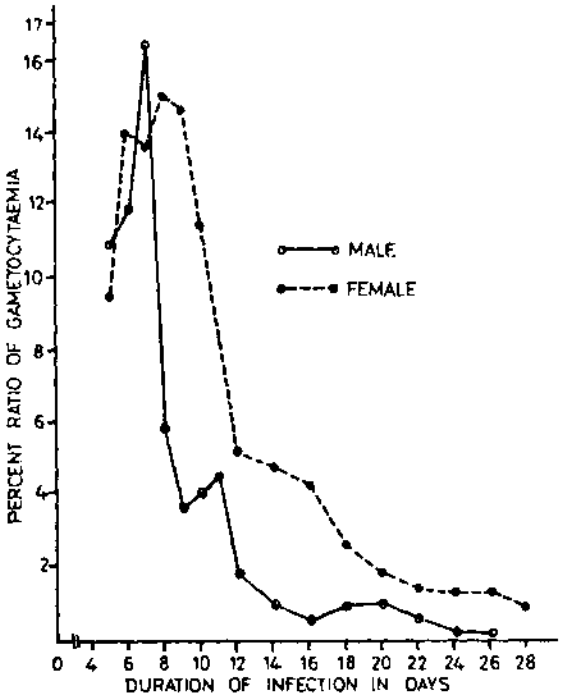

Fig. 3: Graph showing ratio of gametocytes to total parasites in male and female *M. natalensis*

peak was observed on Day 11 (0.51 ± 0.04%). The number of gametocytes again started decreasing from Day 12 onwards. The third peak of gametocytaemia was observed on Day 20 (0.58 ± 0.05%) followed by decline till death.

Ratio of gametocytes to asexual parasites in both sexes of *M. natalensis*

The ratio of gametocytes to asexual parasites has been shown in Fig. 3. In female *Mastomys* the ratio of gametocytes to asexual parasites was maximum on Day 8 (15.07 ± 0.72%) and on Day 9 (14.79 ± 1.00%) followed by gradual decline through second peak on Day 16 (4.27 ± 0.28%) till death on Day 28 (0.89 ± 0.21%). In male animals similar pattern was observed; the ratio was maximum on Day 7 (16.45 ± 1.44%) followed by decline, through second peak on Day 11 (4.45 ± 0.35%) and third peak on Day 20 (1.02 ± 0.09%).

DISCUSSION

The strain of *P. berghei* (NK 65) used in this study killed all the animals at peak parasitaemia suggesting lesser immunological interference against parasite multiplication. The course of parasitaemia was similar in both the sexes of *M. natalensis*. The percentage of parasitaemia was however significantly higher ($P < 0.05$) in males on Day 5 (1.08 ± 0.12%) to Day 9 (6.80 ± 0.39%), Day 12 (17.92 ± 0.78%) and Day 20 (55.83 ± 1.14%). On other days the parasitaemia was higher in males though not significant. In both sexes rate of multiplication of parasites was high during initial stage of infection. Afterwards as the infection increased, rate of multiplication declined suggest-

ing that a large number of normal reticulocytes were not available for invasion by the merozoites.

Regarding the course of gametocytaemia in both sexes on Day 5 no significant difference was observed but later the percentage of gametocytaemia was significantly higher ($P < 0.01$) in females compared to that in males throughout the course of infection. In males the peak of gametocytaemic period was observed between 7th to 20th day while in females it was from 10th to 20th day. Two generations of gametocytes appeared, one on Day 6 and the other between Day 14 and Day 18. In males three waves of gametocytes appeared, on Day 6, 10 and 14. Afterwards new gametocytes were not observed till death. This suggests that gametocytes were not formed continuously in the blood.

Our observations also support the findings of Garnham (1931). He reported that in *P. falciparum*, crescents appeared in blood in waves. The appearance of gametocytes at intervals might be due to rhythmic conversion of merozoites into progametocytes. It was observed that time taken by a gametocyte to attain maturity was 3–4 days. In case these gametocytes were not taken by the mosquito host, they remained in the circulation for a certain period and then started degenerating. A second generation of gametocytes emerged thereafter. The reason for occurrence of two waves of gametocytes in females and three in males could not be explained in absence of further results.

The ratio of gametocytes in relation to total parasites in the circulation was higher during the first peak. Although the absolute or total number of gametocytes in the second peak was higher, the ratio of gametocytes to total parasites was low. These gametocytes of the second wave showed signs of degeneration. The chromatin of these forms was fragmented while the cytoplasm became highly vacuolated and scanty. The pigment grains underwent clumping and seemed blacker; all this appeared to be due to degeneration. Hawking *et al.* (1971; 1972) reported circadian rhythms in maturation and infectivity of many malaria parasites. This phenomenon helps in transmission of the parasite. Landau *et al.* (1979) also reported that gametocytes of *P. yoelii nigeriensis* can establish infection in mosquitoes only at a certain age.

The present account reports that females produced more gametocytes as compared to the males. It has also been ascertained that there are definite peaks of gametocytes in the circulation. Further studies on the comparative infectivity of the gametocytes of different peaks are in progress and would be reported soon.

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5-HT, 5-HIAA and Related Enzymes in *P. berghei* infected Rats

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Vasomotor changes occur in malaria; some vessels dilate and others constrict. Endogenous vasoactive substances such as 5-hydroxytryptamine (5-HT) may be involved. Changes in the level of tryptophan hydroxylase, 5-hydroxytryptophan decarboxylase (5-HTP decarboxylase), monoamine oxidase (MAO) and 5-hydroxyindoleacetic acid (5-HIAA) were examined in *P. berghei* infected rat tissues and compared with control values. 5-HT content of liver, lung, spleen and brain was significantly decreased; it was increased in kidney and intestine. Tryptophan hydroxylase level of liver, lung, spleen, heart and brain was decreased. 5-HTP decarboxylase level of liver, lung and spleen was decreased and that of intestine and brain was increased in infected rats. In infected rats MAO level increased significantly in spleen, brain, lung and plasma but it is lowered in kidney, liver and intestine. 5-HIAA level of liver, lung and spleen was significantly increased; that of intestine and brain was decreased.

INTRODUCTION

Increased capillary permeability and vasomotor disturbances are common features of the pathophysiology of malaria (Maegraith, 1948). But the mechanisms involved are yet to be fully ascertained. Active endogenous substances may possibly be involved, of which 5-hydroxytryptamine (5-HT) is one. Aviado and Sadavongvivad (1970) showed that lung 5-HT level decreased in *P. berghei* infected mice. The tissue level of 5-HT reflects a dynamic equilibrium between 5-HT formation and catabolism. Alteration in 5-HT formation or its catabolism would tend to be reflected in tissue 5-HT level. Hence, a study of tryptophan hydroxylase, 5-HT level, 5-HTP decarboxylase (forming enzyme) monoamine oxidase (catabolising enzyme) and 5-HIAA (end metabolite of 5-HT, which would reflect the situation indirectly) appeared to be of interest in this context as no information in this regard in *Plasmodium berghei* infected rat is available.

The present investigation was designed to examine the changes in the level of tissue 5-HT, tryptophan hydroxylase, 5-HTP decarboxylase, monoamine oxidase and 5-HIAA in *P. berghei* infected rats.

MATERIAL AND METHODS

Charles Foster strain male rats (80 -100 gm) were used throughout. Infection of *P. berghei* was maintained by injection passage in male rats. Blood smears were obtained on alternate days for staining with Leishman to determine the percentage of parasitized erythrocytes. Parallel

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control experiments were run in which normal saline had replaced infected blood. Animals with parasitaemia between 10–50% were sacrificed. Blood and tissues (liver, lung, spleen, kidney, intestine, testis, heart and brain) were collected from both control and infected animals for the estimation of 5-HT, tryptophan hydroxylase, 5-HTP decarboxylase, monoamine oxidase, and 5-HIAA.

Tissue 5-HT and 5-HIAA were determined after the method of Curzon and Green (1970). 5-HT was expressed as μg/gm tissue. Tissue 5-HIAA was expressed in terms of μg 5-HT/gm tissue. Blood 5-HIAA was determined according to Udenfriend *et al.* (1959). Tryptophan hydroxylase was estimated according to Gal Martin and Patterson (1973) and activity was expressed as n mole tryptophan hydroxylated per mg protein/hr. 5-HTP decarboxylase was assayed according to Lovenberg (1971) and was expressed as μg 5-HT/mg protein. Tissue MAO was determined after the method of Green and Haughton (1961) and activity was expressed as enzyme unit/mg protein/hr. Plasma MAO was determined after the method of McEwen (1963) and activity was expressed as enzyme unit/gm protein/hr. Protein was estimated after Lowry *et al.* (1951).

RESULTS

In *P. berghei* infected rats, as compared with control animals 5-HT content of liver, lung, spleen and brain was significantly decreased; intestine and kidney 5-HT levels were enhanced. Values obtained from testis, heart and blood were not significantly altered (Table 1). Table 2 shows the tryptophan hydroxylase level in tissues of control and infected rats. Tryptophan hydroxylase level of liver, lung, spleen, heart and brain was significantly decreased but tryptophan hydroxylase level of intestine, kidney and testis was not altered in infected rats. In infected rats,

Table 1. 5-HT content (5-HT μg/gm tissue) in control and *P. berghei* infected rats

Animals	Tissues								
Rats	Liver	Lung	Spleen	Kidney	Intestine	Testis	Heart	Brain	Blood*
Control	0.21	0.65	1.37	0.50	1.2	0.09	0.27	0.80	38.0
(10)	± 0.3	± 0.06	± 0.1	± 0.09	± 0.07	± 0.01	± 0.04	± 0.02	± 3.0
Infected	0.06	0.29	0.64	0.75	2.3	0.12	0.22	0.5	37.0
(10)	± 0.009	± 0.02	± 0.07	± 0.04	± 0.04	± 0.01	± 0.02	± 0.02	± 5.0
P	<0.001	<0.001	<0.001	<0.05	<0.01	>0.05	>0.05	<0.01	>0.05

Results expressed as the mean ± S.E.; number of animals in parentheses.
P – Significance of the difference between infected and control animals.
*(μg/100 ml)

Table 2. Tryptophan hydroxylase (n mole tryptophan hydroxylated/mg protein) in control and infected rats

Animals	Tissues							
Rats	Liver	Lung	Spleen	Heart	Brain	Kidney	Intestine	Testis
Control	18.6	7.33	15.0	11.33	4.7	4.91	8.13	9.5
(10)	± 1.33	± 0.4	± 0.62	± 0.88	± 0.63	± 0.48	± 0.65	± 0.4
Infected	10.5	2.87	6.24	6.3	1.88	6.0	7.0	8.47
(10)	± 0.42	± 0.43	± 0.31	± 0.73	± 0.62	± 0.68	± 0.54	± 0.55
P	<0.001	<0.001	<0.001	<0.001	<0.001	>0.05	>0.05	>0.05

Results expressed as the mean ± S.E.; number of animals in parentheses.
P – Significance of the difference between infected and control animals.

Table 3. 5-hydroxy tryptophan decarboxylase level (μg 5-HT formed/mg protein) in control and infected rats

Animals	Tissues							
Rats	Liver	Lung	Spleen	Intestine	Brain	Kidney	Testis	Heart
Control	1.19	0.8	0.19	0.10	1.26	1.41	0.15	0.24
(10)	± 0.11	± 0.06	± 0.01	± 0.02	± 0.02	± 0.02	± 0.01	± 0.006
Infected	0.56	0.32	0.07	0.22	1.92	1.3	0.11	0.20
(10)	± 0.1	± 0.03	± 0.009	± 0.03	± 0.10	± 0.03	± 0.007	± 0.01
P	<0.001	<0.001	<0.001	<0.001	<0.01	>0.05	>0.05	>0.05

Results expressed as the mean ± S.E.; number of animals in parentheses.
P = Significance of the difference between infected and control animals.

Table 4: Monoamine oxidase level (enzyme unit/mg protein/hr) in control and infected rats

Animals	Tissues								
Rats	Liver	Lung	Spleen	Kidney	Intestine	Brain	Testis	Heart	Plasma*
Control	11.6	3.25	0.95	2.5	14.7	5.57	1.5	3.2	8.0
(10)	± 0.7	± 0.14	± 0.16	± 0.4	± 0.36	± 0.25	± 0.15	± 0.12	± 0.88
Infected	4.71	5.82	3.25	1.3	11.2	8.8	1.6	3.4	27.0
(10)	± 0.28	± 0.37	± 0.57	± 0.1	± 1.1	± 0.84	± 0.1	± 0.23	± 5.5
P	<0.001	<0.01	<0.001	<0.01	<0.05	<0.01	>0.05	>0.05	<0.001

Results expressed as the mean ± S.E.; number of animals in parentheses.
P = Significance of the difference between infected and control animals.
*Plasma (Enz. unit/gm protein).

Table 5. 5-HIAA content (5-HT μg/gm tissue) in control and infected rats

Animals	Tissues								
Rats	Liver	Lung	Spleen	Intestine	Brain	Kidney	Testis	Heart	Blood*
Control	0.12	0.22	0.24	0.50	0.14	0.10	0.08	0.10	0.02
(10)	± 0.01	± 0.01	± 0.03	± 0.01	± 0.02	± 0.01	± 0.009	± 0.004	± 0.002
Infected	0.28	0.44	0.46	0.29	0.08	0.08	0.06	0.009	0.03
(10)	± 0.01	± 0.005	± 0.002	± 0.02	± 0.005	± 0.009	± 0.009	± 0.009	± 0.001
P	>0.001	>0.001	>0.001	>0.001	>0.001	<0.05	<0.05	<0.05	<0.05

Results expressed as the mean ± S.E.; number of animals in parentheses.
P = Significance of the difference between infected and control animals.
*(μg/ml)

5-HTP decarboxylase activity of liver, lung, and spleen was significantly decreased and that of intestine and brain was significantly increased. There were no significant changes in respect of kidney, testis and heart (Table 3). Table 4 shows the MAO level in tissues of control and infected rats. In the infected group MAO level of lung, spleen, brain and plasma was significantly increased and that of liver, kidney and intestine was significantly lowered. Testis and heart MAO levels were not altered in infected rats. In *P. berghei* infected rats, 5-HIAA level of liver, lung, and spleen was significantly increased; that of kidney, heart and blood was not altered. 5-HIAA level of intestine and brain was significantly decreased as compared to control values (Table 5).

