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Volume 19

Number 1

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CONTENTS

(contd.)

Short Notes

Indian Journal of Malariology
Vol 19, June 1982, pp 1-4

Plasmodium berghei: in vitro Microtechnique for Preliminary Screening of Antimalarials

S. SINHA[1], A. GAJANANA[1], VIJAY KUMAR SINGH[1] and A.N. RAI CHOWDHURI[1]

In vitro microtechnique for testing antimalarials using short-term cultures of *Plasmodium berghei* has been described. The inhibition of maturation was the basis for assessing antimalarial activity. Dose-response curves for chloroquine, quinine and mefloquine were determined. Experiments with mefloquine resistant strain indicated utility of the test in comparing the effect of drugs in normal and resistant strains.

INTRODUCTION

Search for simple and reproducible *in vitro* tests for screening large number of compounds economically has assumed importance with growing interest to synthesise new antimalarials. Earlier studies using *Plasmodium berghei* for screening compounds *in vitro* have been based on biochemical methods (Cenedella and Saxe, 1967; Richards and Williams, 1973). This paper reports a simple *in vitro* microtest where inhibition of maturation to schizont was the basis for testing antimalarials.

MATERIAL AND METHODS

Medium— RPMI-1640 complete medium (Rai Chowdhuri *et al.*, 1979) adjusted to pH 7.5 with 5% sodium bicarbonate was used.

Antimalarials— Stock solutions of chloroquine phosphate (Bayer's Resochin, 30 mg base/ml) quinine bihydrochloride (Bengal Immunity, 240 mg base/ml) were injectable solutions. Stock solution of mefloquine hydrochloride (0.9 mg base/ml) was prepared in the laboratory, sterilized by filtration and stored at 4° C. The dilutions were prepared from stock solutions in complete medium and used in the test.

Parasites— NICD strain of *Plasmodium berghei* susceptible to chloroquine, quinine and mefloquine (Ramakrishnan *et al.*, 1957; Kazim *et al.*, 1979) was maintained in adult albino rats. This strain is designated as normal strain (NS). Weanling rats inoculated with a mefloquine resistant strain (MRS) (Kazim *et al.*, 1979) of *P.berghei* were received from CDRI, Lucknow.

In vitro microtest— Blood was collected in heparin (20 I.U./ml) from donor rats with 1 to 2 per cent parasitaemia. Tests were put up in sterile multi well polysterene plates (Laxbro, Pune) with 6x10 mm flat bottom wells, essentially by the method of Rieckmann *et al.*, (1978). Each well received 100 mcl of complete medium with (test) or without (control) drugs and 10 mcl of parasitised blood. Duplicate wells were used for each drug concentration as well as controls. The plates were incubated at 37° C for 16-18 hr (except for those used in time-course experiments). Thin smears were prepared from pre-incubation samples and from each well after incubation, stained with Giemsa/JSB stain and differential counts of 100 asexual parasites from each smear were recorded. The number of schizonts in drug samples was expressed as percen-

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tage of controls. The effective dose was the lowest concentration of the drug which inhibited maturation by greater than 90 per cent (ED>90). Concentrations of drugs have been expressed as ng per mcl of blood in culture.

RESULTS

Time course studies— In order to determine the optimum period of incubation, smears prepared from cultures of NS at 12, 16, 20 and 24 hours were examined and the mean values of maturation of 9 experiments were 39, 76, 90 and 95% respectively. In 24 hours' samples many mature schizonts ruptured while preparing smears which interfered with counting. Therefore, in subsequent 75 different experiments cultures were incubated for 16 to 18 hours as a standard procedure. Results (Fig. 1) showed that the mean percentage maturation was 84±SE 1.3 with a range of 45 to 100%.

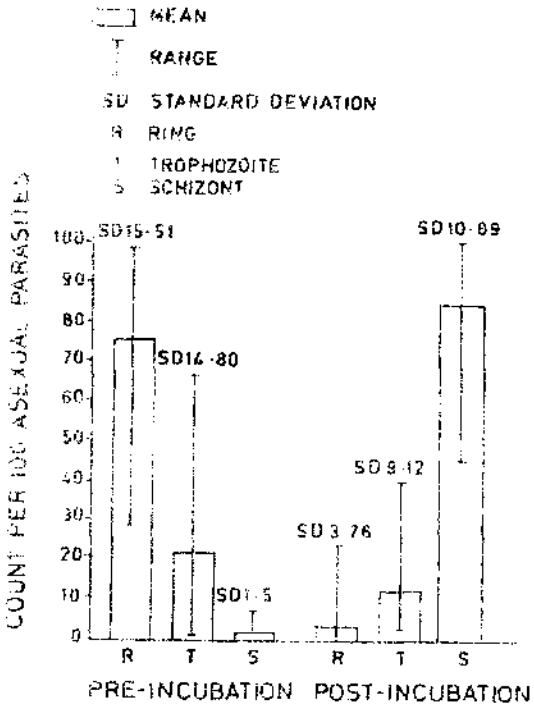

Fig. 1 Differential counts of pre-and post-incubation samples of *P.berghei* (NS) cultured *in vitro* (Data of 75 experiments)

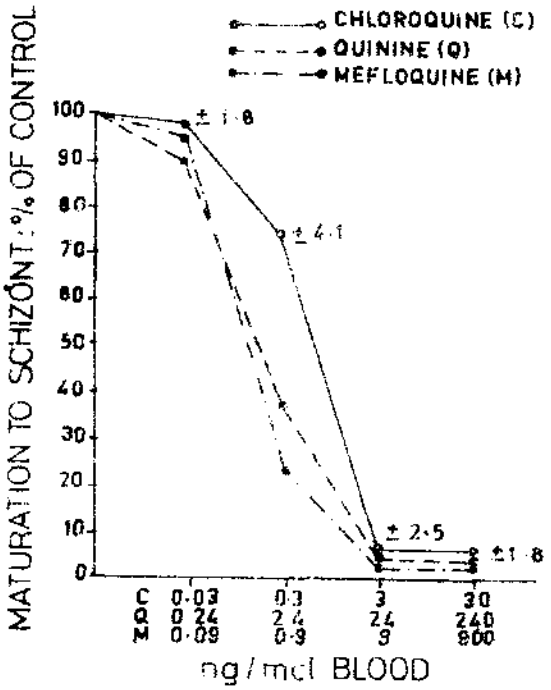

Fig. 2 Dose response curves for chloroquine, quinine and mefloquine against *P.berghei* (NS) *in vitro* (Each point represents mean values of 9 and 2 experiments with chloroquine and mefloquine respectively and 1 experiment with quinine. Numbers denote standard errors.)

Dose response studies— In the first series of experiments the effect of 10-fold increasing concentrations of chloroquine, quinine and mefloquine against NS was studied (Fig. 2). ED>90 were 3, 24 and 9 ng respectively. A further 10-fold increase beyond these drug concentrations did not bring about appreciable difference ("tailing off effect"). Further experiments with narrow ranges (Fig. 3) showed that the ED>90 were 3.12 and 2.2 ng respectively. Effect of mefloquine against MRS and NS given in Fig. 4 indicated that ED>90 of MRS (900 ng) was 100 times greater than that of NS (9 ng).

Comparison of maturation and infectivity of *P.berghei* (NS) treated with chloroquine *in vitro*— Cultures were set up in normal medium and medium containing 1.5 and 3.0 ng of chloro-

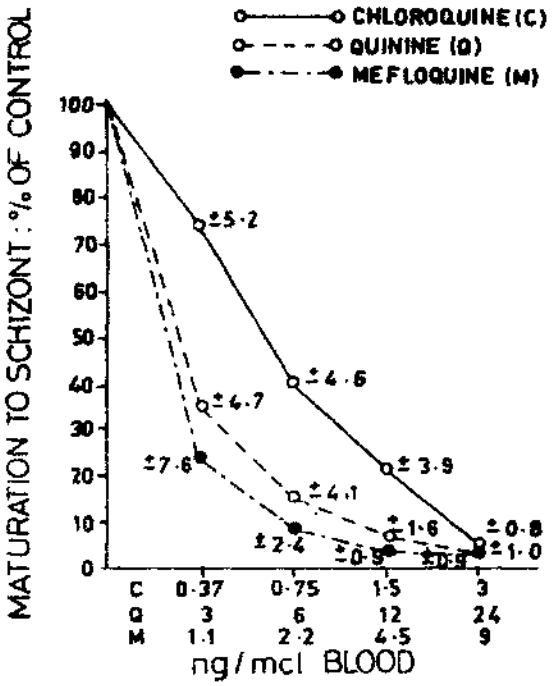

Fig. 3 *P.berghei* (NS) *in vitro*: Effect of antimalarials.(Each point represents mean values of 22, 9 and 5 experiments with chloroquine, quinine and mefloquine respectively. Numbers denote standard errors.)

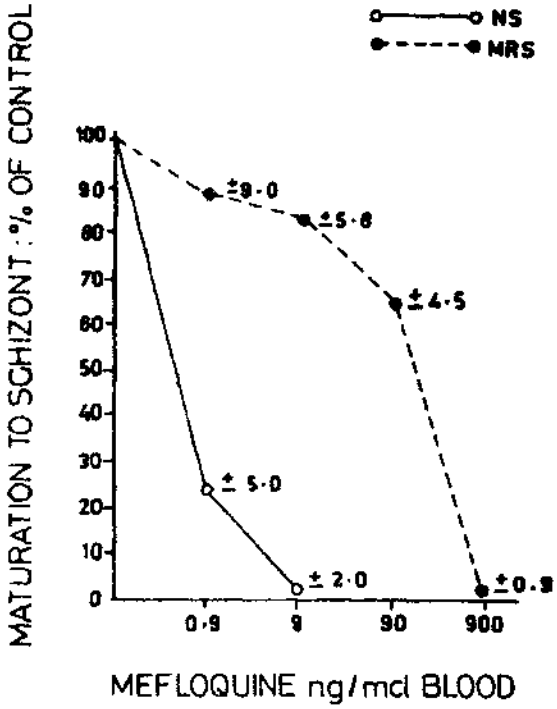

Fig. 4 Susceptibility of NS and MRS of *P.berghei* to mefloquine *in vitro*. (Each point represents mean values of 3 experiments. Numbers denote standard errors.)

quine using 4 wells for each concentration. After incubation for 16 hours smears prepared from two wells under each category were examined for schizonts. The contents of other two wells were pooled and washed once with 0.85% saline. The cells after resuspending in 0.5 ml of saline were inoculated in equal quantities to groups of 3 mice each. Results (Fig. 5) showed that there was a dose-related inhibition of growth and corresponding dose-related prolongation of pre-patent periods.

DISCUSSION

Since *P.berghei* follows an asynchronous multiplication *in vivo* (Garnham, 1966), it was essential to collect blood at a specified time from donors a few days in the beginning of parasitaemia to ensure maximum number of rings and trophozoites. ED>90 was taken as the end point because of the "Tailing off" effect seen in dose-response studies. This might be due to presence of a small percentage of late trophozoites and early schizonts in the inocula which grew to next stage before the action of drugs.

Richards and Williams (1973) reported that in *P.berghei* the concentration of chloroquine which inhibited 3H-leucine incorporation by 90% (IC 90) was about 60 mcg/ml medium which was equivalent to 43 ng/mcl of blood in their culture system. Cenedella and Saxe (1967) used 0.5 to 5 mM concentration of quinine as standards for demonstrating reduced metabolic activity. But in our studies the ED>90 for chloroquine was 3 ng/mcl of blood and for quinine it worked to be 0.03 mM. The results suggested that the test is quite sensitive. Further it was interesting to note that these concentrations represented approximately the blood levels in rodents attained after administration of thera-

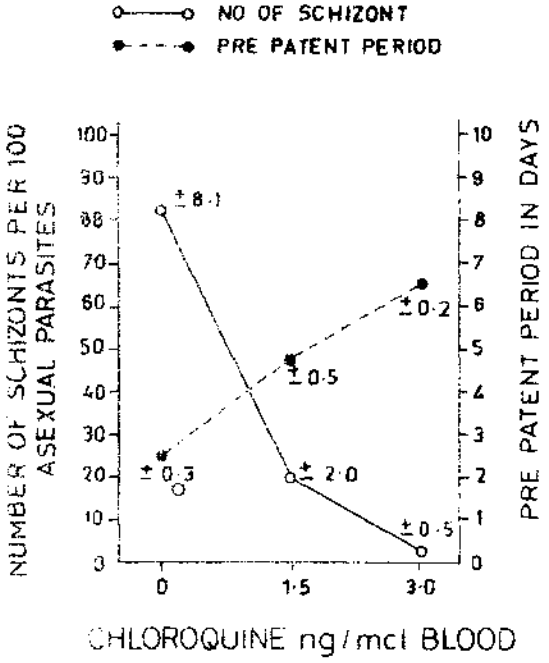

Fig. 5 Effect of chloroquine on *P.berghei* (NS) *in vitro*: Comparison of Schizont maturation and pre-patent period in mice (Each point represents mean values of 2 experiments. Numbers denote standard errors.)

peutic doses (Thompson *et al.*, 1967; Mu *et al.*, 1975). However, further work may be needed to compare relative sensitivity of different methods using the same strain of *P.berghei*.

Differences between NS and MRS in susceptibility to mefloquine *in vitro* is in agreement with the *in vivo* studies (Kazim *et al.*, 1979) where the minimum effective dose of mefloquine for MRS was about 60 times greater than that for NS. Thus the test can be used for characterization of resistant strains.