DISCUSSION

Vasomotor disturbances are well-known in malaria. Vascular changes of malaria may vary from one regional circulation to another. This may involve vasodilation in one area (cerebral vessels), while there may be vaso-constriction in others (kidney, liver), depending on the vessels involved (Maegraith and Alexander, 1972). In view of vascular changes, any alterations in vasoactive substance such as 5-HT would potentially be of interest.

In mice, infected with *P. berghei*, 5-HT level was reported to be decreased in lung (Aviado and Sadavongvivad, 1970) but 5-HT levels in other tissues have not been reported. No information is available on enzymes participating in the process of formation and destruction of 5-HT. Changes in this respect are potentially of interest because the dynamic equilibrium between 5-HT forming and 5-HT catabolising enzymes would be reflected in the 5-HT level, which in turn would affect vasomotor status.

Reduced tryptophan hydroxylase and 5-HTP decarboxylase level would tend to lower 5-HT and so will enhanced MAO level. Increased catabolism would tend to be reflected in higher 5-HIAA level. In lung and spleen, decrease in tryptophan hydroxylase level, 5-HTP decarboxylase level as well as a rise in MAO level are likely to contribute to reduced tissue 5-HT level. With raised 5-HTP decarboxylase level and lowered MAO and 5-HIAA level, one would expect enhanced tissue 5-HT level, and such is the case in intestine.

In liver, lowered tryptophan hydroxylase and 5-HTP decarboxylase level would tend to reduce 5-HT level but decreased MAO level would tend to raise 5-HT level. The algebraic sum of these two opposing influences would determine the outcome. In the event, lowered activity of tryptophan hydroxylase and 5-HTP decarboxylase appear to overshadow the decreased MAO activity and the net result was a decreased 5-HT level. This data does not explain the increased hepatic 5-HIAA level. MAO converts 5-HT to 5-hydroxyindole acetaldehyde which is catabolised partly by aldehyde reductase (to 5-hydroxytryptanol) and partly by aldehyde dehydrogenase (to 5-HIAA). Reduced aldehyde reductase may "divert" greater amount of 5-hydroxyindole acetaldehyde to the action of aldehyde dehydrogenase pathway, and greater formation of 5-HIAA. This speculation will have to await more information. Another possibility is greater formation of 5-HIAA level via transamination of tryptophan.

In brain, tryptophan hydroxylase level was decreased (tending to lower 5-HT level) and 5-HTP decarboxylase level was increased (tending to raise 5-HT level). Raised MAO level would tend to lower brain 5-HT. In brain, tryptophan hydroxylase activity changes appear to overshadow the effect of 5-HTP decarboxylase level changes; this would explain the reduction of brain 5-HT level. The overall vasodilation status of brain vessels would be an algebraic sum of vasodilator and vasoconstrictor inputs. 5-HT tends to constrict brain vessels, hence, its lowering would lead to an overall brain blood vessel dilatation. Vasodilator substance histamine was increased in blood and brain which would tend to dilate brain vessels (Bhattacharya and Lahiri, unpublished data).

In plasma of infected rats, MAO level was increased while blood 5-HT level remained unaltered. This is somewhat difficult to explain unless one accepts that the increase is somehow offset.

Changes of 5-HT level may be due to 5-HT metabolism changes caused by parasites. Moreover, vasomotor changes occurring in malaria may be significant in the context of renal and intestinal circulations. Small doses of 5-HT given subcutaneously to rats showed constriction of glomerular arteries (Page, 1958). Schneckloth *et*

al. (1957) showed that 5-HT infused into normal people reduced renal blood flow and glomerular filtration rate. Raised renal 5-HT level found in the present experiment may possibly lead to renal vasoconstriction. Constriction of the renal small blood vessels was demonstrated in advanced stages of *P. knowlesi* malaria (Maegraith, 1948). Cortical ischaemia, reduction in glomerular blood flow and consequent reduction in glomerular filtration was shown (Maegraith, 1948).

It should also be borne in mind that renal vasoconstriction may trigger, via renin, increased formation of angiotension II which may correct the general fall of blood pressure in malaria.

Migasena and Maegraith (1969) showed that in *P. berghei* infected monkeys intestinal wall arterioles were constricted; there was also less absorption of amino acids fats and xylose. Increased 5-HT level might possibly contribute to this change.

While it appears that 5-HT plays some part in the development of malaria pathophysiology, one may only speculate whether fall in the 5-HT level of liver, lung, spleen and brain is due to the parasite metabolising 5-HT.

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Microdot ELISA: Development of a Sensitive and Rapid Test to Identify the Source of Mosquito Blood Meals

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A rapid and sensitive microdot ELISA on nitrocellulose membrane for visual identification of the source of mosquito blood meal (MBM) has been developed. In this method MBM collected on filter papers are eluted with PBS and then spotted onto a nitrocellulose (NC) membrane, the remaining protein sites are blocked, the NC is then soaked in a solution of peroxidase labelled anti (Ig) human antibody solution. Finally, the immune complexes are made visible by incubation with substrate, 4 chloro-1-naphthol.

The method is highly sensitive, reproducible and suitable for the rapid identification of hosts of haematophagous insects in field laboratories. The technique is simple and does not require expensive equipment.

INTRODUCTION

Identification of the source of mosquito blood meals is an important parameter in epidemiological studies, particularly, in determining the role of mosquitoes in disease transmission. Several serological methods are available for host identification viz., ring test (Weitz, 1960), agar gel diffusion (Crans, 1969), precipitin test (Tesh *et al.*, 1971) and latex agglutination test (Boorman *et al.*, 1977). The immunohistochemical method of immunofluorescence has been advocated by Gentry *et al.* (1967) and Mckinney *et al.* (1972). The simplest and most commonly used precipitin test lacks specificity and sensitivity. A major limitation is that a relatively large amount of blood meal is needed. If the arthropod ingests very little blood, or most of the blood meal is digested, precipitin test is not suitable. Also, mulitiple hosts are not identified (Eliagon, 1971). Though agglutination method offers greater sensitivity and specificity, the results are often variable, and not suitable as a routine test. These methods often fail to discriminate closely related hosts. Immunofluorescent technique is very reliable and highly sensitive but it requires the use of sophisticated equipment (Mckinney *et al.*, 1972). Advantages and limitations of various techniques for host blood meal identification have recently been reviewed (Washino and Tempelis, 1983).

Immunoelectrophoresis and gel diffusion technique (Collins *et al.*, 1983; 1986) has been found to be more sensitive. Immunoelectrophoresis requires good laboratory facilities. Therefore it is not suitable for rapid field assay. The method also has the disadvantage of strong cross-

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reaction to heterologous sera (Washino and Tempelis, 1983).

Recently, interest has been focussed on enzyme linked immunosorbent assay (ELISA) (Burkot *et al.*, 1981; Edrissian and Haffizi, 1982; Linthicurn *et al.*, 1985; Ailus, 1985; Service *et al.*, 1986) because of its excellent specificity, sensitivity and reproducibility. It can detect 57 ng of serum protein (Burkot *et al.*, 1981) or 0.02 μl of blood spotted on filter paper (Service *et al.*, 1986). Sandwich ELISA method has excellent specificity and sensitivity for detection of multiple hosts (Service *et al.*, 1986). The method, however, is quite expensive since it involves the use of ELISA reader without which the method would be very slow with limited adaptability in the field.

We have developed a microdot ELISA on nitrocellulose paper for host identification of mosquito blood meals which shares the sensitivity and precision of ELISA technique. The method is quick, cheap, easy to perform and ideally suited for field use. The method can identify hosts from a smear of 0.1 μl blood with excellent specificity and comparable sensitivity.

MATERIAL AND METHODS

Microdot ELISA

Material and reagents

1. Nitrocellulose paper (0.2 μm pore size) from Advanced Microdevices, Ambala, India.
2. Peroxidase conjugated rabbit immunoglobulins (IgA, IgG, IgM, Kappa, Lambda) to human, made in Denmark by DAKO catalog no. P 212, Lot 125, Exp. Dec 91.
3. Bovine serum albumin, Fr V powder Sigma A 8022.
4. Tween-20 Sigma No. P 1379.
5. 4-chloro-1-naphthol Sigma No. C 8890.
6. Peroxidase conjugated rabbit immunoglobulins to cow, made in Denmark by DAKO catalog No. P 159.

Solutions

1. Eluting buffer: 0.01M PBS, pH 7.4.
2. Washing buffer: TBS-Tween, pH 7.5, 20 mM Tris, 500 mM NaCl, .02% Tween 20.
3. Blocking solution:
   1% BSA in TBS, pH 7.5.
4. Conjugate solution:
   Peroxidase conjugated rabbit immunoglobulin to human and cow (1:800) in blocking solution.
5. Substrate solution:
   The substrate for the HRPO conjugate was prepared fresh by dissolving 6 mg 4-Cl-l-naphthol in 2 ml methanol; added to 10 ml 0.1 M Tris-HCl, pH 7.6 containing 100 μl of 3% H_2O_2.

Specimens of blood samples

Two groups of specimens were used for standardization of the technique. One group contained samples from mosquitoes fed on human and buffalo killed at various times after feeding, then squashed on the filter paper (5 samples for each time interval). The second group contained blood samples of human, buffalo, cow, goat, sheep, pig and chicken origin. All the specimens were spotted on Whatman filter paper No. 1, dried at 37°C for 1 hour and kept in dessicator at 4°C. All the specimens collected from field were similarly preserved.

Preparation of eluates

A punch was used to standardize the size of filter paper disc for elution of a size which fits the wells of a microtitre plate. The punch was made in the middle of a blood spot. Blood meals were eluted in 100 μl of PBS, pH 7.4 at room temperature for 2 hours. In case of blood from different hosts elution was done with 200 μl of PBS. Buffalo and

human fed mosquito blood absorbed on filter paper was used as control in each assay.

Test conditions

The optimal conditions for the test which yielded the maximal values with homologous IgG and minimal values with heterologous IgG were determined by chequer board titrations as described by Voller *et al.* (1980). Rabbit anticow and antihuman HRPO conjugates were used at 1:600, 1:800 and 1:1000 dilutions. 1:800 dilution gave the optimum test for both the conjugates.

Description of the microdot ELISA assay

1. Nitrocellulose membrane (8x12 cm) is dipped in PBS solution for 10 minutes, blotted and dried at 37°C for 1 hr.
2. A clone master template (Hyclone) was used to transfer samples (~4 μl) from the wells of microtitre plate onto the prewashed nitrocellulose (NC) membrane dried at 37°C for 10 mins.
3. Sample spotted NC was washed with TBS-Tween (.02%) fifteen minutes each with gentle shaking thrice. It is important that the membrane should be washed thoroughly in order to minimise the background due to nonspecific binding.
4. The membrane was incubated with 12 ml of 1% BSA-TBS for 1 hour at 37°C for blocking nonspecific protein binding sites.
5. After removing the blocking solution NC was incubated with 10 ml of peroxidase labelled antispecies Ig conjugate for 1 hr. at 37°C.
6. The conjugate was aspirated off and the NC was washed thoroughly as explained in step 3.
7. 10 ml of freshly made substrate solution was used to dip the NC membrane in a covered tray. After 10 to 20 mins. in the dark, purple dot or ring appeared for positive sample and the enzyme reaction was stopped by washing with a large volume of water.

Reading of results

The positive homologous control dot should be coloured as an indication that all the reagents are functioning and the negative control dot should be colourless. The colour in the individual sample dots was assessed visually. Any dots with light purple to intense purple were considered as weakly positive (+), positive (++), strongly positive (+++), most strongly positive (++++) reactions and colourless dot recorded as negative (−).

RESULTS

Sensitivity

The test system for human blood was evaluated for sensitivity as follows; 10 μl of human blood was absorbed on a Whatman filter paper No. 1 dried at 37°C, stored overnight and eluted with 200 μl PBS at room temperature for 2 hrs. Serial dilutions of this eluate were made in PBS (1:10–1:10,000) and these were tested by microdot ELISA method for human blood (Table 1). The dilutions of the eluate ranging from 1:10 to 1:100

Table 1. Results of microdot ELISA tests on human blood collected on filter paper

Dilution of blood	Qualitative assay
Neat eluate	++
1:10	++
1:100	+++
1:1000	+
1:10000	

gave strong positive reactions for human blood. This indicates that 0.1 μl of blood could be detected on a filter paper disc very easily.