The dose related inhibition of maturation and corresponding dose related prolongation of pre-patent periods in mice (Fig. 5) suggested reduced number of viable parasites. A similar observation has been made by Richards and Williams (1973).

The *in vitro* microtechnique described in this communication thus offers a simple, economical and reproducible method for evaluation of antimalarials.

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Thanks are due to Shri Mohd. Illyas Khan, Jai Siya Ram and Smt. Ammini Sivaraman for their technical assistance. Mefloquine and mefloquine resistant strain of *P.berghei* were kindly provided by Dr. G.P. Dutta, Assistant Director, CDRI, Lucknow. The Study was financially supported by ICMR.

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Impact of DDT Spraying on Malaria Transmission in Villages with Resistant *Anopheles culicifacies*

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Impact of DDT spraying on malaria transmission was evaluated in Haryana villages with DDT resistant *Anopheles culicifacies*. Spraying resulted in marked reduction of vector densities and the incidence of malaria. There was no difference in spraying of villages with DDT at 2 dose levels i.e., 1 g and 2 g per m², as monitored by the entomological and epidemiological indices. The study also revealed that *A. subpictus* was the most dominant species followed by *A. culicifacies*, and *Plasmodium falciparum* infection rate in the community was extremely high.

INTRODUCTION

The first report of increased tolerance to DDT in *A. culicifacies* came from Gujarat in 1957 (Rahman *et al.*, 1959). In the decade that followed DDT resistance in *A. culicifacies* had become widespread (Raghavan *et al.*, 1967). During 1967-69 there were 96 units under persistent attack phase and in 44 units tested, *A. culicifacies* was resistant to DDT in 35 units and to DDT and HCH in 5 units (Rao, 1970). Focal outbreaks occurred during these years which became more serious and extended year after year. During 1968, out of a total of 393.25 units, 71.385 units involving a population of 91 million were reverted to spraying (Madhok, 1967). The problem of DDT resistance was tackled by HCH spraying, and when resistance to DDT and HCH developed, control was achieved by malathion spraying. The total quantities of insecticides used in 1980-81 under NMEP were 15250 mt (75%) DDT, 2500 mt (50%) HCH and 11890 mt (25%) malathion. Malathion requirements of the NMEP have increased considerably and may further increase in the coming years. Change of insecticides is a costly proposition. Spraying of DDT to cover 10 lakh population costs Rs.14.01 lakhs and with HCH 28.20 lakhs (2-fold increase) and with malathion Rs.177.02 lakhs (12.6-fold increase) and the cost of newer insecticides is many times more. Use of malathion has resulted in triple resistance i.e., to DDT, HCH and malathion in certain areas (Roy *et al.* 1979 and Rajgopal, 1977), and a change of insecticides is indicated. Also the choice of replacement insecticides is limited. Therefore, optimum use of the available insecticides is indicated both scientifically and economically.

With this background, in some parts of India (though WHO recommended tests show high degree of resistance) the insecticides are being used as they are considered to have epidemiological impact. It has been reported from the field that in some areas after the spray there was decline of cases while in others the impact was questionable. As the cost of insecticides is about 50% of the NMEP's operations, judicious use of insecticides is indicated. Therefore a study on the

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true impact of DDT spraying on the disease in areas showing resistance is of great importance in the present context. Results of this study are reported in this paper.

MATERIAL AND METHODS

Villages of district Faridabad (Haryana state) were selected for this study. The entire district was sprayed with HCH in 1978. State insecticide susceptibility data in 1979 showed that the vector species was resistant to DDT i.e., in Ballabgarh block there was 13.3% mortality of *A. culicifacies* on 4% DDT papers in 1 hour. There was no spray in 1979. During 1980, HCH was sprayed selectively in high incidence areas. In 1981, malathion was sprayed in some areas to control the disease. Therefore villages of Ballabgarh block with a history of high DDT resistance were selected for spraying and villages of block Palwal were held for comparison purposes. These villages are situated at about 50-80 kms south from the MRC office and fall under the jurisdiction of Kurali & Dudhola PHCs. Villages with total population of approximately 13,000 (sections 16, 17, and 18) were selected for spraying with the usual dosage used under NMEP for spraying i.e., $1g/m^2$. A second set of villages with a total population of approximately 13,000 (sections 27, 28, and 29) was selected for spraying with the double dosage i.e., $2g/m^2$. A third group of villages with a total population of approximately 14,000 (sections 45 and 46) was held as control i.e., without spraying (Fig. 1).

All nozzles and spray pumps were checked before spraying. First round of spraying started on 1st June, 1981 and second round on 17th August, 1981. The experimental villages were sprayed in about 3 weeks. In order to achieve $2g/m^2$ surface deposits, every structure was sprayed 2 times i.e., after spraying the entire village it was sprayed second time. All spraying operations were done under the strict supervision and guidance of the study team.

Insecticide susceptibility tests were carried out before the start of the experiment and at 6 week intervals. Adults were exposed on 4% DDT impregnated papers for 1 hour and held under controlled temperature and humidity conditions for 24 hours before recording mortalities (WHO, 1980).

Entomological data was collected from the fixed stations (1 living room and 2 cattlesheds) from each village on monthly intervals. The following methods were used.

(a) Hand catch index: (i) Early morning 2 insect collectors made anopheline collections from each sprayed and control village. Man hour density was calculated from the actual time spent in the collections. (ii) Mosquito collections were made from 9 PM to 5 AM at 1 hourly interval for 15 minutes duration. Actual time of collection was calculated i.e., 120 minutes and the density per man hour was calculated.

(b) Total catch: Early morning pyrethrum space spray collections were made from the sprayed and control villages. Before spraying all openings of the rooms were sealed and a white bed sheet was spread. A commercially available preparation FINIT was sprayed using a hand flit gun. After 15 minutes, knockdown mosquitoes were collected from the bed sheet and identified.

Parasitological investigations were carried out by the MRC staff. Independent active surveillance was carried out every day for the duration of the study. Blood smears from the sprayed and unsprayed sections were prepared from as many fever cases as possible. It was not considered necessary to collect blood from all fever cases in the study areas. Fever cases after the preparation of blood smear were given presumptive treatment at the rate of 600 mg chloroquine adult dose and proportionate dose to the children. Slides were stained with JSB stain and identified under the microscope for the presence of malaria parasite.

RESULTS AND DISCUSSION

Insecticide susceptibility tests revealed that *A. culicifacies* was resistant to DDT. A total of 105

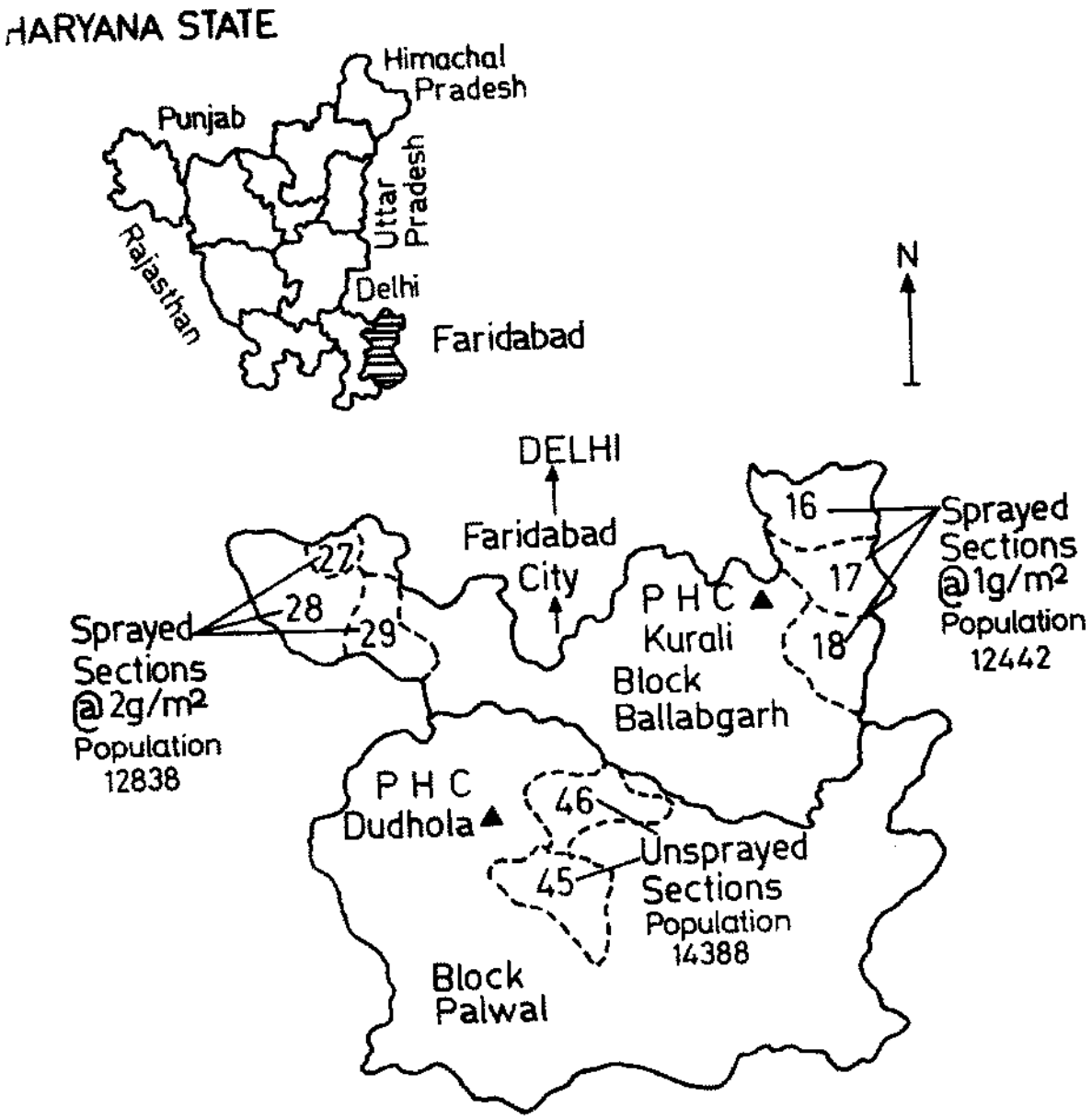

Fig. 1. Study areas in district Faridabad

Table 1-Density of *Anopheles culicifacies* in the experimental villages

Month	Sprayed village (1g/m²) Man hour density Morning	Night	Total catch	Sprayed villages (2g/m²) Man hour density Morning	Night	Total catch	Unsprayed villages Man hour density Morning	Night	Total catch
June	8.4	—	42.3	0	—	0	0.9	—	1
July	1.3	3.32	29.3	1.8	1.66	33.3	21.0	5.25	79.5
August	3.26	10.60	87.3	1.5	4.16	93.0	12.0	23.75	137.5
September	0.16	0.82	14.0	5.5	3.6	29.0	7.8	10.75	78.5
October	0	0.5	33.0	0	0.32	0.7	1.25	1.56	11.0
November	0	1.0	0	0.16	0	1.0	1.0	1.0	14.0

adult females were tested in 4 tests at an interval of 6 weeks. The average mortality was 14.2% (corrected mortality using the Abbott's formula was 11.53%). There was 100% mortality of *A. culicifacies* on 5% malathion papers in 1 hour. During spraying efforts were made to maximize coverage. It was not possible to achieve complete coverage for various reasons. The coverage varied from 65 to 80% with an average of 75%.

Entomological indices collected during the study period are given in Table 1. The three indices viz., morning and night per man hour density and total catches showed decline of vector populations in all the sprayed villages as compared to the unsprayed villages (see Figures 2, 3 and 4). Per man hour density revealed that first round of spraying resulted in marked reduction of vector densities in single and double dose sprayed villages. During the same period there was upward trend in the unsprayed villages (Figure 2). A similar trend in the reduction of *A. culicifacies* was observed in per man hour collection during night and total catches (Fig. 3 and 4). The second round of spraying coincided with the natural reduction in vector populations. There was much greater reduction of vector populations in the sprayed villages to that almost negligible number of *A. culicifacies* were found in the sprayed villages in the months of October & November. During the same period, low densities of *A. culicifacies* were still observed in the unsprayed villages (see Figures 2, 3 and 4). A notable feature of the entomological evaluation was that there was no difference in the response of vector populations to the single dose (1g/m²) and double dose (2g/m²) of DDT spraying.

Relative abundance of different anophelines viz. *A. culicifacies*, *A. subpictus* and *A. annularis* was calculated from the total catches (see Table 2 & Figure 5). It was revealed that *A. subpictus* was the most dominant species of the area followed by *A. culicifacies*. Populations of *A. annularis* were found in small numbers with a peak in August. Spraying of DDT resulted in the reduction of *A. subpictus* and *A. annularis*, and populations of these anophelines behaved in a way similar to *A. culicifacies*. It may however be pointed out that populations of *A. subpictus* were extremely high throughout the study period, and they may be responsible for most of the nuisance. Recently it has been incriminated as vector of malaria near Pondicherry (Panicker *et al.*, 1981) but no evidence of its role in malaria transmission has been found in north India.