Kinetics

Logistically, host identification of field mosquitoes has to be done when most of the blood might have already been digested. To determine sensitivity of the test on blood meals that had

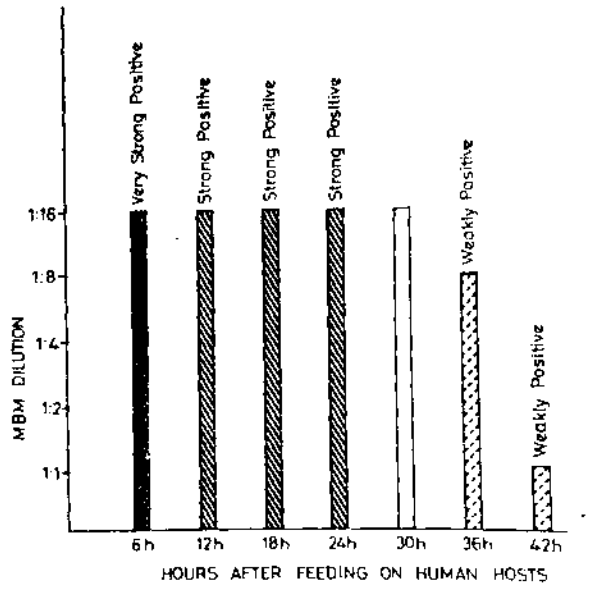

Fig. 1: Identification of blood meals in laboratory reared *A. stephensi* fed on human host, at varying times after blood ingestion, with varying dilutions.

experienced varying degree of digestion we maintained known host fed mosquitoes at room temperature, 28°–30°C and took blood smears at definite intervals. All host identification was correct even at 36 hours after feeding. Five samples for each interval (digestion period) were used: 32 laboratory fed mosquitoes were tested. As the size of the blood meal ingested varied greatly, there was wide variation in spot intensity. After 36 hours, blood meal was dark in colour and very little blood was left. It is known that in tropical conditions blood is commonly digested within 24–48 hours. Only two human fed mosquitoes survived past 36 hours and we identified these correctly. This result (Fig. 1) compares well with sandwich ELISA data reported (Service *et al.*, 1986).

Fig. 1 shows the result of digested MBM with manifold dilutions. MBM was eluted with 100 μl PBS at room temperature for 2 hrs. The eluate was serially diluted; 2 μl aliquots of each dilution were spotted on nitrocellulose and tested with antihuman HRPO conjugate. Upto a sixteen fold dilution, the spot intensity did not diminish perceptibily upto 30 hrs. digested blood meal. Thirty six hours and onward ingested blood meal identification was very weak at lower level of dilutions. It means that a smear of 0.1 μl blood on filter paper was enough to be detectable. This is important, for then the spot intensity with maximally digested MBM will not be compromised. From smears of freshly gorged mosquitoes (blood meal about 30–50 μl of blood) the eluate could be diluted 16-fold without perceptible change in spot intensity.

Specificity

Rabbit antihuman Ig HRPO conjugate was tested against blood and absorbed blood on filter paper from human, cattle and avian hosts for specificity test. Ten μl of absorbed or unabsorbed blood was eluted or diluted up to 200 μl with PBS. At this elution (1:1) volume all hosts were cross-reacting faintly with antihuman conjugate

Table 2. Filter paper absorbed blood screened against antihuman HRPO conjugate by microdot ELISA

Dilution	Human	Buffalo	Cow	Pig	Sheep	Goat	Chicken
1:1	++	+	+	+	+	–	+
1:10	+++	–	–	–	–	–	–
1:100	+++	–	–	–	–	–	–
1:1000	+	–	–	–	–	–	–
1:10000	–	–	–	–	–	–	–

Table 3. Blood screened against antihuman HRPO conjugate by microdot ELISA

Dilution	Human	Buffalo	Cow	Pig	Sheep	Goat	Chicken
1:1	+++	++	++	+	+	–	+
1:10	++++	+	+	–	–	–	–
1:100	+++	–	–	–	–	–	–
1:1000	+++	–	–	–	–	–	–
1:10000	+	–	–	–	–	–	–

except goat. The results are shown in Table 2 and 3.

Filter paper absorbed blood and unabsorbed blood from various hosts were screened at the same dilution. A 10-fold decrease in detectability due to filter paper absorption was evident. A loss of colour intensity and cross-reactivity was also identified when fed mosquito blood meal and absorbed blood were compared.

Double blind test

Extensive 'double-blind' tests on human, rabbit and cow fed mosquitoes were done with anti-human conjugate. The samples taken were of varying digestion period and tested in batches of 35, 45, 64 and 84 samples. Results are given in Table 4. All hosts could be detected correctly. During each assay a known human fed and buffalo fed mosquito blood meal, treated and spotted similarly, was taken as standard against which the spot intensities were matched.

Table 4. Double blind test of different host fed mosquito blood meal identification against antihuman Ig HRPO conjugate

Batch No.	Total samples	Human sample given	Human sample identified
1	84	38	38
2	35	16	16
3	45	17	17
4	64	24	25*

*One cow sample gave false positivity.

Field Tests

Source of blood meals of *A. culicifacies* collected from Nadiad (Gujarat) and Chazarsi and Bhoop Kheri (U.P.) were identified by microdot ELISA. Filter papers spotted with blood meals, stored at 4°C for several months were equally detectable. Two μl of eluate of each blood meal was spotted on duplicate filters and tested with antihuman and anticow conjugate. Results are given in Tables 5 and 6. Table 5 gives the results of microdot assay using rabbit antihuman conjugate and of precipitin tests carried out on the same samples. Among 264 mosquitoes collected from cattlesheds or houses, only 7 had human hosts (microdot ELISA). The higher sensitivity of microdot (2.7%) is evident compared to precipitin test (0.7%).

Table 5. Identification of human blood meal in mosquitoes collected from U.P. by microdot ELISA and precipitin test

Villages	Species	Sample stored for	No. of samples	Microdot ELISA	Precipitin test
Chazarsi	*A. culicifacies*	1 yr.	168	4 (2.4%)	1 (0.7%)
Bhoop Kheri	*A. culicifacies*	7 mths.	96	3 (3.0%)	1 (0.7%)

Table 6. Identification of human blood meal in mosquitoes collected from Nadiad, Gujarat

Species	Nature of dwelling	Blood meals tested (Nos.)	ELISA positive	Microdot ELISA +ve
A. subpictus	Mixed	112	4	4
	Human	82	0	0
	Cattle	96	0	0
A. annularis	Mixed	74	4	4
	Human	11	1	1
	Catttle	23	0	0
A. culicifacies	Mixed	106	6	6
	Human	112	4	4
	Cattle	108	0	0

A comparison of ELISA and microdot ELISA is shown in Table 6. It clearly shows a comparable sensitivity in both the assays. A total number of 654 mosquitoes collected from different dwellings were screened against rabbit antihuman Ig HRPO conjugate, 2.7% were found to be human fed mosquitoes.

DISCUSSION

There has been growing interest in developing a more sensitive and reliable assay for host identification to replace the commonly used precipitin test. Often the blood collected from the field is considerably digested and inadequate for precipitin test. In ELISA, which is more sensitive, both direct and sandwich type of assays have been developed. The latter claims more precision and sensitivity using purified IgG fraction and hence requiring more expense and sophistication. We considered that the sensitivity and precision of ELISA could be retained while disadvantage of cost and sophistication could be eliminated if ELISA principle were translated into a microdot assay on nitrocellulose paper. In microdot, unlike ELISA, the colour intensity does not vary significantly with dilution of blood meal upto a point. This is because antigen is highly concentrated in a very small region, when visual differentiation is not possible. This factor provides a big advantage of sensitivity.

We observed distinct decrease in intensity in case of filter paper eluate, when compared with equal volume of blood. A 10-fold loss of sensitivity was evident. This loss may be due to some proteins irreversibly adsorbed by filter paper. When filter paper absorbed human fed mosquito blood meal and human blood of equal amounts were tested under identical conditions, a loss of sensitivity in case of MBM was observed. At the initial step of MBM elution no cross-reactivity was observed when screened with antihuman conjugate. A conjugate dilution (1:800) was optimal, background interference was reduced to a minimum. However, if the blood amount was inad-

equate even .01 μl to start with, we experienced no difficulty in correct identification. Usually detection time after feeding of mosquitoes is taken as a guide for sensitivity and reliability of blood meal assay. The detection depends upon blood meal size and hence on rate of digestion. Service *et al.* (1986) rightly points out that it is difficult to compare different studies on blood meal assay, since the rate of digestion depends upon various factors such as temperature, type of mosquito, age, type of blood, and even humidity. The temperature, which is the most crucial factor, is often not mentioned. It is known that in tropical conditions, blood meals get completely digested within 24–48 hours, yet we could detect human blood meals in more than 3/4th gravid mosquitoes after 36 hours of digestion. This compared well with sensitive fluorescent antibody test of Mckinney *et al.* (1972), which detected human blood in exquiscine faeces at 44 hours post fed period (temperature not mentioned). Sandwich ELISA assay of Service *et al.* (1986) could detect blood meals in 50% mosquitoes after 44 hours of feeding. So far we have screened 1300 mosquitoes from the field of which only 2% appeared to have human hosts. Only recently a report on DOT-PAP assay of mosquito blood meal (Lombardi and Esposito, 1986) on nitrocellulose paper has appeared. The method uses double antibody technique, and PAP (double complexes of peroxidase-anti-peroxidase) as detector. The method claims greater sensitivity than ELISA and removal of possible cross-reactivity by diluting the second antibody with a mixture of animal serums. However, working with known laboratory fed mosquitoes, they could not detect blood meal after 28 hours of digestion. With this limitation, the method is cumbersome and costlier.

We consider microdot ELISA described here as considerably superior to precipitin and agglutination assays and comparable to ELISA and fluorescence antibody test in precision and sensitivity. Microdot is the method of choice for field use as it is simple, quick and does not require any special equipment or skill. Costwise the method is cheaper than other tests except precipitin. According to our calculation, expense will be less than 30 paise per sample. However, if labour cost is added to this material cost (considering 1000 samples or more can be screened by microdot per day in comparison to 100 samples by precipitin test) the method becomes very competitive with precipitin test.

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Effect of Zinc diet on Xanthine oxidase activity of Liver of Mice infected with *Plasmodium berghei*

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A study has been made of the effect of zinc administration on xanthine oxidase activity and lipid peroxides levels in the stress organs of the host, mouse in *Plasmodium berghei* infection.

The results of this new approach clearly show the ability of zinc to suppress the above two interrelated biochemical parameters and thus protect the stress organs against injury in this infection.

INTRODUCTION

A systematic study of the host biochemistry in *Plasmodium* infection revealed gross injury at molecular level to the host stress organ (Sharma *et al.*, 1978b; Sharma *et al.*, 1979) probably caused by the abnormally high levels of free radicals/free radical generating systems (FRGS) induced by the parasite. One of these FRGS in the present study was found to be xanthine oxidase (Sharma *et al.*, 1978a; Pryor, 1976; Anonymous, 1979) which was coupled with low activity of the counteracting enzyme system of superoxide dismutase (SOD) (Sharma *et al.*, 1979) favouring the stress organ injury. One of the biological approaches to counteract the above injury could be to suppress the formation of xanthine oxidase in the infected host. A survey of literature suggested that this could be made possible by zinc administration to the host (Dennis and Dorothy, 1962). Accordingly a study was devised on these lines which has corroborated the logic of the above premise and has thus shown that a diet containing zinc could significantly mitigate the stress organ injury in *Plasmodium berghei* infection.

MATERIAL AND METHODS

Zinc oxide (BDH) was mixed with pulverised standard animal house pellet diet at a concentration of 0.02, 0.05 and 0.1% levels. Pilot experiments were carried out with each of the above diets employing 10 animals in each case for a period of 15 days. The diet with 0.02% zinc oxide incorporated into it was found to be well tolerated as there were no mortalities and it was used in our experimental studies.

Four week old albino mice, each weighing 18–20 gm and subdivided into four groups were used in the present study. The groups of animals, one on zinc diet and the other on normal animal house diet without zinc oxide, were challenged

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intraperitoneally with 10^5 RBC parasitised with *Plasmodium berghei* each. The remaining two groups served as uninfected controls; one of which was fed the zinc oxide diet.

The course of parasitaemia was followed by examining the blood smears on alternate days. The feeding of experimental diet coincided with the challenge. Three animals from each group were sacrificed on the seventh day when parasitaemia was 3–6% in infected control group. On the corresponding day parasitaemia was 2–4% in infected + zinc treated group. On fourteenth day parasitaemia was 10–22% in control group on normal diet and 8–18% in zinc treated group. (The parasitaemia had reached a level of 15–25% within 18–20 days in the untreated infected group).

After sacrificing the animals, liver was collected, immediately washed with normal saline, weighed, and kept in deep freeze till further use. A 10% homogenate of the frozen tissue, using teflon homogenizer, was prepared in cold potassium phosphate buffer 0.039M (pH 7.4) for the determination of xanthine oxidase (Litwack *et al.*, 1953), lipid peroxide (Utley *et al.*, 1967), ferric iron (Fortune and Mellon, 1938) and proteins (Lowry *et al.*, 1951).

RESULTS

It may be seen from the fourteen days experimental data (Figs. 1–4 from the data of Table 1) that in uninfected animals zinc treatment caused a fall of 25.26% (NS) in the xanthine oxidase activity, while there was a perceptible decline of 14.67% (NS) in iron concentration in liver. However, the lipid peroxides levels were found to be somewhat lower than the normal ones by 7.24% in this treatment.

Plasmodium berghei infection caused a highly significant increase in the iron and lipid peroxides concentrations and xanthine oxidase activity, each by 58.76%, 39.48% and 81.40% respectively (P being <0.01 in each case).

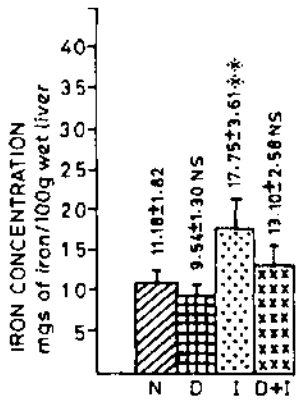

Fig. 1: Influence of zinc oxide on iron concentration of host (mouse) liver in *Plasmodium berghei* infection:
N —Normal group
D - Drug treated group
I —Infected group
D + I —Drug treated and infected group
Scale —2 divs. = 1 mg iron present 14 days experimental data of Table 1.

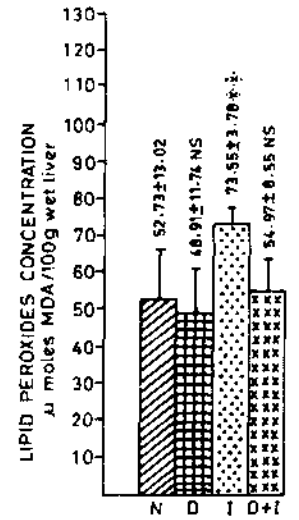

Fig. 2: Influence of zinc oxide on lipid peroxide concentration of host (mouse) liver in *Plasmodium berghei* infection:
N —Normal group
D —Drug treated group
I —Infected group
D + I —Drug treated and infected group
Scale —1 div. = 1 μ mole MDA formed 14 days experimental data of Table 1.

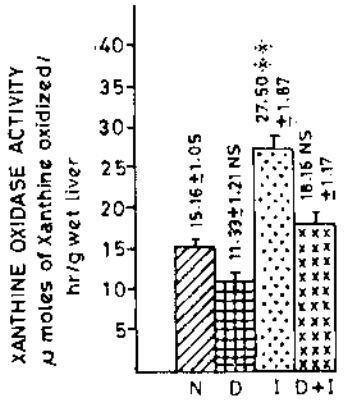

Fig. 3: Influence of zinc oxide on xanthine oxidase activity of host (mouse) liver in *Plasmodium berghei* infection:
N—Normal group
D—Drug treated group
I—Infected group
D+I—Drug treated and infected group
Scale—2 divs. = 1μ mole xanthine oxidase 14 days experimental data of Table 1.

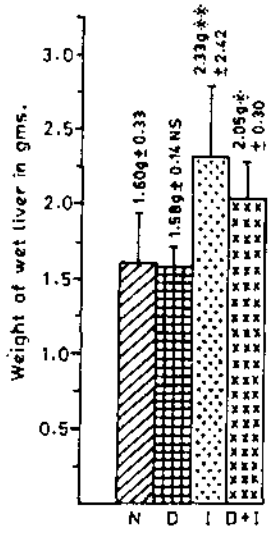

Fig. 4: Influence of zinc oxide on wet weights of host (mouse) liver in *Plasmodium berghei* infection:
N—Normal group
D—Drug treated group
I—Infected group
D+I—Drug treated and infected group
Scale—1 div. = 25 mg wet liver 14 days experimental data of Table 1.

NS implies that the difference is not significant statistical ($P > 0.05$).
* implies that the difference is statistically significant ($P < 0.05$).
** implies that the difference is statistically highly significant ($P < 0.01$).

The administration of zinc in the diet to the animals infected with *Plasmodium berghei* exerted a marked lowering (modulating) effect on the three biochemical parameters. The iron concentration approached the corresponding normal value, that is, from 158.76 to 117.17 (NS), while each of lipid peroxides and xanthine oxidase values exhibited a remarkable fall on zinc administration in case of the infected animals, the corresponding decline being 139.48% to 104.25% (NS) and 181.40% to 119.97% (NS) with respect to normal. Therefore, it may be seen that malarial infection leads to an increase in the xanthine oxidase activity in liver, as a result of which lipid peroxides levels exhibit an elevation in their levels, suggestive of a damage to the stress organs, the two effects being mitigated by zinc administration.

The profile of the biochemical/physical parameters viz., iron, lipid peroxides, xanthine oxidase and tissue wet weight of the stress organ, liver, after a period of seven days infection was practically similar to the fourteen days profile as shown in Table 1. It may also be noted that tl zinc containing dietary regime practically d: not influence the course of malarial infectic under the present experimental conditions.

DISCUSSION

If raised levels of lipid peroxides in the stre: organs of the host in *Plasmodium berghei* i fection are an index of biochemical injur (Sharma *et al.*, 1979) the present objective of m tigating stress organ injury obviously has bee greatly realized in the sense that, as expecte zinc in the diet (Dennis and Dorothy, 1962) coul modulate the disturbing action of infection o

Table 1. Effect of zinc on certain biochemical parameters of host (mouse) in *Plasmodium berghei* infection

Average of 7 days old observation for six animals processed for each parameter in each group				Average of 14 days old observations for six animals processed for each parameter in each group			
Uninfected untreated grp. (normal)	Uninfected Zn treated group	Infected untreated group	Infected Zn treated group	Uninfected untreated group	Uninfected Zn treated group	Infected untreated group	Infected Zn treated group
Iron concentration, Fe^{+++} mgs/100 gms of wet liver							
12.33 ± 2.46	9.51 ± 2.10 NS	16.35 ± 3.48 **	12.13 ± 1.75 NS	11.18 ± 1.82	9.54 ± 1.30 NS	17.75 ± 3.61 **	13.10 ± 2.58 NS
(100)	(77.13)	(132.60)	(98.36)	(100)	(85.33)	(158.76)	(117.17)
Lipid peroxide concentration, μ moles MDA formed/100 gms of wet liver							
50.06 ± 15.06	48.17 ± 13.69 NS	72.21 ± 11.81 **	55.34 ± 12.33 NS	52.73 ± 13.02	48.91 ± 11.74 NS	73.55 ± 3.78 **	54.97 ± 8.55 NS
(100)	(96.22)	(144.25)	(110.55)	(100)	(92.76)	(139.48)	(104.25)
Xanthine oxidase activity, μ moles of xanthine oxidized to uric acid/hr/gm. of wet liver							
11.00 ± 1.41	8.33 ± 1.03 NS	22.16 ± 1.47 **	14.83 ± 1.17 NS	15.16 ± 1.05	11.33 ± 1.21 NS	27.50 ± 1.87 **	18.16 ± 1.17 NS
(100)	(75.73)	(201.45)	(135.00)	(100)	(74.74)	(181.40)	(119.79)
Average activity of xanthine oxidase per hr./gm wet liver (Total contents of Xanthine oxidase μ moles/wet liver)† in six livers × average wt. of six wet livers in each group							
1720	12.10 NS	40.35 **	23.09 NS	24.34	17.97 NS	64.02 **	37.33 NS
(100)	(70.35)	(234.59)	(134.24)	(100)	(73.83)	(262.98)	(153.37)
Weight of wet liver in gms.							
1.563 ± 0.248	1.452 ± 0.129 NS	1.821 ± 0.097 **	1.557 ± 0.321 *	1.606 ± 0.330	1.586 ± 0.144 NS	2.328 ± 2.428 **	2.056 ± 0.305 *

The values in parentheses are % with respect to normal; NS $P > 0.05$ statistically not significant; *$P < 0.05$ statistically significant; **$P < 0.01$ statistically highly significant; †Mean value of x.o activity x mean value of wet liver weight.

host biochemistry as monitored by the three biochemical parameters of lipid peroxides; xanthine oxidase and iron concentration/content in the liver.

Thus, the new approach of alleviating injury to the stress organ, liver, in malaria has proved that the premise of counteracting stress organ damage is valid. Injury is alleviated by neutralizing the presently studied causative factor(s) of such damage (namely, the free radical generating system of xanthine oxidase) (Sharma *et al.*, 1978a) through zinc administration to the host animal, this being indexed by a fall in the lipid peroxide and xanthine oxidase levels in the liver. This would undoubtedly suggest that the highly reactive and therefore undesirable free radicals (Pryor, 1976; Anonymous, 1979; Chance, 1979) induced/engendered in malarial infection through various biochemical processes directly or indirectly by the parasite, one of which is undoubtedly xanthine oxidase, can be modulated probably to the advantage of the host.

The success of this approach would encourage one to suggest that other supplementary approaches may be devised to counteract the damage caused to the stress organ in malarial infection by neutralizing the harmful causative factors/or their effects.

As a plausible explanation of the wholesome effect of zinc on the stress organ injury in *Plasmodium* infection, it may be stated that zinc in the diet may be suppressing the formation/functioning of the free radical generating system (FRGS) (Dennis and Dorothy, 1962), one of which, namely xanthine oxidase, is the topic of the present study. This in turn would imply the formation of lower amounts of the highly reactive and therefore the dangerous free radicals under the influence of this infection, manifested as the protective effect of zinc to the stress organs.

In conclusion, the present study corroborates the premise that the host stress organ injury in *Plasmodium* infection is being partly caused by FRGS—xanthine oxidase, because a counteraction of the latter has been observed to lead to the mitigation of the injurious effect of infection on the host stress organs.

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Detection of *Plasmodium vivax* in Human Blood using Synthetic DNA Probe

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Oligonucleotides representing repetitive sequences in the genes coding for CS proteins of *P. falciparum* and *P. vivax* have been synthesised, radiolabelled and tested as probe in DNA-DNA hybridization assay on nitrocellulose filters. These show desirable specificity and the vivax probe was used in a simple dot-blot assay for detecting parasites in patients' blood.

INTRODUCTION

Malaria is detected by microscopic examination of stained blood smears. The technique is slow and to cope with the workload, technicians often resort to reducing the time of examination. This compounded by monotony and strain to the eyes leads to inaccuracies. In a recent study (Choudhury *et al.*, 1987), slides of blood smears collected from 180 PHC laboratories were re-examined at a central laboratory (MRC). Results showed that PHC technicians missed a large number of positive smears. Out of 491 Pf and 234 Pv infections the PHCs detected only 106 Pf and 88 Pv cases. Missed cases could be potential sources of transmission and may lead to serious or protracted illness. In endemic areas, during peak transmission season the backlog of slide examination is at times 2/3 months or more. DNA probe based on a published repetitive sequence of *P. falciparum* (McLaughlin *et al.*, 1985) has been developed. So far, no such probe for *P. vivax* has been reported. Only recently, the gene coding for CS protein of *P. vivax* (Pv) has been cloned and shown to include code for 19 tandem repeats of a nonapeptide (Arnot *et al.*, 1985). We have synthesized the published oligonucleotide sequence corresponding to the nonapeptide and tested its ability to detect vivax parasite in patients' blood. For comparison of specificity and cross-reactivity, we synthesized the reported 21 mer oligonucleotide probe for *P. falciparum* (Pf) also.

In this preliminary report, we show that the published vivax repetitive sequence is specific and can be used in hybridization assay.

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MATERIAL AND METHODS

P. falciparum (FAN strain) maintained in culture by Trager and Jensen (1976) method at Malaria Research Centre, Delhi was used at 8% parasitaemia level. Slide positive patients blood for *P.*

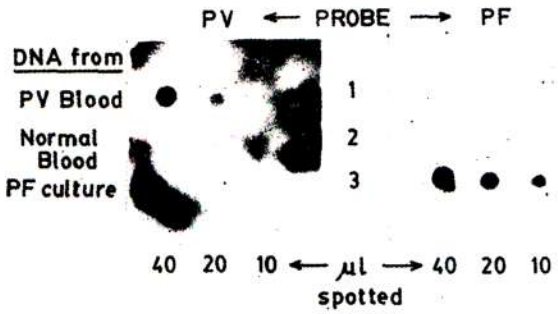

Fig. 1: Autoradiography of DNA extracted from blood samples as described in text, spotted on duplicate nitro cellulose filters and hybridized with ^{32}P labelled synthetic DNA probe Row 1: Pv extract; Row 2: Human leucocyte DNA; Row 3: Pf culture extract.

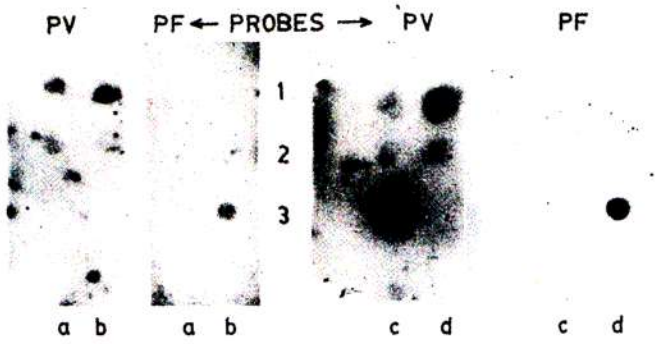

Fig. 2: 5 µl blood samples spotted on duplicate filters, hybridized *in situ*, with ^{32}P-labelled synthetic DNA probe (Pf & Pv) Row 1,2: Vivax infected patient's blood. Samples in a,b are same as in c,d; Row 3: b,d; *P. falciparum* culture blood, a-old, and lysed blood sample, c-patient blood with 2% parasitaemia.

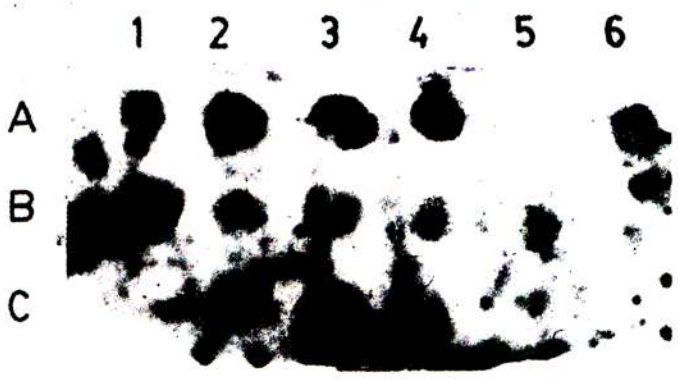

Fig. 3: Spot hybridization of human blood sample (10 µl) with radiolabelled synthetic DNA Pv probe. Rows A,B and C (columns 1-3): Patient's blood with 0.3 to 0.5% parasitaemia: Row C: Column 4, 2% parasitaemia patients blood: 5, Pf culture; 6, normal blood.

vivax was collected from endemic areas around Delhi. The parasitaemia ranged from 0.3 to 0.5% except one sample which had 2% parasitaemia.