Results of the parasitological investigations are given in Table 3. In the initial stages i.e., May to August, surveillance was not good as most of the staff was being recruited and trained. Therefore data in table 3 has been shown from September to November. The slide positivity rate (SPR) was reduced to about half in DDT sprayed villages (sprayed with 1g or 2g/m² DDT), whereas SPR remained at about the same level in the unsprayed villages. There was therefore a definite reduction in the incidence of the disease as a result of spraying. Parasitological results sub-

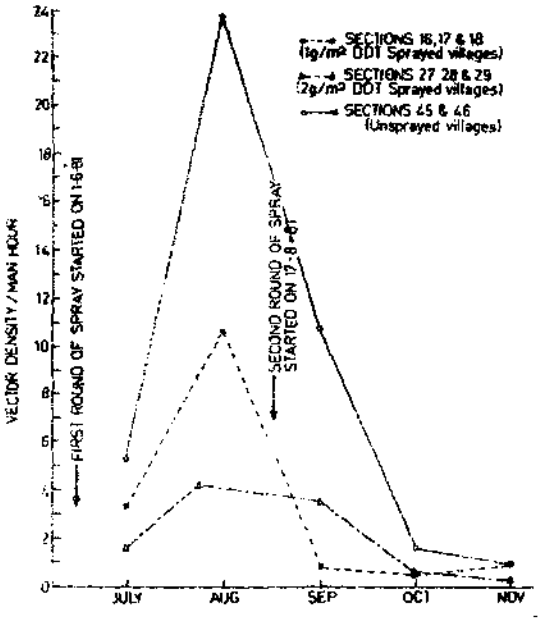

Fig. 2. Man hour density of *A. culicifacies* in the morning hand catches.

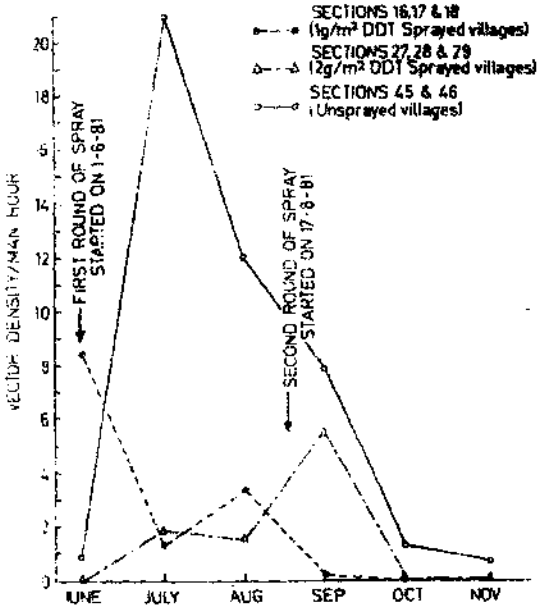

Fig. 3. Man hour density of *A. culicifacies* in the night hand catches.

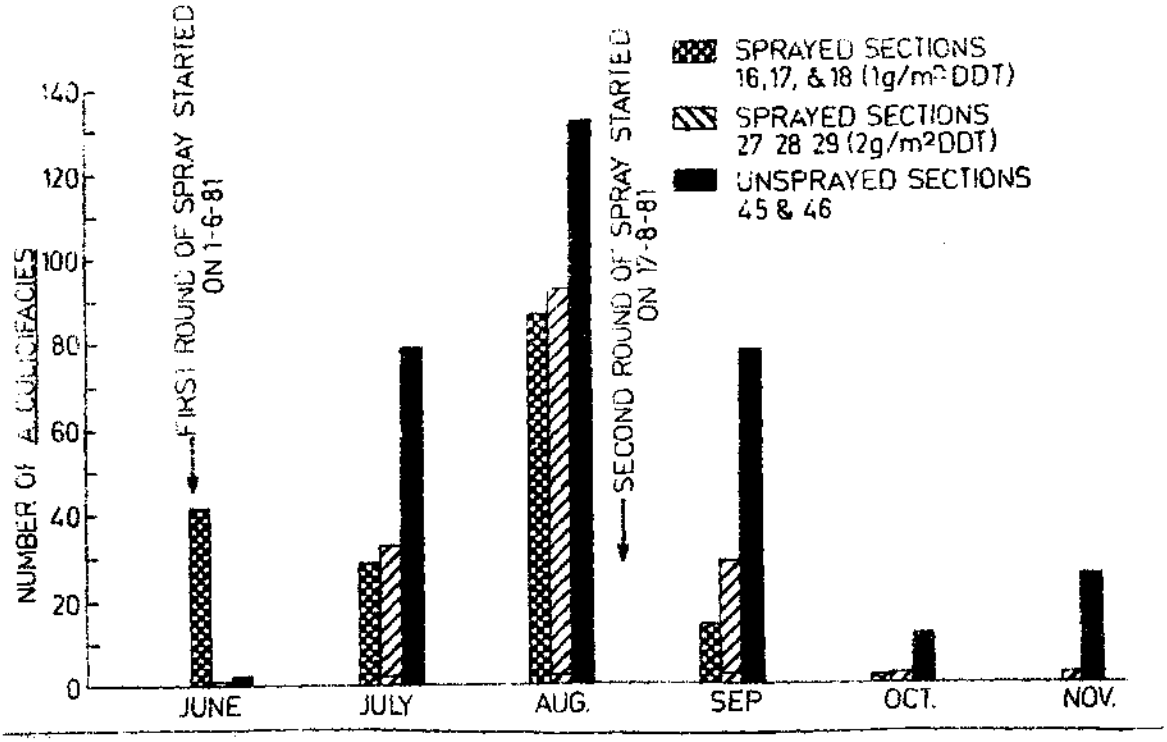

Fig. 4. Total *A. culicifacies* collected in 2 cattlesheds and 1 room by pyrethrum space spray method.

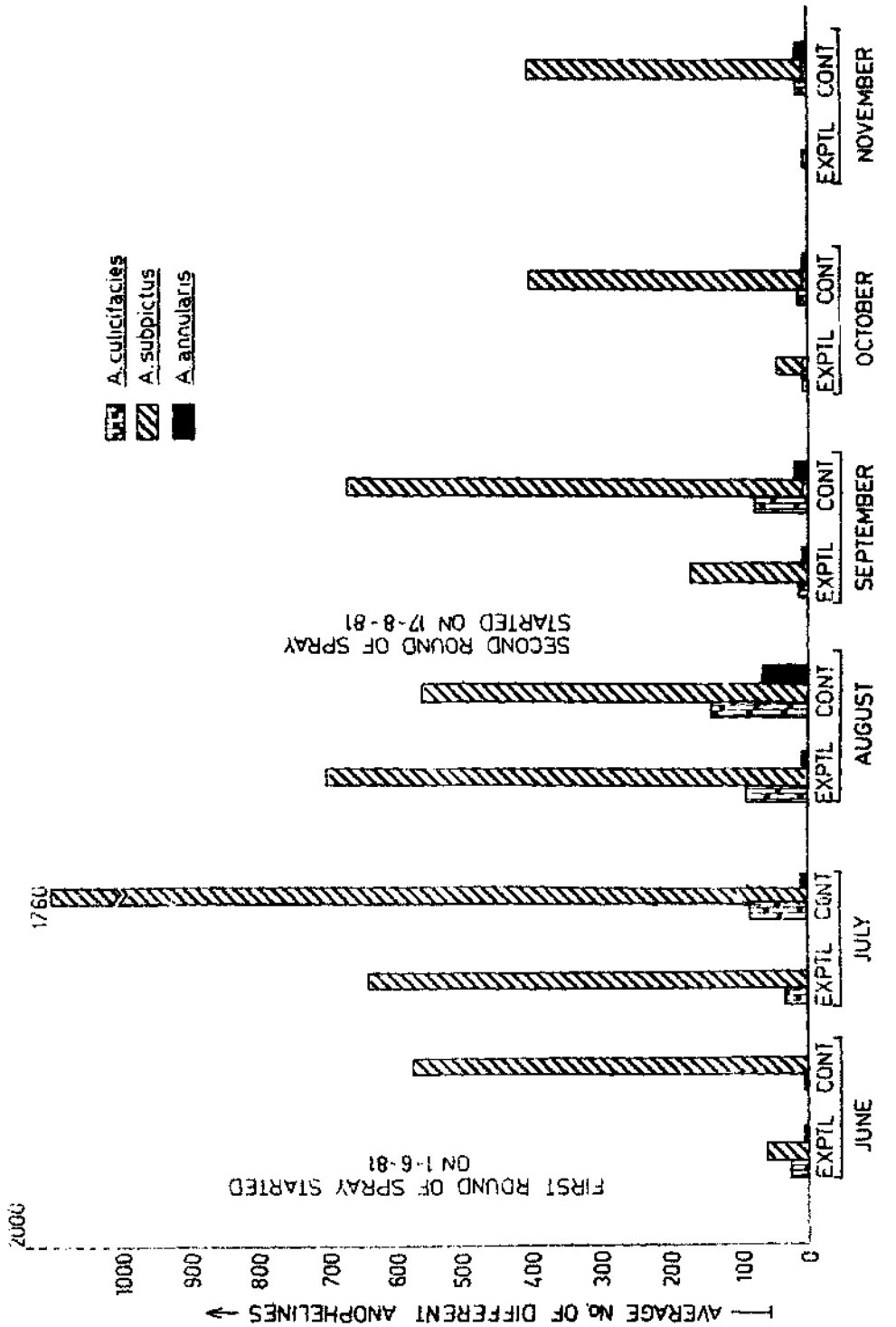

Fig. 5. Histogram based on total catches from 2 cattlesheds and 1 room showing relative density of anophelines in the study areas.

Table 2 — Dominant Anophelines found in pyrethrum space spray collections*

Month	Sprayed areas			Unsprayed areas		
	A. annularis	*A. culicifacies*	*A. subpictus*	*A. annularis*	*A. culicifacies*	*A.subpictus*
June	1.33	21.6	59.5	0	1.0	576.0
July	0.33	31.33	646.0	10.0	84.5	1780.5
August	6.66	90.66	705.16	65.5	137.5	565.0
September	16.66	21.5	171.5	22.0	78.5	676.5
October	1.66	0.5	47.66	6.5	11.0	418.0
November	0	0.5	4.0	16.0	14.0	418.5

* Average collection of mosquitoes per village. Data includes total number of mosquitoes collected from 1 room and 2 cattlesheds.

Table 3 — Epidemiological situation in the study areas

DDT dosage	Section	Population	September		October		November	
			B.S. Examined	S.P.R	B.S. Examined	S.P.R	B.S. Examined	S.P.R.
1g/m²	16	4766	92	16.3	220	5.0	151	1.32
	17	3516	142	15.6	144	7.64	81	6.16
	18	4160	66	4.5	61	8.80	123	11.38
	Total	12442	300	13.33	425	6.35	355	5.97
2g/m²	27	5535	396	34.3	329	20.65	119	29.4
	28	3823	523	36.7	305	40.65	132	25.00
	29	3480	437	42.3	299	20.10	165	9.09
	Total	12838	1356	37.9	933	28.29	416	19.94
Control	45	7030	506	45.4	77	41.56	285	41.75
(No spray)	46	7358	410	48.2	242	49.01	—	—
	Total	14388	916	46.72	319	47.03	285	41.75

Note: 1st round of DDT spraying started from 1-6-81 and second round from 17-8-81
The insecticide coverage varied from 65 to 80% with an average of 75%.
B.S. = Blood smear
SPR = Slide positivity rate

substantiated the entomological observations that there was no difference in villages sprayed at two dose levels. Studies also revealed a high incidence of *P. falciparum* malaria in study villages. *P. falciparum* rate in the sprayed and control villages pooled together was 19.2% in July (24 out of 125 cases), 62.4% in August (292 out of 468 cases, 61.6% in September (605 out of 982 cases) 32.6% in October (365 out of 442 cases) and 78% in November (174 out of 223 cases).

The study clearly brings out the usefulness of DDT in the resistant areas. A good coverage would provide adequate epidemiological impact and reduce the morbidity at a much lower cost than envisaged by the use of replacement insecticides.

ACKNOWLEDGEMENTS

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Help and cooperation of the technical staff of the project is gratefully acknowledged.

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Blocking of Malarial Transmission by a Gamete Vaccine Against *Plasmodium berghei NK-65*

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Effect of a gamete vaccine was studied in hamsters infected with *P.berghei*. Animals were immunized with three weekly intramuscular doses of irradiated gametes followed by a parasite challenge. Mosquitoes feeding on the immunized hamsters during patent blood infection showed a complete or nearly complete arrest of oocyst formation. This technique of active immunization had a minimal effect on the development of the asexual parasitaemia. Thus active immunization against sexual stages of *Plasmodium berghei NK 65* using irradiated gametes as the immunogen is capable of inducing effective transmission-blocking immunity even without any adjuvant.

INTRODUCTION

Malaria still remains one of the major public health problems in the tropics as vector eradication and chemotherapy programmes have met with serious difficulties. Attention is now being diverted to immunotherapy. Studies pertaining to successful immunization mainly involving use of the sporozoites (Nussenzweig, 1977 and Clyde *et al.*, 1975) and asexual blood stages (Brown *et al.*, 1970; Mitchell *et al.*, 1975 and Sympson *et al.*, 1974) give considerable support to the feasibility of a malarial vaccine. Recently, immunization using formaline treated or X-irradiated gametes of avian malaria *Plasmodium gallinaceum* (Gwadz, 1976 and Carter and Chen, 1976), rodent malaria *P.yoelii* (Mendis and Targett, 1979) have resulted in an effective transmission-blocking immunity reducing oocyst production in the mosquito vector. Similar studies with *P.knowlesi* infection in rhesus monkeys (Gwadz and Green, 1978) have demonstrated transmission-blocking immunity as well as resistance to asexual stages using Freund's adjuvant.