Using a Pharmacia Gene assembler based on phosphoramidite chemistry we synthesized the 27 nucleotide long sequence, 5' dGACAGAGCAGATGGACAGCCAGCAGG A, of *P. vivax* (Arnot *et al.*, 1985) and the 21 mer, 5' dAGGTCTTAACTTGACTAACAT, of *P. falciparum* (Franzen *et al.*, 1984; McLaughlin *et al.*, 1985). The deprotected oligomers were purified on a Pharmacia FPLC system, first on a pro-RPC column with the terminal DMT group remaining intact, then on a MONO Q anion exchanger after removal of the DMT group. Using 0.1 μg of the oligomer, the 5' ends were labelled with 32p using 32p-ATP (AMERSHAM) and T4-polynucleotide kinase (BRL). Labelled probes were precipitated at -20°C overnight in 70% ethanol and 0.3 M Sodium acetate and washed with 70% ethanol twice. Incorporation of the label was checked by spotting on DE81 paper (Maniatis *et al.*, 1982), which were 1.8×10^8 cpm/μg for Pf probe and 2.5×10^7 cpm/μg for Pv probe.

Application of blood samples (5 μl) on nitrocellulose filters and the treatments prior to hybridization were performed as described by Pollack *et al.* (1985). The baked filters were first prehybridized for 3 hours in a plastic bag at 42°C and then hybridized with radiolabelled probe for 12–16 hours. The prehybridization mix contained 5x Denhardt's solution, 5x SSC and 2x SSPE (Maniatis *et al.*, 1982) with 100 μg/ml of denatured salmon sperm DNA. The prehybridization solution was mixed with 50% Dextran sulphate containing 0.5% SDS in a ratio of 4:1 and 5×10^5 counts of the probe to form hybridization solution. After hybridization, filters were extensively washed successively with 2x SSC, 0.5% SDS, and 0.1 SSC, 0.1% SDS. Washed, air dried filters were covered in Saran Wrap and exposed to INDU X-Ray film at -70°C, with enhancer screen. Exposure varied from 1–3 days depending on the counts on the filters.

RESULTS

Pv infected patients blood, normal blood and falciparum culture filtrate were treated essentially as described by Franzen *et al.* (1984). Washed blood cell pellets (Pv 100 μl, Pf 50 μl) were incubated for 1 hr with proteinase K (100 μg/ml) and equal time with RNAse A (50 μg/ml) in KI buffer (Pollack *et al.*, 1982) containing 0.2% SDS at 37°C. Solution was diluted to 1 ml and extracted with phenol twice and once with chloroform-isoamylalcohol (4:1). The solution was left overnight in 70% ethanol and 0.3 M Sodium acetate at -20°C. After centrifugation, the pellet was washed with ethanol and resuspended in 200 μl of 10 mM NaOH. Aliquots of 40, 20 and 10 μl were spotted on duplicate nitrocellulose filters and hybridized with Pv and Pf probes. The results shown in Fig. 1 demonstrate clearly the species specificity of these probes; none of them hybridized with the normal human DNA, and Pf probe hybridized with the culture extract only. The 21 mer Pf probe has been reported to detect 0.1 ng of falciparum DNA in overnight exposure McLaughlin *et al.*, 1985). The vivax probe here detects about 10 ng of DNA. This, of course, does not imply the sensitivity limit of the probe as sensitivity depends upon the efficiency of labelling, specific activity of the probe, efficiency of hybridization experiments, copy number of repetitive sequence and exposure time.

When hybridization was done with untreated blood, spotted directly on nitrocellulose filter, Pv infected patients blood hybridized only with Pv probe without cross hybridization with Pf culture or normal blood. A lysed Pv blood sample, which was stored in the freezer for months and underwent repetitive freezing and thawing, failed to hybridize with Pv probe (Figs. 2 a,b). Another set of duplicate filters, where the old sample was replaced by a sample of high parasitaemia (2%)

gave a very strong signal with Pv probe but not with Pf probe (Figs. 2 c,d). Normal blood used as negative control did not hybridize.

Fig. 3 shows autoradiogram of vivax infected patients blood samples (10 μl) hybridized with Pv probe. All slide positive samples (0.3 to 0.5% parasitaemia) could be detected easily. Two missing spots (a 5 and c 1) are due to the fact that during lysis these blood samples floated away as intact blood clots without leaving adequate smear for *in situ* hybridization. Small sample amount and immediate lysis can prevent this.

DISCUSSION

The results show that the synthetic DNA probes based on repetitive sequences can specifically discriminate between *P. falciparum* and *P. vivax*. Mclaughlin *et al.* (1985) reported that falciparum DNA cross hybridizes with vivax and babesia DNA; and vivax genomic probe, possibly due to host DNA contamination, cross hybridized with as little as 0.1 ng human DNA. Genomic probes in their large size contain nonunique additional sequences of parasites and plasmids, which may cross hybridize with host or other DNA. In analysis of clinical samples absolute specificity is more important than sensitivity. Synthetic DNA probes appear to be superior in this regard (McLaughlin *et al.*, 1985) as it would carry the desired and defined sequence only.

The end labelled synthetic DNA probes have been shown to be, understandably, 5 times less sensitive than the nick translated genomic probes (McLaughlin *et al.*, 1985). Nevertheless, sensitivity can be improved by more efficient hybridization assay using large amount of probe, or more exposure time. Genomic clones are difficult to prepare in large quantities. Also, specific repetitive clones may not be stable and may bear the risk of spontaneous deletions or other mutations. Synthetic DNA, on the other hand, can be made easily in large amounts and will always have the desired sequence and advantage of low background.

The importance of correct diagnosis for administration of radical treatment and interruption of transmission cannot be overemphasised. Where rapid screening of a large number of samples is necessary DNA probes will provide desirable speed, accuracy and sensitivity. However, labelling with radioisotopes is a serious limitation for field use. Alternative approaches, labelling with fluorescent or enzyme markers, are yet to be developed and assessed for their sensitivity. Efforts in this direction have started in various laboratories, including ours. When such probes are fully developed, one technician should be able to reliably screen at least 1000 samples per day. Hybridization assay would need only simple equipment like a water bath shaker and vacuum oven and with falling prices of synthetic oligonucleotides made on automated synthesisers the method may ultimately become cost effective.

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SHORT NOTES

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Studies on the Role of Indigenous Fishes in the Control of Mosquito Breeding

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Rural malaria control is based primarily on the spraying of residual insecticides to interrupt transmission and treatment of malaria cases to eliminate the parasite reservoir. The bio-environmental strategy of malaria control being employed in Nadiad lays primary emphasis on control of mosquito breeding combined with intensive fever surveillance. Among biological control agents, fishes occupy the foremost position due to their effectiveness in the control of mosquito breeding, and the ease with which they can be mass produced, transported and released in a variety of habitats.

Fish fauna survey in 20 experimental villages of Nadiad taluka revealed the presence of 27 types of fishes. The fishes were identified by the keys of Hora and Mukerji (1937) and Jhingran (1975). These fishes were found in temporary pools, wells, a variety of ponds, seepage water, canal and river etc. Eight fishes were distributed widely in many water bodies. Two species of top minow (*Aplocheilus lineatus* and *Aplocheilus panchax)* and guppies (*Poecilia reticulata)* were most commonly encountered. In semi-permanent water bodies 3 additional types of fishes were found. Permanent water bodies had the richest fauna i.e., 24 species of fishes, whereas the canal and river yielded 19 and 16 types of fishes respectively (Table 1). Though the fishes of India have been studied quite extensively (Prasad and Hora, 1936) and there are several accounts of their distribution, habitat and feeding habits e.g., freshwater fishes of Bombay and Karnataka (Gideon *et al.*, 1937) this is the first account of fish fauna of Kheda district. Of particular interest was the wide distribution of *Aplocheilus lineatus, A. panchax* and *Poecilia reticulata* which have been well documented as excellent larvivorous fishes and of these *A. lineatus* was reported to be the fish most suitable for the control of mosquito breeding in a variety of habitats (Hora and Nair, 1938; John, 1940).

During 1985 hatcheries established in the village ponds in 20 villages produced enough guppies for 100 villages. Our field experience shows that production of guppies is easy to accomplish. Although top minnow fishes can also be produced and transported with equal ease, these fishes are heavily preyed upon by a variety of birds. This decimates their population in natural habitats.

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Table 1. Fishes of study villages

Species	Temporary	Semi-permanent	Permanent	Canal	River
Aplocheilus lineatus	+	+	+	+	+
Aplocheilus panchax	+	+	+	+	+
Ambassis nama	+		+	+	+
Chela bacaila	+		+	+	+
Catla catla	+	+	+	+	+
Cirrhina latia	+	+	+	+	+
Cirrhinus mirgala	+	+	+	+	+
Cirrhinus reba	+	+	+	+	+
Channa punctatus	+	+	+	+	+
Channa sp.	+	+	+	+	+
Glossogobius sp.	+	+	+	+	+
Heteropneustes fossilis	+	+	+	+	+
Labeo rohita	+	+	+	+	+
Mastocembelus armatus	+	+	+	+	+
Mastocembelus pancalus	+	+	+	+	+
Mystus punctatus	+	+	+	+	+
Mystus seenghala	+	+	+	+	+
Notopterus notopterus	+	+	+	+	+
Ompok bimaculatus	+	+	+	+	+
Poecilia reticulata	+	+	+	+	+
Puntius sp.	+	+	+	+	+
Puntius stigma	+	+	+	+	+
Puntius ticto	+	+	+	+	+
Rasbora daniconius	+	+	+	+	+
Trichogaster fasciata	+	+	+	+	+
Wallago attu			+		
Xenentodon cancila				+	+
Total	8	11	24	19	16

Laboratory tests were conducted in plastic containers (6 litres) containing 5 fishes each (except *Notopterus notopterus* [1 fish] and *Mastocembelus pancalus* and *Cyprinus carpio* [4 fishes each]). Four hundred IV instar anopheline larvae were introduced in the containers on the first day, those that survived 24 hours later were counted and the number made upto 400 again. The experiment lasted four days.

Tests on larvivoracity revealed (Table 2) that two fishes viz., *Mastocembelus pancalus* and *Xenentodon cancila* did not consume any larvae *Poecilia reticulata* fed on an average 40.5 larvae per day while all other fishes consumed 60 to 80 larvae per day. *Mystus seenghala* consumed 132 larvae per day. *Notopterus notopterus* consumed 371 larvae per day which was the highest number recorded among the 16 species tested.

For outdoor experiments cement tanks (80 × 80 × 75 cm) were filled three fourths with water. Eight types of fishes were tested on *Culex* and *Anopheles* larvae. Mosquito larvae were introduced in the tanks and their density was estimated (average of 5 dips) daily for 6 days.

Results of outdoor experiments are given in Table 3. The study revealed that feeding capacity of fishes was reduced in presence of

Table 2. Laboratory tests for larvivoracity

Fishes	Size in cms.	Fishes (Nos.)	Number of mosquito larvae consumed					
			Day 1	Day 2	Day 3	Day 4	Total	Average*
Aplocheilus lineatus	2.5–3.0	5	400	400	334	400	1534	76.6
Aplocheilus panchax	2.5–3.0	5	396	260	175	354	1185	59.2
Chela bacaila	3.0–4.0	5	400	400	400	400	1600	80.0
*Cyprinus carpio***	2.0–5.0	4	400	400	400	400	1600	80.0
Channa punctatus	6.0–10.0	5	399	400	400	400	1599	80.0
Cirrhinus reba	3.0–4.0	5	391	382	400	400	1573	78.6
Glossogobius sp.	3.0–3.5	5	260	327	191	400	1178	58.8
Mastacembelus pancalus	5.0–5.0	4	0	0	0	0	0	0
Mystus seenghala	4.0–7.0	3	386	396	400	400	1582	132.0
Notopterus notopterus	15.0	1	360	367	393	365	1485	371.0
Poecilia reticulata	2.5–3.0	5	350	100	130	330	810	40.5
Puntius sp.	3.0–3.5	5	320	363	388	400	1471	73.6
Puntius ticto	4.0–5.0	5	400	400	400	400	1600	80.0
Rasbora daniconius	3.0–4.0	5	400	400	400	400	1600	80.0
Trichogaster fasciata	4.0–6.0	5	400	400	400	400	1600	80.0
Xenentodon cancila	21.0–23.0	5	0	0	0	0	0	0

* Average larvae consumed/day/fish

** Chinese or common carp

aquatic vegetation, although the margins were clean and the fishes were able to reach all corners of the tank. The larval density was reduced to very low numbers in a 5 to 6 day period. *Mystus seenghala* consumed the larvae very quickly followed by *Puntius ticto*. The known larvivorous fishes *Aplocheilus* and *Poecilia* also reduced the mosquito densities in ponds to low levels but these fishes were found inferior to *Mystus* and *Puntius*. In the absence of vegetation, the preference was more pronounced for *Anopheles* than *Culex* larvae. This may be due to better visibility of *Anopheles* larvae which float on the water surface. In the absence of vegetation, the rate of consumption was high and almost all the larvae were eaten away in four days (Table 3). Though *Gambusia* has been credited for reducing malaria transmission in the Ukraine (Gerberich and Laird, 1968), WHO (1982) and Hulbert *et al.* (1972) disfavour it because of its adverse ecological impact. Fisheries departments in India also discourage the use of *Gambusia*. It was not found in rural areas of Kheda district, although it is used in the urban malaria scheme in Gujarat. *Gambusia* was not studied in the present experiments.

Field experiments were carried out to study the impact of cleaning the margins of ponds. Larval density was ascertained using a dipper 24 hours before and after cleaning the margins and results were compared against ponds with unclean margins.

Results of field studies revealed that there was considerable reduction in the larval density within 24 hours i.e., average reduction in density was 52%, 39% and 28% in January, February and September respectively (Table 4). The importance of cleaning the margins was therefore clearly demonstrated and incorporated as an important measure in the biological control of mosquito breeding using fishes (Sharma and Sharma, 1986).