The present work reported in this paper relates to an effective immunization of hamsters against sexual stages of *P.berghei NK 65* using X-irradiated gametes. Use of an adjuvant was not a necessary prerequisite.

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MATERIAL AND METHODS

Animal— Six week old hamsters of either sex were used as vertebrate hosts and *Anopheles stephensi* were used as vector for cyclic transmission. Anopheles colony is being maintained in the laboratory under standard conditions.

Parasite— *P.berghei* NK 65 was maintained by cyclic transmission through vector *A.stephensi*. Parasites from the first vector transmitted infection in hamsters were used for propagating infection in experimental animals.

Media— (a) Suspended animation medium (SAM) composition:
(i) 0.21% Tris;
(ii) 0.96% NaCl;
(iii) 0.2% Glucose
adjusted to pH 7.4.

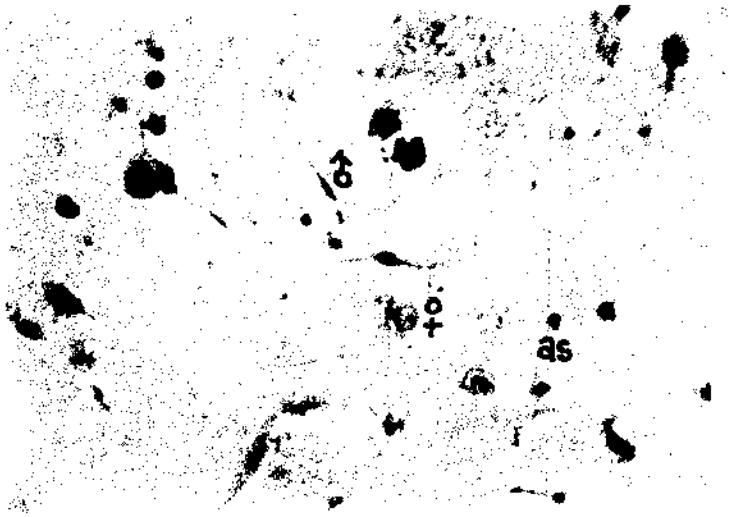

Fig. 1 Gamete preparations used for immunization stained with Giemsa stain (x 1600). ♂ -microgamete, ♀ - macrogamete and as- asexual blood stage of the parasite.

(b) Gamete releasing medium (GRM) composition:
- (i) 2.5 volumes of 5% NaCl:
- (ii) 2.5 volumes of 10% glucose:
- (iii) 20 volumes of 1.46% $NaHCO^3$:
- (iv) 100 volumes of inactivated hamsters/rat serum adjusted to pH 8.0.

Isolation of gametes— Blood from hamsters with 25-40% parasitaemias and 1-2% gametocytes of *P.berghei* NK 65 was collected by cardiac puncture in heparinized tubes. Gamete isolation was achieved as described by Carter and Chen (1976) with minor modifications. Heparinized, infected blood was mixed with 50 volumes of SAM; the suspension was centrifuged at 500 g for 5 minutes. The pellet was resuspended in three volumes of GRM, incubated for 35-40 minutes at 25°C to facilitate complete gametogenesis and then centrifuged at 500 g for 5 minutes. The supernatant was then recentrifuged at 18000 g for 15 minutes and the pellet resuspended in 0.5 ml SAM. The 18000 g pellet contained mainly gametes (micro and macro) some asexual blood stages of the parasite, red cells and acellular debris. The relative amount of gamete and non-gamete material was counted by examining Giemsa stained smears of the pellet (Fig. 1). The material used for immunization contained about 45-50% microgametes, 20% macrogametes and 30-35% asexual parasite stages.

Immunization of hamsters— 18000 g pellet was suspended in SAM and x-irradiated with 20 k Rad. The concentration of microgametes in the suspension was counted using haemocytometer. A group of six, 6 weeks old hamsters were immunized with three doses of $5x10^5$, $1.5x10^6$ and 3.5×10^6 microgametes respectively at seven days interval by intramuscular injection (Table 1).

Parasite challenge— 12 days after the last immunization dose, the immunized and an equal number of age-matched control hamsters were inoculated i.p. with 10^7 parasitised red cells. Thin blood films were made daily, stained with Giemsa and the parasitaemia and gametocytaemia counted per 10^4 red blood cells.

Table 1 — Immunization of hamsters with irradiated (20k Rad) gametes of *Plasmodium berghei NK 65* and result of subsequent challenge with 10^7 PRBC

No.	No. of gametes and days of immunization			Challenge with PRBC on Day*	Prepatent period in days	Percent peak parasitaemia
115	1×10^8	1.5×10^8	3.5×10^8	27	4	25
116	"	"	"	27	2	40
117	"	"	"	27	4	20
118	"	"	"	27	4	25
119	"	"	"	27	4	25
120	"	"	"	27	4	20
121	C O N T R O L			—	1	40
122				—	1	40
138				—	2	40
139				2	2	40
140				—	2	45
141				—	2	50

* Day 27 after 1st immunization and day 12 after last immunization.

Infection to mosquitoes— Groups of 20 female *A.stephensi* mosquitoes were caged for each hamster and kept fasting overnight prior to infective blood meal. Immunized and control hamsters showing initial 2-5% parasitaemia (Table 2) on day 7 after challenge were anaesthetised and kept on the top of 12 mosquito cages. After 30 minutes, blood feeding was obvious from the abdominal swelling of the mosquitoes. The unfed mosquitoes were excluded from the experiments.

Oocyst count— On day 9 after infective blood meal, 10 female mosquitoes' guts were dissected from each cage and stained with 5% Giemsa for 15 minutes. The number of oocyst per mosquito gut were counted.

RESULTS

Course of patent parasitaemia— Immunization of hamsters against sexual stages of *P.berghei NK 65* using x-irradiated gametes had little effect on the ultimate course of patent blood infection. All the immunized and the control hamsters succumbed to infection between 16 to 18 days after challenge. However, there was an increase in the prepatent period from 2 to 4 days in the immunized animals. Peak parasitaemias achieved in all immunized hamsters on days 4 to 14 compared well with the peak parasitaemias in the control. However, a marginal difference was observed on day 15 when the peak parasitaemia was lower in the immunized group (26.6+ 6.19%) versus 41.0+ 6.0%), but had no effect on the survival of the animals (Fig.2; Table 1).

Immunization did not affect gametocyte production & morphology. The gametocyte counts relative to asexual parasites were similar in both immune and control hamsters.

Infectivity to mosquitoes— Ten female mosquitoguts from each group of mosquitoes were examined on day 9 after the infective blood meal for oocyst production. Mosquito-guts from all the six groups of mosquitoes fed on unimmunized hamsters during patent infection developed large number of oocysts (Fig.3). The total number of oocysts in individual mosquito-gut varied from 51-151. As shown in Table 2, the mean number of oocysts in each group of

Table 2 Oocyst production in mosquitoes fed on immunized and unimmunized hamsters after challenge with 10^7 PRBC.

No.	% parasitaemia* on day of feeding mosquitoes	Mean number of oocysts per mosquito gut in each group
Immunized		
115	2	0
116	2	1.10
117	2	0
118	4	0.82
119	4	0.70
120	5	0
Unimmunized		
121	5	81.1
122	5	73.0
138	2	81.9
139	3	76.2
140	4	73.9
141	4	78.3

*% parasitaemia on day 7 after challenge with 10^7 PRBC.

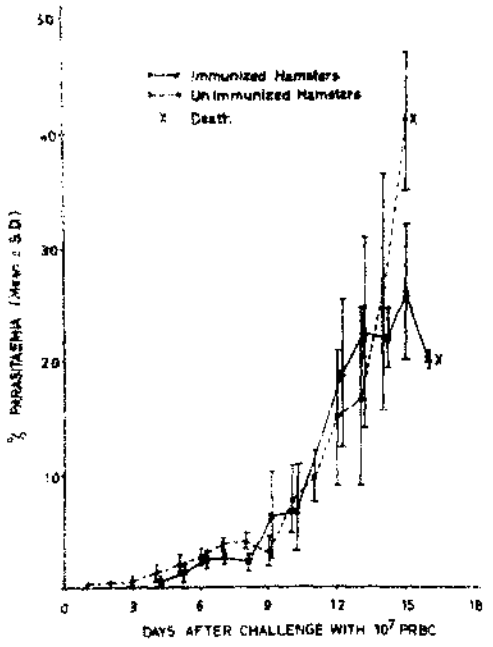

Fig.2 Effect of Gamete vaccine on the course of *P.berghei NKGS* infection.

mosquitoes ranged between 73-81.9 per mosquito-gut.

On the other hand, amongst mosquitoes fed on immunized hamsters, in three out of six groups, mosquitoes failed to develop oocysts. While in the remaining three groups a few mosquitoes developed a small number of oocysts; the yield of oocysts from individual mosquito gut varied from 0.7 to 1.1. Table 2 represents the mean numbers of oocyst per mosquito-gut fed on immunized hamsters and unimmunized hamsters. Thus, in our experimental studies immunization of hamsters, against sexual stages using x-irradiated gametes without any adjuvant showed a complete or nearly complete arrest of oocyst production during the period of patent infection.

DISCUSSION

Immunization against sexual stages of the malarial parasite *P.gallinaeum* in chickens (Gwadz, 1976; Carter and Chen, 1976) and *P.yoelii* in mice (Mendis and Targett, 1979) using irradiated or formaline treated gametes had shown effective

Fig 3. Gut of a mosquito fed on unimmunized hamster.
Note: Oocyst formation in the gut.

transmission-blocking immunity. In the present study immunization of hamsters in three weekly intramuscular doses by x-irradiated gametes of *P.berghei NK 65* parasite and challenge with parasitised red cells 12 days after the last immunization dose resulted in effective transmission-blocking immunity. This type of vaccination had very little effect on the development of the asexual stages of the parasite in blood. Similar observations had been made by Carter and Chen (1976) in chickens. The development of gametocytes was also uninterrupted in the immunized animals as also observed by Gwadz(1976); i.e., gametocytes from immunized and nonimmune animals, when washed and resuspended in normal plasma or serum, exflagellated and the gametes sustained activity. Although the immunized hamsters revealed a mild increase in the prepatent period (4 days as compared to 2 days in the control), yet the peak parasitaemias were not significantly different in the two groups except on day 15. The peak parasitaemia on this day in the control hamsters was 41.0+6.0% as compared to 26.6+6.19% in the immunized hamsters. Yet the infection was lethal in both the groups and all the animals succumbed to infection within 16-18 days of challenge. Using a similar schedule of immunization of mice against sexual stages of *P.yoelii* parasite, Mendis and Targett(1979) demonstrated resistance to asexual stages as well and complete reduction of oocyst production. The contamination of the asexual stages in the gamete vaccine might have been responsible for the resistance of host to parasite in their study or the gametes might have protective antigens in common with the asexual stages. Gwadz and Green (1978) also demonstrated the induction of effective transmission-blocking immunity alongwith resistance to asexual stages in rhesus monkeys immunized against sexual stages of *P. knowlesi* parasite using gametes emulsified in Freund's complete adjuvant. The observed differences could be due to the differing patterns of immune responses to sexual stages in the various animal models and the species of plasmodia investigated.

Immunization of hamsters against sexual stages of *P.berghei NK 65* parasite using crude gamete preparations consitently suppressed infectivity to mosquitoes during patent blood infection. The crude gamete preparations contained both female and male gametes. Mosquitoes allowed to

feed on immunized hamsters, during patent blood infection showed complete or nearly complete arrest of oocysts production in their guts. Carter *et al.* (1979a) have demonstrated that purified gamete preparations from which the gametes of one or the other sex were absent, were poorly immunogenic when compared with the mixed gamete preparation.

Carter *et al.* (1979b) have also highlighted the *mechanism* of oocyst suppression where ookinete formation was totally suppressed when the immune serum was present from the time of initiation of gametogenesis up to the time of fertilization. Immune serum had no effect on the development of ookinete once fertilization had occurred *in vitro*. These observations suggest that transmission-blocking immunity is mediated by the neutralization of extracellular gametes prior to fertilization in mosquito midgut by antigamete antibodies ingested by the mosquitoes in blood meal (Carter and Chen, 1976) The precise nature of the antibodies participating in the blocking of parasite transmission is not known. Gamete antibodies in the serum of immunized animals immobilize the male gametes within seconds of exflagellation. This almost immediate immobilization of fresh gametes could limit fertilization and possibly explain the consequent reduction of oocysts in mosquitoes. Gwadz(1976) showed that immobilizing activity resided in the IgG fraction of immune serum. Recently, Carter *et al.* (1979b) identified two types of antibodies in the serum of immunized animals against sexual stages of the malaria parasite: (i) microgamete surface-fixation type of antibodies (SF) and (ii) gamete agglutinating type of antibodies (AG). AG type of antibodies were found frequently even when immunization had been inadequate to suppress transmission of blood infection to mosquitoes. In contrast to AG antibodies, SF type of antibodies were directly related to effective transmission-blocking immunity.

Although, previous studies on *P.knowlesi* using gamete vaccine have revealed that the use of adjuvant was mandatory, yet in *P.berghei NK 65* as in avian plasmodia effective immunization could be achieved without any adjuvant. It would be of immense interest to clearly define the protective antigens and to specify the antibodies using hybridoma technique.

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Seroepidemiology of Human Malaria: Indirect Haemagglutination Test Using the *Plasmodium knowlesi* Antigen

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Using Plasmodium knowlesi antigen and glutaraldehyde-treated sheep erythrocytes, an indirect haemagglutination (IHA) test was used in the diagnosis of malaria. Results revealed that *P. knowlesi* can be successfully used in IHA test in human malaria. The study also revealed that Shahjahanpur and Mathura had long standing malaria infection and Unnao had fresh focus of infection

INTRODUCTION

Resurgence of malaria has brought into prominence importance of the development of reliable serological tests. These tests can be used in the diagnosis of clinical cases as well as in the seroepidemiological studies. The indirect haemagglutination (IHA) test is simple and inexpensive and has been used in the diagnosis of clinical cases of malaria and in the detection of malaria antibody titres (Meuwissen *et al.*, 1972; Meuwissen 1974; WHO 1974; Agarwal *et al.*, 1981). Though human *Plasmodium falciparum* antigen is considered ideal for IHA test (Meuwissen *et al.*, 1973; Mathews *et al.*, 1975), several workers have successfully employed simian malaria (*P.knowlesi*) antigen for serological studies in human malaria (Kagan *et al.*, 1969; Agarwal *et al.*, 1981). Since human malaria antigen is not available in sufficient quantity for the large scale seroepidemiological surveys of malaria, the Indian Council of Medical Research task force recommended the standardization and evaluation of *P.knowlesi* antigen for serodiagnosis of human malaria. We, therefore, prepared the antigen from *P.knowlesi* and successfully used this in the IHA test for screening sera collected from slide positive malaria cases, cases of pyrexia of varied origin, random hospital patients and healthy subjects. Results of this study are reported in this paper.

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MATERIAL AND METHODS

Antigen— Soluble *P.knowlesi* antigen used for sensitization of sheep erythrocytes was prepared from experimentally infected rhesus monkey according to the procedure described by Agarwal *et al.* (1981). Briefly, schizont infected erythrocytes were separated using Ficoll-Conray gradient. Schizonts were obtained by saponin lysis of the infected erythrocytes and stored at —196° C in liquid nitrogen. On the day of test, an aliquot of these schizonts was disrupted by ultrasonication and the soluble fraction was used as antigen (Meuwissen *et al.*, 1972; WHO 1974). Same batch of antigen was used throughout the studies reported in this paper.

Sera: Serum samples of 155 slide positive malaria patients collected at the time of slide examination from the field at Unnao, Shahjahanpur, Mathura and Lucknow districts of Uttar Pradesh were tested by the IHA test. Out of these cases 101 suffered from *P.falciparum* infection, 51 from *P.vivax* and 3 from the mixed infection. Control groups consisted of sera from (i) 28 patients of pyrexia of varied origin (ii) 118 random hospital patients, and (iii) 130 healthy subjects. All the controls included were slide negative for malaria. The sera were stored at —20°C and inactivated at 56°C for 30 minutes prior to test.

IHA Test:— IHA test was carried out in the 96 well (U-bottom) Cooke No. 1-220-6 permanent lucite microtire plates. Antigen coated sheep RBC pretreated with glutaraldehyde were prepared essentially as described by WHO (1974). The test was performed and results were recorded as suggested by Meuwissen *et al.* (1972). The controls included a known positive and a known negative serum in each series of the test.

RESULTS

Results of the IHA test using glutaraldehyde treated SRBC's are summarized in Table No. 1. At an IHA titre of 1 in 32 which was taken as the diagnostically significant titre (Agarwal *et al.* 1981), 84.5% of the malaria patients were found positive while only 7.14, 4.23 and 1.53% of the cases of pyrexia of varied origin, random hospital patients and healthy subjects respectively yielded positive IHA results. The IHA test was positive in 89.1% cases of *P.falciparum* infection as compared to 74.5% cases of *P.vivax* infection. Geometrical mean reciprocal titres (GMRT) of 234.1, 1.724, 4.571, and 3.712 were obtained for malaria patients, pyrexia patients, random hospital patients and healthy persons respectively. Among patients with *P.falciparum*, *P.vivax* and mixed infection, GMRT of 293.6, 121.3 and 1024 respectively, were obtained.

Locality-wise break-up of the IHA results (Table 2) indicated that 33.3% cases of malaria in Unnao, 98.18% in Shahjahanpur, 80% in Mirzapur, 97.36% in Mathura and 72.72% in Lucknow, gave positive IHA reaction. Of the *P.falciparum* cases, 40% from Unnao, 97.61% from Shahjahanpur, 72.72% from Mirzapur and 97.36% from Mathura were found positive. Of the *P.vivax* cases, 25% from Unnao, 100% from Shahjahanpur, 84.21% from Mirzapur, 66.6% from Mathura and 72.72% from Lucknow gave positive IHA result. All the three cases of mixed infection were positive.

DISCUSSION

Progress in the seroepidemiological studies on malaria was slow because of the non-availability of suitable antigens in the country. The present work extends our earlier study (Agarwal *et al.* 1981) and confirms that in an endemic situation, *P.knowlesi* antigen can be successfully employed in the IHA test for human malaria. This is in contrast to the findings of Chandanani *et al.* (1981) who reported low positivity of IHA test (47.2%) among malaria patients using *P.knowlesi* antigen.

Results of the present study are suggestive of the subtle differences in the period prevalence of malaria in different localities as indicated by differences in the seropositivity and GMRT's. Thus, an overall seropositivity of above 97% in Shahjahanpur and Mathura areas (Table 2) tends to demonstrate these areas were centres of long-standing malaria infection. This fact is further corroborated by the very high GMRT i.e. 566.2 in Shahjahanpur cases and 547.8 in Mathura cases. In contrast, low seropositivity in Unnao cases (33.3%) together with low GMRT (9.699) indicates that this area represented a fresh focus of malaria infection.

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Table 1 — Reciprocal IHA titres obtained among different groups using glutaraldehyde treated sheep erythrocytes sensitized with *P. knowlesi* antigen.

Study Group	No. of cases	GMRT	No. of cases showing reciprocal IHA titre											
			4096	≥2048	≥1024	≥512	≥256	≥128	≥64	≥32	≥16	≥8	≥4	≥2
1. Malaria patients	155	234.1	6 (3.87)	21 (13.55)	58 (37.41)	87 (56.12)	103 (66.45)	114 (73.54)	116 (74.84)	131 (84.51)	132 (85.16)	143 (92.25)	154 (99.25)	155 (100)
a. *P.falciparum* infection	101	293.6	1 (0.99)	11 (10.89)	41 (40.59)	62 (61.38)	74 (73.26)	80 (79.20)	82 (81.18)	90 (89.10)	90 (89.10)	95 (94.05)	101 (100)	101 (100)
b. *P.vivax* infection	51	121.3	5 (9.8)	9 (17.64)	15 (29.41)	22 (43.13)	26 (50.98)	31 (60.78)	31 (60.78)	38 (74.50)	39 (76.47)	45 (88.23)	50 (98.04)	51 (100)
c. Mixed infection	3	1024	Nil	1 (33.3)	2 (66.6)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
2. Pyrexia of varied origin	28	1.724	Nil	Nil	Nil	Nil	Nil	Nil	Nil	2 (7.14)	2 (7.14)	2 (7.14)	5 (17.85)	11* (39.28)
3. Random hospital patients	118	4.571	Nil	Nil	Nil	Nil	Nil	Nil	2 (1.69)	5 (4.23)	20 (16.94)	39 (33.05)	75 (63.55)	118 (100)
4 Healthy subjects	130	3.712	Nil	Nil	Nil	Nil	Nil	Nil	Nil	2 (1.53)	10 (7.69)	31 (23.84)	73 (56.15)	130 (100)

Figures in parentheses indicate cumulative percentages

GMRT = Geometric mean reciprocal titre

Titres of 1 in 32 were considered positive

*Out of 28 cases of pyrexia of varied origin, 17 cases showed a titre of less than 1 in 2.

Table 2 — Locality-wise breakup of reciprocal IHA titres obtained from 153 malaria patients using glutaraldehyde treated human erythrocytes sensitized with *P. knowlesi* antigen

Locality	No. of cases	GMRT	No. of cases showing reciprocal IHA titre											
			4096	≥2048	≥1024	≥512	≥256	≥128	≥64	≥32	≥16	≥8	≥4	≥2
1. Unnao	18	9.699	Nil	Nil	Nil	Nil	Nil	Nil	Nil	6 (33.3)	6 (33.3)	12 (66.6)	17 (94.4)	18 (100)
2. Shah-jahanpur	55	566.2	1 (1.8)	7 (12.72)	25 (45.45)	41 (74.54)	51 (92.72)	54 (98.18)	54 (98.18)	54 (98.18)	54 (98.18)	54 (98.18)	55 (100)	55 (100)
3. Mirzapur	30	65.49	Nil	Nil	1 (3.3)	5 (16.6)	9 (30)	15 (50)	16 (53.3)	24 (80)	24 (80)	27 (90)	30 (100)	30 (100)
4. Mathura	41	547.8	1 (2.43)	7 (17.07)	24 (58.53)	33 (80.48)	35 (85.36)	37 (90.24)	38 (92.68)	39 (95.12)	39 (95.12)	39 (95.12)	40 (97.56)	41 (100)
5. Lucknow	11	580.5	4 (36.36)	7 (63.63)	8 (72.72)	8 (72.72)	8 (72.72)	8 (72.72)	8 (72.72)	8 (72.72)	9 (81.81)	11 (100)	11 (100)	11 (100)

Figures in parentheses indicate cumulative percentage values.
GMRT = Geometric mean reciprocal titre.

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Karyotypic Variations in *Anopheles culicifacies* Complex

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Cytogenetical studies revealed that the Y-chromosome is submetacentric in species A and acrocentric in species B of the taxon *Anopheles culicifacies*. This finding can be used in the identification of sibling species as an alternative to polytene chromosome method.

INTRODUCTION

Anopheles culicifacies is an important vector of malaria in the Indian sub-continent. In this taxon, Green and Miles (1980) found 2 fixed paracentric inversions in one population which also had unidirectional sterility with the other population. This finding resulted in splitting of the taxon, *A. culicifacies* into two sibling species i.e., species A and species B. The one originally described by Saifuddin *et al.*(1978)was termed species A and the other species B.

Species A has been incriminated as vector of malaria in two localities around Delhi (Subbarao *et al.*,1980) and species B was experimentally incriminated (M.R.C. Annual Report, 1980). Further, a high sporozoite rate was found in *A. culicifacies* during long term epidemiological and entomological studies in Haryana villages (D.S. Choudhury, personal communication). It was therefore important to study the distribution and the vectorial status of the two sibling species in different ecological frames of the country. For such a study an easier and alternate method of identification was essential. The ovarian polytene technique was difficult during those months when adult females of the required ovarian stage were few in the field. Cytogenetical studies with *A. culicifacies* revealed some interesting differences in the male mitotic chromosomes of species A and species B. In a few strains, X-chromosomal polymorphism was also observed. Results of this study are reported in this paper.

MATERIAL AND METHODS

A. culicifacies strains of Mandora (Haryana), Basantpur (Haryana), Okhla (Delhi), Aurangabad (Maharashtra) and Sirolifarm (terai, Nainital district, U.P.) were used in this study. These strains were colonized in our laboratory following the procedures developed by Ainsley, 1976 & Ansari *et al.*, 1977. Pure colonies of species A and species B were established with the help of polytene chromosome identification. Mitotic chromosomes of these strains were prepared from the neurogonial cells of the IV instar larvae by squash technique of Breland (1961) with slight

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modifications. The larvae were placed in 0.01% colchicine for 1 - 1½ hours and the brain tissue was removed, stained in 2% lactoaceto -orcein for 10 minutes and squashed. To ascertain the diploid number, metaphases of both the species were scored. Chromosomes were karyotyped and measured for the construction of a composite idiogram for all the strains studied. They have been classified according to Levan *et al.* (1964).

RESULTS AND DISCUSSION

A total of 2983 well spread metaphases from 60 females and 69 males (Table 1) were counted to establish the diploid number and study the karyotypic variations for the construction of the idiogram (fig 1, 2 & 3). The karyotype of species B consists of two pairs of autosomes and a sex chromosomal pair, the sex chromosomal mechanism being XX in the females and XY in the males as described for species A by Sakai *et al.* 1977.

Table 1 Number of male and female larvae utilized and the metaphases scored.

Strain used	Number of larvae examined		No. of metaphases scored	
	♂	♀	♂	♀
Mandora	20	10	584	221
Hasanpur	12	12	185	225
Dekhla	10	16	112	96
Aurangabad	15	15	412	448
Sirsilfarm	12	13	297	403
Total	69	80	1590	1393

In all the strains examined the autosomal pairs 2 and 3 were metacentric with an arm ratio of 1:1.3 and 1:1 respectively. Chromosome 2 was the largest pair and comprises 36-39% of the total chromosomal length (TCL) of the haploid genome. Chromosome 3 was the second largest pair comprising of 32-35% TCL. The sex chromosome pair was the smallest, the X-chromosome varying from 25-27% of TCL. However, in species B strain of Aurangabad, chromosome 3 constituted 28% of TCL and was much smaller than the sex chromosomal pair (XX) which comprised of 35% of TCL. This rare instance of the X-chromosome being larger than the chromosomal pair 3 may be the result of a translocation of chromosomal material from chromosome 3 to X chromosome during the course of evolution. Studies are in progress to examine this. Genetic crosses are being made between Aurangabad strain and Delhi strain to study the hetrozygotes, if any. G-banding studies are also in progress to confirm whether there is any translocation between X and the chromosomal pair 3. The details of karyotypic analysis are summarized in

Table 2– Karyotypic analysis of *An. culicifacies* strains.