Kheda district has a large number of ponds. Fish fauna of these ponds is rich but these ponds also support moderate to heavy mosquito breeding. The ponds were never cleaned or properly maintained by the village Panchayats. Although fishes were being collected from these ponds and occasionally auctioned by the Panchayats the production was negligible. The ponds were

Table 3. Larvivoracity test of fishes in cement tanks

Species	Size in cms.	Fishes (Nos.)	Mosquito Species	Larval density per dip						
				Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
(with aquatic vegetation)										
Aplocheilus panchax	2.5-3.0	5	An.	35.0	32.5	28.2	23.2	19.2	14.4	9.2
			Cx.	10.8	4.4	2.2	2.0	1.6	1.2	1.0
Channa punctatus	6.0-10.0	5	An.	38.2	17.6	15.2	9.0	7.6	5.2	3.6
			Cx.	13.0	3.2	2.2	1.8	1.2	1.0	1.0
Cirrhinus reba	3.0-4.0	5	An.	28.0	27.4	20.4	15.2	5.6	3.6	2.0
			Cx.	25.0	23.6	19.8	12.6	5.4	1.4	1.0
Heteropneustes fossilis	7.0-12.0	5	An.	26.2	12.4	4.0	1.6	1.2	0.4	0
			Cx.	1.0	1.0	1.0	0.6	0.4	0	
Mystus seenghala	4.0-7.0	2	An.	47.2	35.4	28.0	13.2	5.0		
			Cx.	5.6	4.2	1.0	0.4	0		
Poecilia reticulata	2.5-3.0	5	An.	33.8	32.0	30.4	22.6	13.4	10.6	5.4
			Cx.	7.6	4.0	2.2	2.2	1.2	1.0	0.2
Puntius ticto	4.0-5.0	5	An.	35.8	8.2	5.0	2.2	1.0	0	
			Cx.	8.4	5.2	1.0	0.6	0		
Trichogaster fasciata	4.0-6.0	5	An.	31.8	12.8	10.6	5.2	4.2	3.0	2.2
			Cx.	7.2	1.8	1.6	1.0	1.0	1.0	1.0
Control			An.	33.3	31.2	30.6	28.2	26.0	24.4	21.2
			Cx.	23.2	19.2	18.0	15.4	14.2	12.0	10.2
(without aquatic vegetation)										
Aplocheilus panchax	2.5-3.0	5	An.	54.0	28.4	19.2	7.2	2.4		
			Cx.	67.8	62.6	53.2	50.6	18.2		
Channa punctatus	6.0-10.0	5	An.	28.4	5.6	2.0	0			
			Cx.	53.6	33.2	3.4	0			
Cirrhinus reba	3.0-4.0	5	An.	43.6	20.2	0.4	0			
			Cx.	60.4	52.0	32.2	29.0	9.4		
Heteropneustes fossilis	7.0-12.0	5	An.	74.8	2.6	0				
			Cx.	48.2	1.0	0				
Mystus seenghala	4.0-7.0	2	An.	51.8	16.4	3.0	0.8	0		
			Cx.	51.8	36.0	22.4	19.6	6.6		
Poecilia reticulata	2.5-3.0	5	An.	54.2	36.0	21.4	5.6	1.6		
			Cx.	48.6	43.5	40.6	38.2	8.0		
Puntius ticto	4.0-5.0	5	An	39.2	12.4	0				
			Cx.	43.0	35.2	4.6	2.2	0		
Trichogaster fasciata	4.0-6.0	5	An.	63.2	8.2	2.2	1.2	0		
			Cx.	66.6	45.4	15.0	0			
Control			An.	52.4	50.2	47.6	46.0	44.8		
			Cx.	66.4	64.8	63.2	61.4	47.0		

cleaned and guppies were introduced to control mosquito breeding. A scheme for the composite culture of food fishes and prawns along with larvivorous fishes was implemented in 8 village ponds. The ponds were thoroughly cleaned of all types of fishes. Fries, fingerlings and juvenile prawns purchased from the inland fisheries department were then introduced and fish food was occasionally provided for optimum growth. At the end of one year the ponds were auctioned.

Table 4. Impact of cleaning the margins of ponds on the larval density of mosquitoes

Area (1985)	No. of sites examined	No. positive	Results based on dips (Nos.)	Average larval density of positive sites									
				Before cleaning margins					24 hrs. after cleaning margins				
				I	II	III	IV	Total	I	II	III	IV	Total
A. Experimental													
January	48	28	133	2.36	0.79	0.23	0.06	3.43	1.18	0.42	0.10	0.09	1.79
February	37	17	85	1.34	0.29	0.18	0	1.81	0.44	0.27	0	0	0.71
September	66	41	213	9.76	3.58	1.15	0.40	14.89	2.87	0.82	0.43	0.12	4.24
B. Control													
January	23	22	117	3.35	4.11	3.27	1.90	12.63					
February	14	13	65	6.9	17.63	5.84	2.94	33.32	No change was observed in larval				
September	20	20	83	43.0	32.8	23.21	7.49	106.50	density				

The production of edible fishes with indigenous larvivorous fishes has motivated the village communities and for the first time composite culture is being practised on a large-scale to control mosquito breeding and improve the village economy. Eight village ponds were selected for food fish culture out of which four ponds had to be abandoned due to drought conditions and large-scale poaching. Remaining four ponds yielded about 10 tons of food fish worth approximately Rs. 1 lakh.

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Malaria Prevalence in Patients attending Primary Health Centres in ten districts of Uttar Pradesh

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A study to monitor the seroepidemiological profile of 10 districts in UP was launched in the non-transmission season during November and December 1983, November and December 1984 and January 1985. The study was carried out in Almora, Baharaich, Basti, Deoria, Gonda, Gorakhpur, Kheri, Nainital, Pilibhit and Pithoragarh districts (Fig.1). Blood samples were collected from all the PHCs of the above 10 districts on the basis of first come first served, regardless of the illness or fever. Finger prick method was used to collect blood on Whatman filter paper No. 3 strips for seroepidemiological surveys. From the same person a thick and a thin blood smear was also prepared for examination of malaria parasite. A total of 50 blood samples were collected from each PHC during November—December 1983 and 150 during November—December 1984 and January 1985. The slides were fixed and stained with JSB and examined by the PHC technicians. These slides along with their results were transferred to the Malaria Research Centre (MRC) for cross-checking. While results of seroepidemiological surveys are being published in another paper, results of the parasitological surveys and cross-checking are reported here.

Results of blood smear examination by MRC technicians are given in Table 1. A large number of slides were poorly stained or unsuitable for examination. The study revealed extremely high incidence of malaria since the slides were randomly collected from all types of patients reporting to the PHCs during the non-transmission season. The western districts consisting of Nainital, Kheri and Pilibhit had high malaria endemicity as compared to eastern districts viz., Deoria, Gorakhpur, Basti, Gonda and Baharaich. The remaining two districts viz., Pithoragarh and Almora also had relatively less malaria. It was also notable that mixed infections were very few i.e., only 4 out of 453 positive cases.

Results of blood smear examinations at the PHCs were compared with the MRC results (Table 2). There was a high rate of discrepancy in the results. The PHC technicians missed a large number of positive blood smears. There were 725 positive blood smears but the PHCs recorded

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Table 1. Results of blood smear examination

S. No.	District	Total PHC	B.S. Collected: Late Nov-Dec. 1983						B.S. Collected: Nov-Dec.1984—Jan 1985					
			PHCs surveyed	B.S. examined	Total positive	Total Pf	SPR	SFR	PHCs surveyed	B.S. examined	Total positive	Total Pf	SPR	SFR
1.	Almora	17	10	460	18	11	3.9	2.39	4	448	6	2	1.3	0.44
2.	Baharaich	19	19	938	33	23	3.5	2.45	18	2124	23	18	1.1	0.84
3.	Basti	32	20	938	44	16*	4.6	1.69	26	3076	182	75	5.9	2.43
4.	Deoria	32	29	1436	39	26	2.7	1.81	21	2546	40	22	1.57	0.43
5.	Gonda	25	17	774	21	13	2.7	1.67	19	3063	29	17	0.94	0.55
6.	Gorakhpur	31	28	1380	45	21*	3.2	1.52	24	1653	15	10	0.9	0.60
7.	Kheri	15	13	623	57	45	9.1	7.22	12	1397	81	69	5.7	4.93
8.	Nainital	14	10	492	112	89*	22.7	18.08	7	1153	225	117*	19.5	15.35
9.	Pilibhit	7	7	348	77	63*	22.1	18.10	5	632	84	67	13.3	10.60
10.	Pithoragarh	11	7	343	7	3	2.0	0.87	10	1394	8	4	0.57	0.28
	Total	203	160	7740	453	310	5.8	3.95	126	17486	693	460	4.1	2.63

*One case was positive for mixed (Pv & Pf) infection.

Fig. 1: Map of Uttar Pradesh showing districts (solid circles) from where the blood smears were collected.

194, missing 521 positive slides (71.9%). Among the positive blood smears there were 491 Pf and 234 Pv but the PHC found 106 Pf and 88 Pv thereby missing out 78.4% Pf and 62.4% Pv infections. No mixed infection was found by the PHC technicians. There was, therefore, very

Table 2. A comparison of results of slides examined at the MRC (A) and PHCs (B)

District	B.S. examined	Total Positives		Total Pf		SPR		SFR	
		A	B	A	B	A	B	A	B
1. Almora	270	11	4	4	1	4.07	1.48	1.48	0.37
2. Baharaich	2591	47	18	34	21	1.81	0.69	1.31	0.46
3. Basti	1010	88	3	14	0	8.71	0.29	1.38	0.00
4. Deoria	2603	55	14	34	7	2.11	0.53	1.30	0.26
5. Gorakhpur	1336	32	3	20*	0	2.39	0.22	1.49	0.00
6. Gonda	564	11	0	6	0	2.01	0.00	1.06	0.00
7. Kheri	1328	116	58	93	36	8.73	4.36	7.00	2.71
8. Nainital	996	218	35	171*	24	22.88	3.51	17.16	2.40
9. Pilibhit	810	132	48	108*	23	16.29	5.92	13.33	2.83
10. Pithoragarh	1737	15	11	7	3	0.86	0.63	0.40	0.17
Total	13245	725	194	419	106	5.47	1.46	3.70	0.80

*One case was positive for mixed (Pv + Pf) infection

Note : B.S. collected from many PHCs were not checked by the PHC technicians and therefore the number of slides available for comparison were less than the slides examined at the MRC.

poor blood smear examination at the PHCs. It may be noted that as per the antimalaria drug policy of the NMEP, all cases of fever are given presumptive treatment with 600 mg chloroquine. The presumptive treatment generally clears the parasitaemia in vivax malaria patients. Chloroquine is also gametocytocidal for *P. vivax* and therefore its administration would also interrupt further transmission of the disease. Relapse cases would however continue to occur because *P. vivax* cases missed due to poor identification would not receive radical treatment. In contrast missed cases of falciparum malaria may become serious and the course of treatment in these cases may be completely misdirected resulting in prolonged illness and complications. In falciparum malaria, presumptive treatment is not sufficient to clear the parasitaemia and chloroquine is not gametocytocidal. Therefore, transmission would continue uninterrupted. The importance of correct diagnosis and administration of complete radical treatment is of paramount importance in the treatment and interruption of malaria transmission. It is however noteworthy to mention that afebrile or asymptomatic malaria cases would not receive antimalarials and these cases would constitute an important reservoir in the community.

Studies in U.P. Terai by Sharma *et al.* (1983; 1984) and Malhotra *et al.* (1985) showed that *P. malariae* had completely disappeared and even after intensive surveillance not even one case of *P. malariae* was encountered over a period of 7-8 years. It was also interesting to note that till 1980, although malaria had increased tremendously in Terai, almost all malaria cases were due to *P. vivax* except isolated cases of falciparum malaria (Sharma *et al.*, 1983). During 1980-84, the incidence of *P. falciparum* increased tremendously and now there are well defined peaks of vivax and falciparum malaria (Sharma *et al.*, 1983; Malhotra *et al.*, 1985). Similar information about other districts is not known, but it is likely that in many areas falciparum malaria has increased considerably over the years. Earlier studies in Sonepat, Haryana and Nainital districts substantiate this finding. The proportion of asymptomatic and afebrile malaria cases was also high (Sharma *et al.*, 1983; Choudhury *et al.*, 1983).

Fulminating epidemics of malaria have been recorded in the past from UP and Punjab. It was

estimated that before the launching of the National Malaria Eradication Programme (NMEP) in 1958, about three fourths of the entire rural areas of the state were endemic for malaria (NMEP, 1960). At that time intensive spraying and adequate anti-parasitic measures uprooted the disease and large parts of the state showed spectacular improvement in the malaria situation. Unfortunately, due to technical, administrative and financial constraints malaria resurgence occurred in late 1960s, (Sharma and Mehrotra, 1986). The resurgence of malaria was aided and abetted by poor spraying and unsatisfactory laboratory services. *Inter alia* there is an urgent need of strengthening the laboratory services by periodical training and re-training of the technicians and strict supervision. At the same time it is important that research on modern methods of parasite identification is intensified to provide more accurate and rapid diagnosis of the parasite positive cases. The availability of DNA probes has considerably improved these prospects (Franzen *et al.*, 1984; Pollack *et al.*, 1985; Mclaughlin *et al.*, 1985; Barker *et al.*, 1986). DNA probes may provide speedy and reliable identification, thus enhancing appropriate and timely treatment of malaria cases. Further developments are required by the use of marker enzymes instead of the radioactive compounds for these techniques to be applicable at the periphery. But until such time all efforts should be directed to improve the quality of slide preparation, staining and reliable microscopical examination of blood smears. The study brought out that (i) the slide examination at the PHC level was far below the acceptable standards and there was an urgent need of training the technicians to improve the performance of laboratory services, and (ii) incidence of malaria was extremely high in most PHCs, particularly in Nainital and Pilibhit districts in view of the fact that blood smears were collected from all types of patients during the non-transmission season.