Strain	Percentage of total chromosomal length Chromosome			
	2	3	X	Y
Mandora (Species A)	37.96 (1.3)	34.51 (1.1)	27.84 (2.)	25.50 (2.5)
Hasanpur (Species A)	39.52 (1.4)	34.82 (1.2)	26.05 (2.5)	25.13* (2.5)
Dekhla (Species B)	37.75 (1.3)	34.8 (1.1)	28.0 (2.0)	26.9**
Aurangabad (Species B)	37.74 (1.4)	28.5 (1.0)	35.04 (2.0)	27.78**
Sirsilfarm (Species B)	38.36 (1.2)	32.7 (1.0)	30.76(XLHM) (2) 26.0 (XLHM) (2) 35.77 (XLHT) (2.5) 38.0 (XsHT)	26.18**

* Submetacentric

** Acrocentric

XLHM = X large, homomorphic

XsHM = X small, homomorphic

XsHT = X small, heteromorphic

XLHT = X large, heteromorphic

Note: The figures in parentheses indicate the arm ratio.

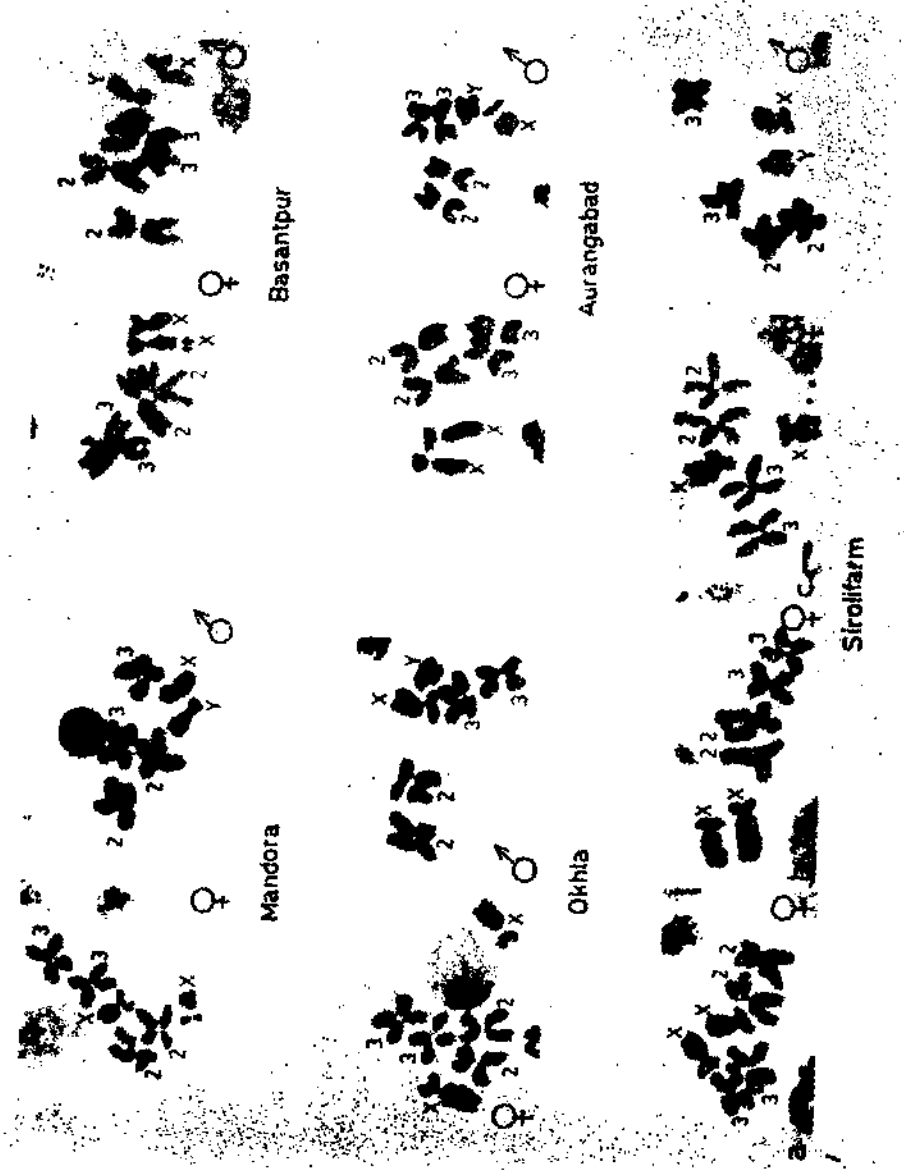

Fig. 1 Mitotic chromosomes from the neurogonial cells of male and female *A. callicfacies* from Mandora, Basantpur, Okhla, Aurangabad and Sirolifarm.

Note:—

In the female of the Basantpur strain, one of Xs (larger one) shows a satellite in the telomeric region of the long arm. In the Sirolifarm strain, three different types of females were found:

(a) Females with two homomorphic large Xs.

(b) Females with heteromorphic Xs - one large & one small

(c) Females with homomorphic small Xs.

Fig. 2. A comparison of the male sex chromosomes from different strains of *A. culicifacies* to show that no difference exists between the Xs of all strains while the Y Chromosome shows structural variation i.e., submetacentric in species A & acrocentric in species B.

Table 2. An idiogram has been constructed on the basis of these measurements

The X-chromosome in all the strains of species A and species B was submetacentric with an arm ratio ranging from 1:2 to 1:2.5. The TCL of the X displayed some variation among the different strains. The Y chromosome in species A was submetacentric with an arm ratio of 1:2.5 and in species B, it was an acrocentric. Therefore, the Y-chromosome can be used in the identification of the species A and B. It may be pointed out that the genomic size (in term of % TCL) of the Y chromosome was almost the same (25-27%) in both the species and the structural variation exhibited may be due to chromosomal rearrangement, possibly by a pericentric inversion. To test the validity of sibling species identification on the basis of centomeric position in Y chromosome we made use of the field material from Basantpur village which had natural populations of species A and species B. Simultaneous preparation of the salivary gland polytenes and mitotic chromosomes from the IV instar larvae of single female cultures were made. Out of the 18 cultures tested by the polytene technique, 12 were identified as species A and 6 species B. The mitotic chromosomes prepared from the brain cells of the males also identified the same 12 cultures as belonging to species A and the other 6 to species B. The method also provided correct identification of laboratory populations of Gopalpur and Pondicherry strains.

An interesting X chromosomal polymorphism was observed in some of the strains. In the females of Basantpur strain, one of the Xs showed a distinct satellite in the telomeric region of the long arm (fig. 1, Basantpur).

In the Sirolifarm strain, three different types of females were observed with reference to the variation in the size of the X chromosomes. In the first category of females, both the X chromosomes were large (XLHM i.e. homomorphic large Xs) comprising of 31% of TCL (fig. 1, Sirolifarm -a) while in the second category one of the Xs was large (XLHT i.e., larger of the heteromorphic females) comprising of 35% of TCL and the other was relatively small (XsHT i.e., small X of heteromorphic females) comprising of 28% TCL (fig. 1, Sirolifarm-b). In the third category of females both X-chromosomes were small (XsHM - i.e., homomorphic small Xs) constituting 26% of TCL (fig. 1, Sirolifarm-C). It was observed that the females with homomorphic large Xs (XLHM) constituted 65% of the population in the sample studied from this strain, while the females with heteromorphic Xs (XLHT XsHT) constituted 35% of the population. Only one individual was found having homomorphic small Xs (XsHM). Explanation for the presence

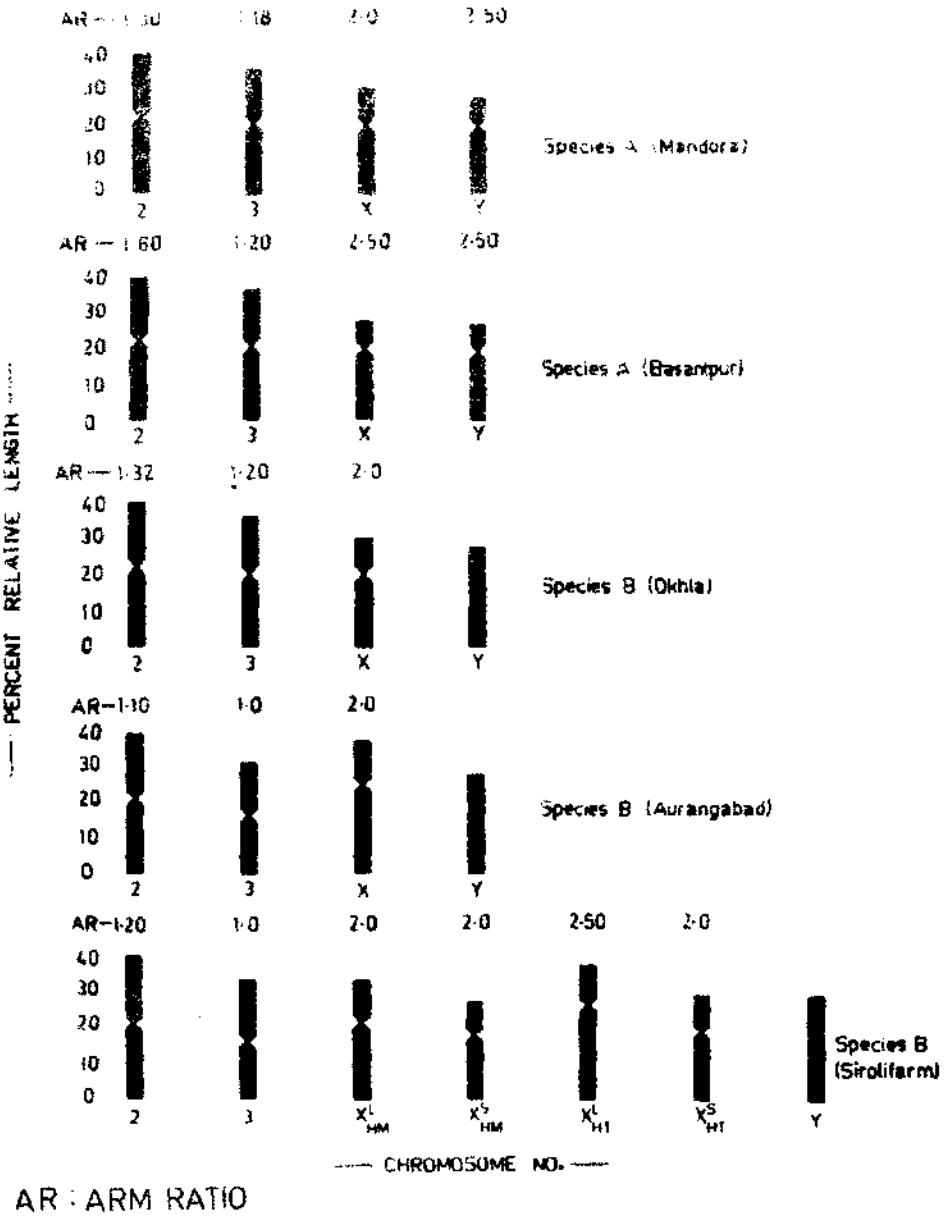

Fig. 3 Composite idiogram of different strains of species A and species B of *A. culicifacies*.

of only such individual is not readily available and further studies are in progress to explain this occurrence.

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Enzyme-linked Immunosorbent Assay Test in the Diagnosis of Human Malaria

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An enzyme-linked immunosorbent assay (ELISA) for seroepidemiology of human malaria has been standardized. Using *Plasmodium falciparum* antigen, sera at a single dilution (1:200) from 344 malaria patients (Group I), 70 patients of varied origin (Group II), 75 random hospital patients (Group III) and 75 normal healthy subjects (Group IV) were tested for the presence of malaria antibodies. At a cut off point equivalent to mean+2SD (95% confidence limit) of normal subjects (Group IV), 100, 0, 9.6 and 12% cases respectively of group I to IV gave positive ELISA reaction. At mean+3SD (99.7 confidence limit), 99.3% cases of group I alone were positive and none of the cases included in Groups II to IV gave positive ELISA reaction at 99% confidence limit.

INTRODUCTION

In view of the resurgence of malaria in the country there is an urgent need to develop sensitive serological tests for the diagnosis of clinical cases, seroepidemiological surveys to monitor the existing malaria antibody levels in the population, detection of new foci of infection, and to assess the persistence of antibody levels in cases given radical curative treatment. Enzyme-linked immunosorbent assay (ELISA) provides a very sensitive test suitable for both diagnosis of clinical cases as well as the seroepidemiological survey (Engvall, 1977; Voller, 1980; Mahajan *et al.* 1981; Voller *et al.* 1980; Quakyi, 1980). Voller *et al.* (1977) reported that the sensitivity of the test was similar to that of radio-immunoassay. Antigen prepared from both *Plasmodium knowlesi* (Voller *et al.* 1975; Mahajan *et al.* 1981) and *P. falciparum* obtained from *Aotus* monkey (Voller *et al.* 1976), or *in vitro* cultures (Spencer *et al.* 1979) have been used earlier for the ELISA test, though the *P. falciparum* antigen has been shown to be more specific for the diagnosis of human malaria. In the present study ELISA test with *P. falciparum* antigen has been applied for screening of sera from malaria positive cases, cases with pyrexia of varied origin, random hospital cases and healthy subjects.

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MATERIAL AND METHODS

Serum samples from 344 slide positive malaria patients (Group I) collected at the time of slide examination were obtained from Unnao, Shahjahanpur, Mathura and Mirzapur districts of Uttar Pradesh. Of these malaria cases, 95 suffered from *P. falciparum*, 45 from *P. vivax* and the remaining 3 had mixed infection with both the parasites. Sera from 70 patients of pyrexia of varied origin (Group II), 75 random hospital patients (Group III) and 75 normal healthy subjects (Group IV) were included as control samples in the study. All control subjects were slide negative for malaria. Serum samples were

stored at −20°C and inactivated at 56°C for 30 min. prior to test.

ELISA was performed in 96 well (flat bottom) micro-ELISA plate, Cooke, No. 1-223-29 (Dynatech Laboratories, Singapore), essentially according to the method described by Agarwal *et al.* (1981), except that plates were coated with *P. falciparum* antigen and the optimal antigen dilution in each well was allowed to undergo air-drying. *P. falciparum*-antigen received as an aqueous extract from Dr. W.E. Collins, Centre for Disease Control, Atlanta, Georgia, U.S.A. was diluted to 1 in 1000 in the carbonate-bicarbonate buffer, this dilution had been found to be optimal for coating the antigen wells as determined by a chequer board titration. All the sera were tested at single dilution of 1 in 200 and horse radish peroxidase labelled anti-human IgG conjugate (Cappel Laboratories, Cochranville, U.S.A.) was used at a dilution of 1 in 2000 which had been found optimal for obtaining clear cut distinction between known positive and negative reference sera in a chequer board titration. Peroxidase activity was estimated using o-phenylenediamine dihydrochloride (OPD) after stopping the reaction with $SN\ H_2SO_4$. After diluting 200 µl of the contents of each well to 3 ml with glass distilled water, the readings were taken as extinction at 492 nm (E_{492}) using Spectronic-20® spectrophotometer. Results were analysed using Student's 't' test.

OBSERVATIONS

For the normal healthy subjects (Group IV), mean E_{492} value of 0.03685 with a standard deviation of 0.011 was obtained (Table 1). Cut-off point for the specific diagnosis of malaria could be any of the three confidence limits determined from E_{492} values of the normal healthy subjects. Thus, at mean+SD (E_{492}=0.04785), all the 143 cases of malaria (Group I), nine of the patients with pyrexia of varied origin (Group II), 31 of 75 (14.66%) random hospital patients (Group III) and 18 of 75 (24%) normal healthy subjects were found positive. At mean+2 SD, (E_{492}=0.05885) positivity of 100, nil, 5.6, and 12% was obtained for Group I to IV, respectively, and at mean+3SD (E_{492}=0.06985), 99.3% cases of malaria were positive while none of the control subjects included in Groups II to IV yielded a positive ELISA reaction (Table 1).

Highest E_{492} values were obtained amongst the patients from Shahjahanpur (mean 0.168±0.042, Table 2) followed by Mathura (mean, 0.144±0.0466). Purva (mean, 0.115±0.047) and Mirzapur (mean, 0.114±0.0237), suggesting that the antibody titres in Purva and Mirzapur were lower compared to Shahjahanpur and Mathura areas.

DISCUSSION

In the conventional ELISA test, the antigen solution (200 µl solution containing one µg. antigen protein) is coated in the wells of micro-titre plate for 18 hrs. in humid chamber at 4°C, which is followed by 1½ hr. incubation with test serum (malaria antibody) and then 1½ hr. incubation with anti-human IgG-peroxidase conjugate. In the final assay, the peroxidase activity is measured with OPD which gives a weak reaction which has to be directly measured without dilution of reaction mixture in the micro-ELISA recorder. In the present study the method of antigen coating was modified so that air-drying of 200 µl of malaria antigen in the wells of micro-titre plates at room temperature for 1-2 days, when followed by standard ELISA procedure, gives an intense colour in the final reaction mixture which could be diluted 15-fold (200 µl of the reaction product to 3 ml in distilled water) and the extinction values (E_{492}) easily recorded with the standard spectrophotometer.

Our results show high degree of sensitivity of ELISA test in the serodiagnosis of malaria. Even at the cut-off point equivalent to the mean+3SD of normal (control subjects) values i.e., E_{492} value of 0.06985, 142 of 143 (99.3%) cases of slide positive malaria were found positive while none of the control subjects (Groups II to IV) gave positive reaction. This higher positive percentage

Table I — Mean extinction values (E_{492}) among various groups and number of positive cases at different cut-off points determined from normal values.

Group	No. of cases	E_{492} (Mean ± SD)	No. of cases positive at		
			Mean + SD* (E_{492} = 0.04785)	Mean +2 SD* (E_{492} = 0.05885)	Mean + 3 SD* (E_{492} = 0.06985)
I. Malaria patients	143	0.1352 ± 0.04082 P<0.001	143 (100%)	143 (100%)	142 (99.3%)
a. *P.falciparum* infection	95	0.1492 ± 0.0049 P<0.001	95 (100%)	95 (100%)	95 (100%)
b. *P.vivax* infection	45	0.1272 ± 0.0317 P<0.001	45 (100%)	45 (100%)	44 (97.77%)
c. Mixed infection	3	0.1723 ± 0.045 P<0.01	3 (100%)	3 (100%)	3 (100%)
II. Patients of pyrexia of varied origin	70	0.0317 ± 0.00477 n.s	Nil	Nil	Nil
III. Random hospital patients	75	0.0400 ± 0.0121 n.s	11 (14.66%)	5 (6.66%)	Nil
IV. Normal healthy subject*	75	0.03685 ± 0.011	18 (24%)	9 (12%)	Nil

*Based on values of normal subjects (Group IV).
SD = Standard deviation
n.s. = non-significant

Table 2 -- Locality-wise break-up of E_{492} values and seropositivity among malaria patients

Locality	Infection	No. of cases	E_{492} (Mean ± SD)	No. of positive cases at		
				Mean + SD* (E_{492}=0.0478)	Mean + 2 SD* (E_{492}=0.05895)	Mean + 3 SD* (E_{492}=0.06985)
1. Unnao (P.H.C. Purva)	*P.falciparum*	10	0.1240 ± 0.0023	10 (100%)	10 (100%)	10 (100%)
	P.vivax	8	0.1127 ± 0.0442	8 (100%)	8 (100%)	7 (87.5%)
	Total	18	0.115 ±0.047			
2. Shahjahanpur (P.H.C. Kalan)	*P.falciparum*	40	0.1706 ± 0.0427	40 (100%)	40 (100%)	40 (100%)
	P.vivax	11	0.158 ± 0.0371	11 (100%)	11 (100%)	11 (100%)
	Mixed	3	0.1723 ± 0.0450	3 (100%)	3 (100%)	3 (100%)
	Total	54	0.168 ± 0.042			
3. Mirzapur (P.H.C. Shaktinagar)	*P.falciparum*	9	0.1020 ± 0.0004	9 (100%)	9 (100%)	9 (100%)
	P.vivax	21	0.1136 ± 0.0245	21 (100%)	21 (100%)	21 (100%)
	Total	30	0.114 ± 0.0237			
4. Mathura (P.H.C. Ral)	*P.falciparum*	36	0.1443 ± 0.0021	36 (100%)	36 (100%)	36 (100%)
	P.vivax	5	0.1404 ± 0.058	5 (100%)	5 (100%)	5 (100%)
	Total	41	0.144 ± 0.0466			

P.H.C = Primary Health Centre; SD = Standard Deviation

* Based on values of normal subjects (Group IV).

obtained in the present study in contrast to 86% positivity reported by Mahajan *et al.* (1981) after visual recording of the data may be attributed to the improvement in the procedure for antigen coating.

Significantly, higher E_{492} values were obtained for the malarious sera compared to the normal healthy controls ($P<0.001$). Shahjahanpur and Mathura not only contributed 66.43% (95/143) of the malarious sera but also recorded the highest E_{492} values (Table 2), indicating long standing exposure of the population to malaria. Relatively low E_{492} values of Unnao and Mirzapur point towards their being more recent foci of malaria. This underlines the importance of ELISA in seroepidemiological studies.

Based on the results of ELISA test with *P. falciparum* antigen, it may be pointed out that extinction values of mean+3SD (E_{492}=0.06985) based on the healthy controls may be used as a rigorous criteria for the diagnosis of malaria cases with active disease and high antibody level while less rigorous criteria of mean+2SD (E_{492}=0.05885) may be used as the cut off point to detect cases of both present and past infection.

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Plasmodium berghei: Regulatory Effects of Spleen Sonicates on the Uptake of Glucose *in vitro* by the Erythrocytes of Albino Rat and *Mastomys natalensis*

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Studies were carried out on the *in vitro* uptake of labelled glucose by erythrocytes of normal and infected albino rats and *Mastomys natalensis*, and of the effect of spleen sonicates from immune and susceptible animals, thereon. In the absence of spleen sonicate, erythrocytes from infected albino rat and mastomys registered 5 and 8 fold increases in glucose uptake respectively compared to erythrocytes from uninfected animals. Spleen sonicates from albino rat in the recovery stage blocked the transport of ^{14}C-glucose in normal or infected erythrocytes from both the species. In contrast, spleen sonicates from infected mastomys at peak parasitaemia facilitated transport. Observations suggest that, depending on the species of susceptible hosts and their immune status, spleen contains, factor s which affect uptake of glucose necessary for growth and development of intraerythrocytic stages of *Plasmodium berghei*.

INTRODUCTION

Since the discovery of *Plasmodium berghei* in Katangan tree rats, *Thamnomys surdaster*, by Vincke (1954) various species of rodents have been tried as an alternate host to this parasite for experimental studies. Albino rats, though susceptible to this infection, have generally been observed to develop sterile immunity. The infection is fatal in albino mice but the intensity of parasitaemia does not seem to be the immediate cause of death of the host. In recent years *Mastomys natalensis* has been found to be very susceptible to *P. berghei* with parasitaemias as high as 70-80% without the development of sterile immunity (Sen *et al.*, 1980). In addition to other possible factors, responsible for the varied susceptibility of different rodent species, penetrability of merozoites and permeability of nutrients, especially glucose into erythrocytes, are very important. The development of "crisis forms" within erythrocytes suggest changes in the erythrocyte permeability to nutrients (Quinn & Wyler, 1980). The spleen might have some effect on the permeability of erythrocytes. Experiments were therefore planned to study the permeability of labelled glucose into normal and infected erythrocytes *in vitro* and the modulating effect, if any, of spleen extract from normal and infected animals thereon. This paper reports the results of such studies.

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MATERIAL AND METHODS

D-glucose-U-^{14}C (specific activity 210 mCi/m-mole) was obtained from the isotope division, Bhabha Atomic Research Centre, Trombay, Bombay, India. All other chemicals used were of analytical grade. The strain of *P. berghei* used in this study was originally obtained from the

National Institute of Communicable Diseases, Delhi, and has been maintained in albino rats and *M. natalensis* by serial syringe passage.

Animals and infection: Eight week old male albino rats (C F strain) and *M. natalensis* drawn from their respective colonies of the Institute animal house were used. These were infected intraperitoneally with 10^6 parasitized erythrocytes, housed in plastic cages and fed with standard pellet diet (Hindustan Lever, Bombay, India) and water *ad libitum*. Parasitaemia was determined in thin blood smears made from tail and blood stained with Leishman's.

Preparation of red blood cell suspension: Blood was drawn into heparin from retro-orbital plexuses of normal and malaria-infected rodents. Cells were separated and washed five time by centrifugation (800 g, for 10 min at 4°C) in chilled phosphate buffered saline (PBS) pH 7.2, 155 mM and adjusted to 30% (V/V) in the same buffer.

Preparation of spleen extract: Spleens from normal and malaria-infected albino rat/mastomys were homogenized with chilled PBS and adjusted to 30% concentration (W/V). In each case the homogenate was centrifuged (800 g, 10 min at 4°C) and the supernatant was sonicated for 2 min at 20 KHz in an ultrasonic cell disruptor (Heat systems – Ultrasonics, Inc., New York, Model W-220F) on ice.

Glucose uptake: Freshly prepared 30% RBC suspensions (5 ml) were incubated with spleen sonicate (1 ml) for 15 min at 37±1°C and transport of radioactive glucose into erythrocytes was studied according to Sherman & Tanigoshi (1974). The method consists of adding 0.1 ml glucose solution containing 1 μCi ^{14}C-glucose (final concentration of sugar was 1 mM) to each flask, incubating for 10 min in an agitating water bath (80-100 strokes/min)set at 37±1°C and terminating the reaction with 5 ml chilled PBS. The contents were emptied into pre-tared centrifuge tubes set in ice, centrifuged for 5 min (20,000 g at 4°C) and 0.2 ml samples of supernatant, representing radioactivity of extracellular fluids, were removed for counting. The remaining supernatant was discarded, the pellet washed four times by centrifugation in chilled PBS (10 ml) and intracellular radioactivity determined by lysing the cells in distilled water (2 ml) followed by precipitation of proteins with TCA (3 ml, 10% W/V) and extraction of supernatant with water-saturated diethylether for removing TCA. Radioactivity of samples was determined in Packard Tri-Carb liquid scintillation counter using scintillation cocktail (0.4% PPO & 0.01% POPOP in toluene and methyl cellosolve, 1:1, V/V). Uptake values were obtained by converting radioactive counts into μmoles glucose taken up/gm dry wt/10 min.