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Daytime Resting Habits of *Anopheles stephensi* in an Area of Calcutta

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Recrudescence of malaria has posed a serious health problem in the city of Calcutta. NMEP reports indicate that malaria has appeared in higher proportion in recent years in comparison to previous years in this city. Siddons (1946), in the past and Hati and Mukhopadhyay (1980) in recent years incriminated *Anopheles stephensi* as the vector of malaria in Calcutta.

Authenticated reports of prevalence of *A. stephensi* in the city of Calcutta were available from the beginning of this century. Several investigators (James, 1902; Stephens and Christophers, 1902; De, 1923; Basu, 1930; Senior White, 1934; 1940; Siddons, 1946; Hati and Mukhopadhyay, 1980) point out that the daytime resting places of this species are somewhat obscure in Calcutta. Hence, an attempt has been made in order to determine daytime resting habits of *Anopheles stephensi* in Calcutta. During a rigorous and continuous search, a peculiar habitat of *A. stephensi* has been identified which is worth reporting.

Different types of possible shelters were visited regularly in the morning hours from April 1985, without any success, but suddenly the insect collectors were able to catch *A. stephensi* from certain temporary human shelters and from a lavatory near the construction work of the Metro railway, in Esplanade area, situated in the heart of the city of Calcutta. The present communication deals with the longitudinal studies for one year (September 1985 to August 1986) on the collection of *A. stephensi* from such habitats and incrimination of the species as the vector of malaria in the present situation.

Twenty three temporary human shelters (*Jhopries*) which were being made by the workers involved in underground construction work for Metro tunnels and a public lavatory of Esplanade area were searched thoroughly for collection of adult mosquitoes by six insect collectors from 0600 to 0800 hrs. The size of the study area was 150 m × 70 m. Each temporary human shelter measured not more than 2.4 m × 1.8 m × 2.4 m and the walls and roofs were made up of corrugated or plain tin, pieces of wood, tarpaulin, bamboo, asbestos or plastic sheets. The entry passages were very narrow and the inside was dark and humid. The public lavatory is a massive cemented structure with concrete roof but the inside was dark and damp.

The study area was littered with heaps of garbage, stone chips, broken pipe lines, iron rods, etc. Discarded tins, cement cisterns for soaking bri-

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Table 1. Collection of mosquitoes from Esplanade area, from September 1985 to August 1986 in the city of Calcutta employing 96 man hours each month

Month	*C. quinque-fasciatus*	*Ae. aegy-pti*	*A. ste-phensi*	*A. va-gus*	*Ar. subal-batus*	*A. sub-pictus*	*C. ge-lidus*	*C. bitaeni-orhynchus*	Total	%
Sept '85	1754	57	69	—	—	3	2	—	1885	11.01
Oct	1211	13	39	—	—	—	—	—	1263	7.4
Nov	1492	9	12	—	—	2	—	2	1517	8.9
Dec	1456	11	4	—	—	3	1	—	1475	8.6
Jan '86	1266	9	—	—	6	—	—	—	1281	7.5
Feb	1365	1	2	45	5	3	—	—	1421	8.3
Mar	1901	—	—	52	1	3	—	—	1957	11.4
Apr	1645	5	4	7	2	2	—	—	1665	9.7
May	972	20	12	4	12	4	—	—	1024	6.0
Jun	857	24	75	6	2	1	—	—	965	5.6
Jul	1343	244	104	3	5	15	1	—	1715	10.0
Aug.	828	53	36	4	18	2	—	—	941	5.5
Total	16090	446	357	121	51	38	4	2	17109	
%	94.0	2.6	2.1	0.7	0.3	0.2	0.02	0.01		100

cks, coconut shells, cut bamboo ends, old tyres, and other unused materials, were scattered here and there. Larvae of *A. stephensi* alone or in association with the larvae of *Aedes aegypti* were detected in the monsoon and post-monsoon periods. Adult collection methods were selected from those suggested by the WHO Expert Committee on Malaria (1962; 1964) and Holstein (1954). The collected mosquitoes were brought to the central laboratory for identification. The salivary glands of each *Anopheles stephensi* was dissected to detect natural infection i.e., sporozoites, if any.

Employing 1152 man hours in a year, a total of 17109 mosquitoes of 8 species were captured (Table 1). *A. stephensi* comprised 2.1% of the total catch. Out of 516 female anophelines, 357 (69.1%) were *A. stephensi*. The mosquitoes were captured from the surface of black umbrella, iron string attached to the lavatory flush, inside and outside of earthen pitchers, *Jhul* (old and new spider webs, impregnated with dust, soot and smoke),nylon or cotton strings, bamboo poles beneath the ceiling, iron pillars, cement walls of lavatory or walls of shelters, below the wooden cots, etc. In Calcutta maximum collection (90.5%) was observed during the monsoon and post-monsoon months i.e., June to October. It is interesting to note that 29.1% (104) *A. stephensi* adults were collected in July (the peak transmission period of malaria in the city) sporozoites were detected in the salivary glands of one specimen in the same month (July). From November 1985 to May 1986 i.e., in the drier seasons, the density was very low and actually no *A. stephensi* was gathered in the month of January and March, 1986. Almost all the shelters were inhabited by the vectors and the maximum number of mosquitoes per shelter/year and per shelter/month varied from 0–72 and 0–26 respectively. Mean per man hour collection of *A. stephensi* was 0.3 (range 0 in January and March 1986 to 1.1 in July 1986). It may however be mentioned that in an ordinary survey to find out the density of *A. stephensi*, our method may not always be feasible due to the huge employment of manpower.

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Dynamics of *P. falciparum* ratio—an Indication of Malaria Resistance or a result of Control Measures?

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It is a well-known fact that the parasite formula is quite a sensitive index of malaria transmission. An increase in *P. falciparum* proportion in the absence of antimalaria measures indicated increased transmission and vice versa. Further observations revealed that when residual spraying was properly done, *P. falciparum* was the first species to disappear (Pampana, 1969).

However, during malaria eradication campaigns, the value of this index lost most of its importance. On the contrary, in malaria control programmes, a complete and careful malaria survey should be carried out, in order to know exactly where malaria transimission takes place and to define various epidemiological characteristics of the infection in different seasons and parts of the country.

In a recent publication on tools for assessment of malaria control, the analysis of malariometric parameters for the purposes of malaria control was undertaken. Specific reference was made to the practical use of the percentage of *P. falciparum* cases (Pf ratio).

It was found that when the Annual Blood Examination Rate (ABER) is high and the Annual Parasite Incidence (API) or the Slide Positivity Rate (SPR) data are available, Pf ratio provides a fair idea of the importance of falciparum malaria. Further, it was observed that this index, however, became highly unsuitable for measurement of the dynamics of *P. falciparum* infection, presumably because under certain situations *P. vivax* would seem to decline faster than *P. falciparum*. As such the amount of *P. falciparum* as reflected by Pf ratio is increasing despite the fall in SfR, which does not indicate deterioration of the *P. falciparum* situation (Ray and Beljaev, 1984).

These observations seem to be in agreement with the available epidemiological data on malaria situation in the countries of the WHO South East Asia Region in general and in India in particular for the last 15 years.

From Table 1, it may be seen that during 1970–77 there was no marked correlation between reduction/increase in the slide falciparum rate (SfR) and Pf ratio. However, over the last 7–8 years, the average rate of increase of Pf ratio has been more than the average rate of decrease of other indexes, such as ABER, API, SPR, SfR. The data was analysed by the use of variance method by using oneway procedure of "SPSS" statistical software and the above difference was found to be statistically significant.

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Table 1. Malaria situation in SEARO and India (1970-1984)*

Year	SEARO				India			
	ABER	SPR	SFR	Pf%	ABER	SPR	SFR	Pf%
1970	8.3	2.4	0.5	19.8	7.8	1.7	0.3	14.8
1971	7.7	3.0	0.6	18.6	7.6	3.3	0.4	11.6
1972	7.7	3.1	0.5	16.5	7.2	3.7	0.4	9.5
1973	7.7	4.5	0.6	14.2	7.6	4.6	0.3	6.8
1974	7.8	6.5	1.2	19.2	8.0	7.0	1.0	14.6
1975	8.6	8.6	1.5	17.4	8.9	10.0	1.4	14.3
1976	8.9	9.7	1.4	14.0	9.5	11.6	1.4	11.7
1977	8.6	7.5	1.0	12.9	9.5	8.3	0.8	9.7
1978	8.7	6.2	1.1	18.3	9.8	6.9	1.0	14.9
1979	8.7	4.6	1.0	22.2	9.7	5.0	0.9	18.3
1980	9.4	4.2	1.2	27.3	10.3	4.3	0.9	20.3
1981	9.3	3.9	1.2	29.8	10.1	4.0	0.9	21.8
1982	9.1	3.3	1.1	33.3	9.7	3.4	0.8	25.3
1983	8.5	3.1	1.0	32.5	9.3	3.1	1.0	32.7
1984	8.6	3.3	1.1	35.1	9.3	3.3	1.0	30.4

*Source: SEARO, 1986

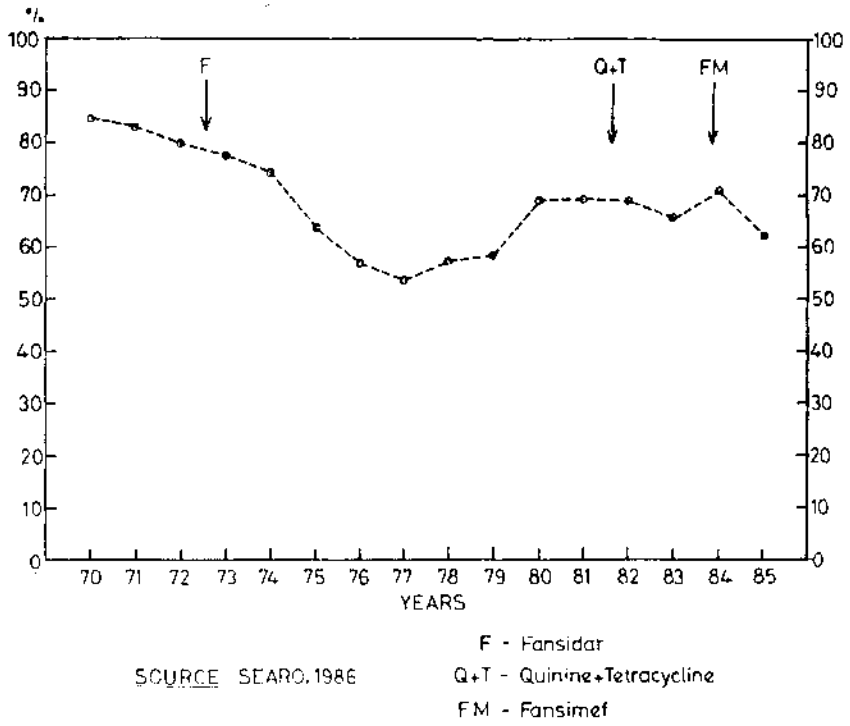

Fig. 1: The dynamics of Pf ratio in Thailand (1970-1985) (Source: SEARO, 1986).

Two hypotheses had been offered for explanation of this progressive imbalance. One was attributed to the widespread use of chloroquine for presumptive treatment, destroying the gametocytes of *P. vivax*, but not *P. falciparum* and thus interfering with the transmission and propagation of *P. vivax* (Ray and Beljaev, 1984; Clyde, 1984). The other hypothesis suggests that this phenomenon is due to widespread malaria resistance to 4-aminoquinolines and other antimalarials.

In Burma, the proportions were approximately 82% for *P. falciparum* and 18% for *P. vivax* since 1980, the ratio being 5:1. This ratio has been stable since 1979; in 1978 it was 3:1, and prior to that year it was 2:1. Considerably more *P. falciparum* (88% of species) was identified in areas under drug or spray operations than under surveillance (82%) or vigilance. Conclusion reached by the local workers was that swing towards *P. falciparum* was attributed to the increasing presence both in geographical distribution and intensity towards RIII of chloroquine resistant parasites (Clyde, 1984).

Available data in a few other countries of the region appear to support the latter view. In this respect, the most interesting observations have been made in Thailand, where malaria resistance to 4-aminoquinolines is a most pronounced epidemiological feature of contemporary malaria in the country.

From Fig. 1, it will be be seen that Pf ratio is quite high in Thailand, where chloroquine resistance of local populations of *P. falciparum* was reported as far back as 1961 (Harinasuta *et al.*, 1962). However, once the new combination of antimalarials fansidar) was introduced throughout the country in 1972–73, there was a marked reduction in Pf ratio. Reduction in values of Pf ratio in Thailand continued upto 1977, with somewhat static value in 1977–78, when resistance to fansidar was reported in many parts of the country.

Since 1979 onwards, there was an upward trend in Pf ratio which continued till 1982, when a combination of quinine and tetracycline replaced the use of fansidar in many parts of the country. The replacement resulted in some reduction of Pf ratio for a short period. An upward trend in Pf ratio was resumed in 1984. However, with an introduction of the combination mefloquine—sulfadoxine—pyrimethamine (fansimef) in early 1985 this trend was controlled (WHO, 1986).

In India, first report on malaria resistance to chloroquine in the Northeast parts of the country relates to the year 1973 (Sehgal *et al.*, 1973). Since then, the spread of malaria resistance occurred throughout the country and Pf ratio was showing an upward trend in subsequent years in spite of the fact that SFR was more or less static.

The possible existence of relationship between Pf ratio and malaria resistance can be traced in malaria situation prevailing in Paralkote Zone of the Dandakaranya (DNK) project in the northwestern part of Bastar district of Madhya Pradesh State, India.

The population of the area is approximately 73,000, of which 54,000 persons are settlers and the rest are tribal. Spraying operations have been conducted by the state in both tribal and settler villages.

Three regular rounds of HCH have been sprayed annually. From Table 2, it can be seen that uptil 1979 there was a positive correlation between the values of API, SPR, SfR and Pf ratio. However, negative correlation was established between above indices since 1980 onwards. Chloroquine sensitivity tests undertaken in DNK project areas in 1981 have revealed that resistance to chloroquine varied from RI to RIII level (Houghton, 1983).

In Nepal, where no evidence of local transmission of *P falciparum* resistant strains was

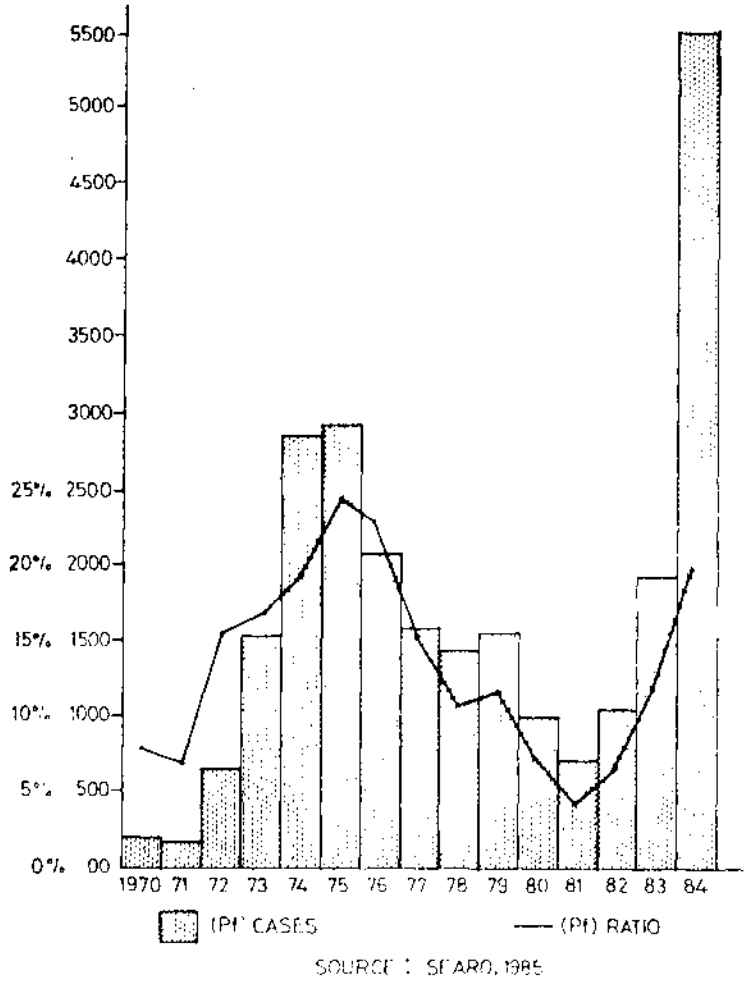

Fig. 2. Pf ratio and Pf cases in Nepal (1970–1984) (Source: SEARO, 1985).

Table 2. Malaria situation in DNK project, Madhya Pradesh (1975–1982)*

Year	ABER	API	SPR	SFR	Pf%
1975	56.2	125.0	22.2	10.7	48.0
1976	72.1	132.4	18.4	6.8	37.0
1977	86.8	139.5	16.1	2.7	17.1
1978	91.5	258.4	28.2	15.0	53.0
1979	104.3	233.1	22.3	10.8	48.3
1980	108.3	216.8	20.0	15.1	75.6
1981	83.6	137.9	16.5	13.6	82.3
1982	61.3	69.9	11.4	9.5	83.3

* Source : NMEP, 1983

established until late 1984, the ratio of Pf showed strong positive correlation with API, SPR and SfR, as can be seen from Fig. 2.

There is, however, some data available in the countries of the Region, which is in agreement with another hypothesis, suggesting that the widespread use of chloroquine for presumptive treatment of *P. vivax* was responsible for alterations of Pf ratio value. A rapid decline in *P. vivax* might be expected, specially in an area where relapse rate of this species is somewhat lower than elsewhere, like in India (Jaswant

Singh *et al.*, 1953; Basavaraj, 1960; Sharma *et al.*, 1973). This situation might be further amplified if *P. falciparum* is resistant to chloroquine in which case *P. vivax* would be reduced much faster than former species.

Since species ratio does not portray the total number of infections, more accurate indicators of the trend are obtained from SPR, SfR and SVR (*P. vivax* Slide Positivity Rate).

Analysing the relevant data for the countries of SEAR and separately for India, it is clearly seen that the decrease in SPR from 1976 through 1984 has been largely at the expense of *P. vivax*, while *P. falciparum* after a small initial fluctuation has held steady with no further reduction in SfR below 0.8–1.0 (Table 3).

Table 3. SPR, SVR and SFR in SEARO and India (1976–1984)*

Year	SEARO			India		
	SPR	SVR	SFR	SPR	SVR	SFR
1976	9.7	8.3	1.4	11.6	10.2	1.4
1977	7.5	6.5	1.0	8.3	7.5	0.8
1978	6.2	5.1	1.1	6.9	5.9	1.0
1979	4.6	3.6	1.0	5.0	4.1	0.9
1980	4.2	3.0	1.2	4.3	3.4	0.9
1981	3.9	2.7	1.2	4.0	3.1	0.9
1982	3.3	2.2	1.1	3.4	2.6	0.8
1983	3.1	2.1	1.0	3.1	2.1	1.0
1984	3.3	2.2	1.1	3.3	2.3	1.0

*Source: SEARO, 1986

Data available for Visakhapatnam district, India, where resistance to chloroquine was established in 1980, indicates that SVR was reduced by about 20 times from 1975 to 1984, while SfR was reduced to the lowest level in 1979 only by 2 folds, and there was an increase in SfR and Pf ratio in subsequent years, while *P. vivax* incidence continued to show downward trend (Table 4).

In Kota district, India, decline in *P. vivax* incidence was also faster than in *P. falciparum*, in spite of the fact, that the latter was found sensitive to 4-aminoquinolines (Table 5).

Table 4. Malaria situation in Visakhapatnam district, India, 1975–1984*

Year	SPR	SVR	SFR	Pf%
1975	8.6	7.8	0.8	9.3
1976	5.7	5.1	0.6	10.5
1977	2.6	2.2	0.4	15.4
1978	1.4	1.1	0.3	21.4
1979	1.3	0.9	0.4	30.8
1980**	1.3	0.8	0.5	38.5
1981	2.2	0.6	1.6	72.7
1982	2.1	0.7	1.4	66.7
1983	1.5	0.4	1.1	73.4
1984	1.7	0.4	1.3	76.5

*Source : NMEP, 1986
***P. falciparum* resistance to chloroquine was established. Source: NMEP, 1986

Table 5. Malaria situation in Kota district, India 1975–1984*

Year	SPR	SVR	SFR	Pf%
1975	8.3	6.1	1.2	14.4
1976	4.7	4.2	0.5	10.6
1977	2.9	2.7	0.2	6.9
1978	2.9	2.7	0.2	6.9
1979	2.4	2.2	0.2	8.3
1980	2.6	2.4	0.2	7.7
1981	3.2	2.7	0.5	15.6
1982	3.1	2.0	1.1	35.6
1983**	2.4	1.3	1.1	45.8
1984	2.6	1.5	1.1	42.3

*Source : NMEP, 1986
***P. falciparum* was found to be sensitive to chloroquine. Source: NMEP, 1986

There could be a few other explanations, apart from above described hypotheses, as to why falciparum/vivax species proportion changed for the last decade in the countries of the Region.

From the past experience it is known that where malaria was stable, usually *P. falciparum* incidence was high and sometimes it was a predominant species of malaria parasite. Under

such a situation, Pf ratio to total cases was always high and might be very close to 100 per cent. Therefore, when malaria transmission is reduced due to effective intervention measures like residual spraying, Pf ratio is not altered. Under such a situation while volume of cases will continue to go down along with SfR, the Pf ratio will remain as high as before (Ray, personal communication).

On the other hand, under unstable malaria situation, a change of Pf ratio could be a warning signal, as these are predominantly vivax areas in most of the years and as a rule *P. falciparum* profile is low. During abnormal epidemic situation, however, this ratio is likely to increase rapidly.

On the whole, it appears that available data from the countries of the WHO South East Asia Region in respect of falciparum/vivax species proportion changes (1976–1984) indicate that this progressive imbalance can not be attributed to any particular factor alone. Therefore, further field observations are required in order to arrive at a definite conclusion i.e., whether the dynamics of Pf ratio can be considered as an indicator of the establishment/expansion of drug resistant populations of *P. falciparum* in certain areas, combined with a heavy exposure to antimalaria drugs, or that interaction of various factors might be held responsible for this phenomenon. This subject may be considered for inclusion under Plasmodium falciparum Containment Programme research component.

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Report of a Case of *P. falciparum* malaria Resistant to Chloroquine and combination of Sulfalene and Pyrimethamine in Delhi

D.S. CHOUDHURY[1], S. SINHA[1], S.K. GHOSH[1], C. USHA DEVI[1] and V.P. SHARMA[1]

A boy aged 8 years in North Delhi attended the Centre's clinic on 24th November, 1986 for treatment of malaria. He had suffered repeated attacks of fever for three months from September to November 1986. His blood smear examination report showed that he was diagnosed positive for malaria although the species was not mentioned. The boy had taken several courses of chloroquine, primaquine and two tablets of Metakelfin[R] (each tablet consisting of sulfamethopyrazine 500 mg + pyrimethamine 25 mg). The latter drug was taken just two weeks before he visited the Malaria Research Centre's clinic.

Blood smear examination carried out at the clinic showed the presence of *P. falciparum* rings. A finger prick sample of blood was collected for micro *in vitro* test for chloroquine sensitivity (WHO, 1981). The boy was given one tablet of Metakelfin and kept under surveillance for one week. He became afebrile after 48 hours and his blood examination on day 7 did not show any parasites. He was given haematinics for anaemia.

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The patient visited the clinic again on 24th December, 1986 with fever. His blood smear examination showed *P. falciparum* rings. A finger prick sample of blood was again collected for micro *in vitro* test for parasite sensitivity against chloroquine, SDX/PYR and qinghaosu. Tests were carried out against combinations of SDX/PYR according to the standard WHO procedure using the WHO charged plates and RPMI 1640 LPLF medium. The qinghaosu plates were charged in the laboratory.

Results of the micro *in vitro* test (Table 1) revealed that the strain was resistant to both chloroquine and SDX/PYR. The MIC of chloroquine was 16 pmol and that of SDX/PYR 5×10^{5} M/6.25^{-7} M. This was approximately 160 times the MIC value of 3×10^{-7} M/3.0×10^{-9} M obtained by Bjorkman and Willcox (1986) on SDX/PYR susceptible strains tested in Liberia, West Africa. Spencer *et al.* (1984) reported MIC value of 3.2×10^{-6} M/3×10^{-7} M in SDX/PYR resistant Indo-China I strain. This was comparable with our results.

Resistance to Fansidar has been reported from different parts of Southeast Asia (Black *et al.*, 1982; Johnson *et al.*, 1982; Sabachareon *et al.*, 1985). Rumans *et al.* (1979) reported resistance against Fansidar from Indonesia. Similar reports

Table 1. Results of WHO *in vitro* micro tests for chloroquine, sulfadoxine/pyrimethamine and qinghaosu

Date of test	MICs of		
	Chloroquine	SDX/PYR	Qinghaosu
24.11.85	16 pmol	NT	NT
24.12.86	16 pmol	5×10^{-5}M/ 6.25×10^{-7}M	2×10^{-9}M

NT: Not tested

have also come up from different parts of Africa (Hess *et al.*, 1982; Bjorkman and Willcox, 1986).

The MIC value of this strain was 2×10^{-9} M against qinghaosu. This was less than the MIC obtained by Thaithong and Beale (1985) in isolates from Thailand i.e., MIC values were 10^{-7} M and 10^{-8} M in different isolates. The strain was therefore sensitive to qinghaosu.

This is the first report of *P. falciparum* resistance from Delhi against chloroquine and metakelfin. In Delhi and other parts of India, combinations of long acting sulfonamides and pyrimethamine are being liberally used in the treatment of malaria diagnosed clinically.

The appearance of resistance to the combination of long-acting sulfonamide and pyrimethamine was imminent. The use of this drug should be restricted to chloroquine resistant *P. falciparum* cases as other drugs such as Mefloquine and qinghaosu are not available in the country and physicians would have to treat patients with quinine.

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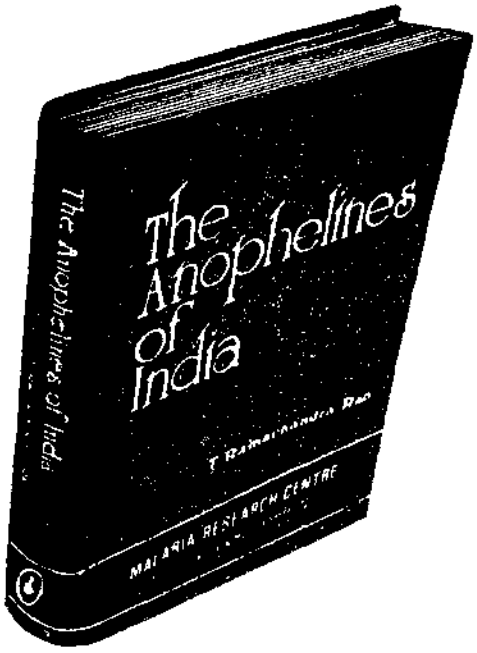

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