RESULTS

The entry of glucose into the normal and the malaria-infected red cells followed Michaelis-Menten kinetics, at low concentrations (below 2 mM). At higher concentrations the entry was simply by diffusion, so 1 mM glucose concentration was used for observing the effects of spleen sonicate on glucose transport. The infected erythrocytes were obtained from animals showing 28-32% parasitaemia. Figs. 1 and 2 depict uptake of glucose by the normal and infected erythrocytes of both the species with or without the spleen sonicate. It would be evident that in the absence of spleen extract, erythrocytes from infected rat and mastomys registered 5 and 8 fold increases in glucose uptake respectively compared to normal animals. Incubation of normal and infected erythrocytes of both the species with spleen sonicate made from normal animals did not exhibit any significant effect on the transport of glucose across the membrane. When spleen sonicates made from albino rats recovering from infection were inoculated with both normal and infected erythrocytes, glucose uptakes were markedly reduced from 0.4 μmoles to 0.18 μmoles and from 2.08 μmoles to 0.7 μmoles respectively. However, incubation of spleen sonicates from infected mastomys (68-70% parasitaemia) with normal and infected erythrocytes caused an enhancement in uptake from 0.2

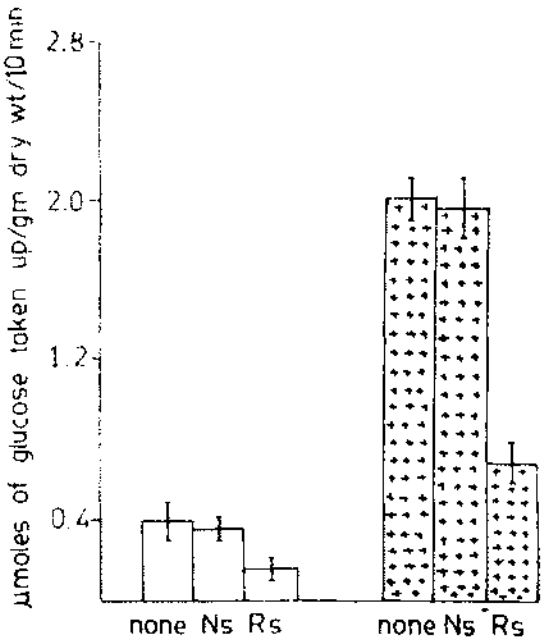

Fig. 1 Uptake of glucose by normal and infected rat erythrocytes with or without spleen sonicate.
Erythrocytes of normal animals.
Erythrocytes of infected animals having 28-32% parasitaemia.
none - No spleen sonicate.
Ns - Spleen sonicate of normal rat.
Rs - Spleen sonicate of recovering rat.
Results represent mean±SE of four determinations.

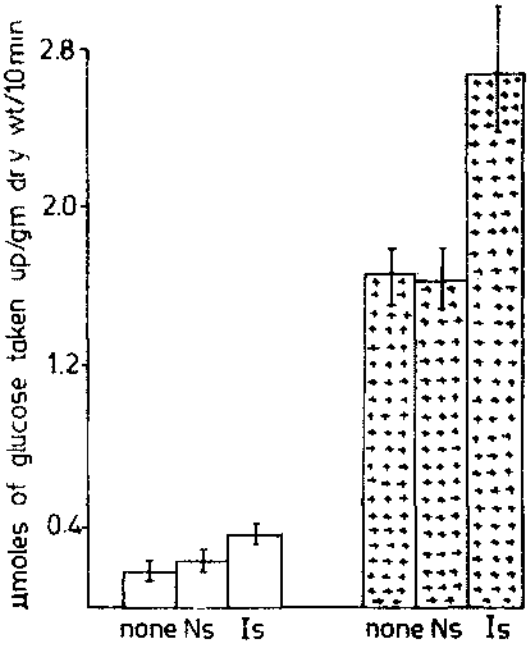

Fig. 2. Uptake of glucose by normal and infected mastomys erythrocytes with or without spleen sonicate.
Erythrocytes of normal animals.
Erythrocytes of infected animals having 28-32% parasitaemia.
none - No spleen sonicate.
Ns - Spleen sonicate of normal mastomys.
Is - Spleen sonicate of infected mastomys having 68-70% parasitaemia. Results represent mean ± SE of four determinations.

µmoles to 0.38 µmoles and from 1.6 µmoles to 2.7 µmoles respectively.

The effect of spleen sonicates of albino rat/mastomys having varying degrees of parasitaemia on glucose uptake of normal and infected erythrocytes would be seen in Figs. 3 & 4. Incubation of normal and infected rat erythrocytes with spleen sonicate from albino rats (10 to 30% parasitaemia) registered more or less similar uptake of 0.4 µmoles and 1.95 µmole respectively (Fig. 2) (c.f. 0.4 µmoles and 2.08 µmoles respectively in the absence of any spleen extract). However, when normal and infected erythrocytes were incubated with spleen sonicate from recovering rats, glucose uptake was reduced to 0.18 µmoles and 0.7 µmoles respectively (Fig. 3). Contrary to this, in mastomys a gradual increase in uptake was observed in normal and infected erythrocytes when incubated with spleen sonicate of animals of varying parasitaemia (10 to 70% parasitaemia). The glucose uptake increased in normal and infected erythrocytes from 0.2 µmoles to 0.38 µmoles and from 1.6 µmoles to 2.7 µmoles respectively (Fig. 4).

Table 1 shows the effect of spleen sonicate on heterologous erythrocytes. When infected mastomys erythrocytes were incubated with spleen sonicate from albino rats recovering from infection glucose uptake came down from 1.6 µmoles to 0.8 µmoles. In contrast incubation of infected

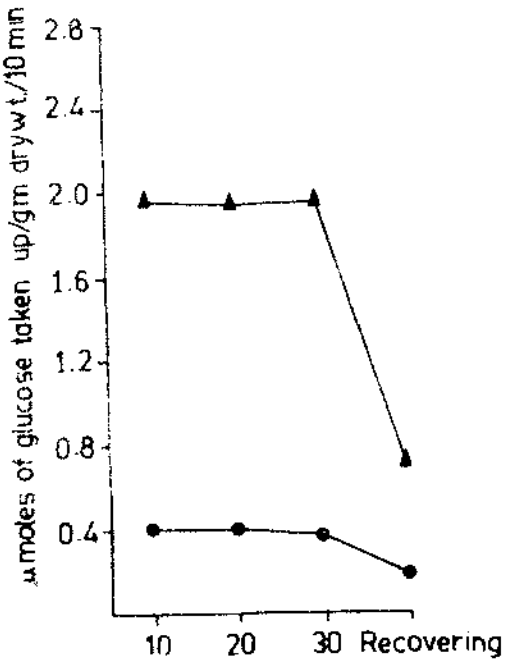

Fig. 3 Effect of spleen sonicates made from albino rat of varying degree of parasitaemia on glucose uptake of normal and infected rat erythrocytes.
Erythrocytes of normal animals.
Erythrocytes of infected animals having 28-32% parasitaemia.
x - axis represents the different levels of parasitaemia of rat.

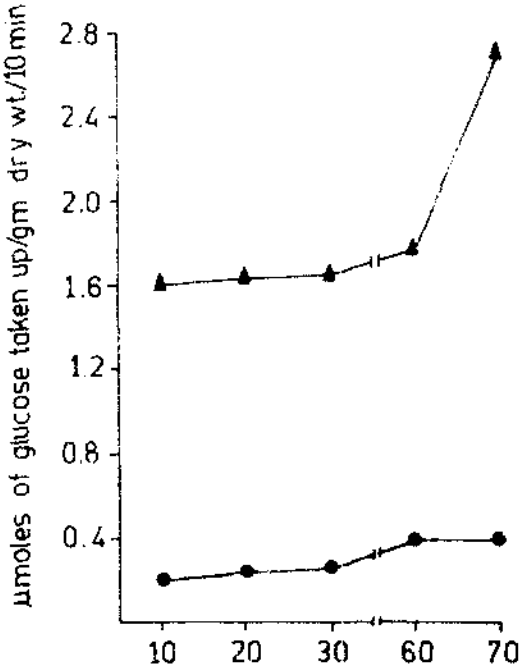

Fig. 4. Effect of spleen sonicates made from mastomys of varying degree of parasitaemia on glucose uptake of normal and infected mastomys erythrocytes.
Erythrocytes of normal animals;
Erythrocytes of infected animals having 28-32% parasitaemia.
x - axis represents the different levels of parasitaemia of mastomys.

rat erythrocytes with infected mastomys spleen sonicate caused an increase in uptake from 2.08 µmoles to 3.01 µmoles. Spleen sonicates from normal rat and mastomys did not show any significant effects on glucose uptake in heterologous erythrocytes.

DISCUSSION

It has been reported that in Plasmodia infected erythrocytes, transport of amino acids (Sherman, 1977) and glucose (Sherman & Tanigoshi, 1974), required for parasitic growth, is considerably increased but no reports are available describing the role of spleen in permeability of the required nutrient especially glucose in them. This permeability differs from host species to species and might be one of the causes responsible for their varying susceptibility to *P. berghei* infection. Now, coming to the role of spleen on erythrocyte permeability, macromolecular mediators such as complement cleavage products of activated splenic macrophages have been suggested to be one of the splenic factors responsible for alteration in erythrocyte membrane transport of metabolic substrates required for parasite growth (Allison & Clark, 1977; Quinn & Wyler, 1980). It would be seen that when permeability of normal erythrocytes from rat and mastomys were studied, the glucose uptake of rat erythrocytes was found to be almost double that of mastomys erythrocytes. In

Table 1. Effect of spleen sonicates on homologous and heterologous erythrocytes.

Spleen sonicate	μmoles of glucose taken up /gm dry wt/10 min	
	*Erythrocytes of infected rats	*Erythrocytes of infected mastomys
None	2.01±0.08	1.6 ± 0.04
Normal rat spleen	1.96±0.08	1.64±0.08
Normal mastomys spleen	1.92±0.04	1.7±0.04
Recovering rat spleen	0.7±0.08	1.8±0.12
**Infected mastomys spleen	3.01±0.28	2.7±0.24

Results represent mean ± SE of four determinations

*Erythrocytes of infected animals having 28-32% parasitaemia were employed.

**Spleen sonicate of infected mastomys having 68-70% parasitaemia.

the present study, when respective spleen sonicates were added to normal rat and mastomys erythrocyte, no change in glucose uptake was observed. When erythrocytes from animals with 28-32% parasitaemia were used, the uptake was 8 times more than normal in mastomys erythrocytes and 5 times in rats (as mastomys is more susceptible to *P.berghei* infection). When respective spleen extracts were added, these values did not differ much in both the species. However, when spleen sonicates from rats with declining parasitaemia was added there was a sharp decrease in glucose uptake of infected rat erythrocytes. In case of mastomys with increasing parasitaemia, the glucose uptake increased and this increase was more significant when spleen sonicates from infected mastomys (with about 70% parasitaemia) was added. To learn if the infected spleen sonicate contains factors, heterologous trials were made. As would be evident from Table 1, when infected mastomys spleen sonicate (68-70% parasitaemia) was added, the glucose uptake of infected rat erythrocytes greatly increased to 3.01 μmoles. In contrast, spleen sonicate from recovering rats significantly inhibited the uptake of glucose by infected mastomys erythrocytes. From the observations made in this study, it can be suggested that infected spleen sonicate contains both immune and nonimmune factors, depending on immune status of the host, one of which decreases permeability, as with the spleen extracts from immune rats and the other which facilitates permeability as from infected mastomys. Allison *et al.* (1978), based on their studies on the role of spleen in protection against murine Babesiosis, inferred that during the phase of recovery from a Babesia infection the rate of transport into infected cells is less than during the phase of rising parasitaemia. Extracts of cells from the spleens of recovered animals or animals made non-specifically immune by BCG inhibited transport. Thus the parasite specifically activates certain transport systems and factors in immune spleen cells inhibit them. This further supports our inference that erythrocyte permeability is the prime factor for the growth and development of malarial parasite within the erythrocyte and that this permeability factor is largely dependent on the spleen. Attempts are now under progress for characterizing the splenic factors responsible for causing these alterations.

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Effect of *Plasmodium cynomolgi* B Infection on the Haematological and Liver Function Test in Rhesus Monkey (*Macaca mulatta*)*

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Sequential haematological and serum biochemical changes in six rhesus monkeys during chronic *Plasmodium cynomolgi* B infection at different mean parasitaemia levels i.e. 2%, 8% and 5% on days 10, 15 and 20 respectively, and during chronic phase of infection were studied upto 40 days. The values for haemoglobin, RBC and PCV showed highly significant decrease ($P<0.001$) on days 10, 15 and 20 respectively. There were no significant changes in liver function tests viz., SGOT, SGPT, LDH and serum bilirubin during *P.cynomolgi* B infection.

INTRODUCTION

Haematological and serum biochemical changes reflecting liver function tests in *P.falciparum* malaria have been studied by several workers (Choudhury and Chakravorti, 1950; Migasena *et al.*, 1978; Wellde *et al.*, 1972 and Cadigan *et al.* 1972), whereas the liver function tests in *Plasmodium vivax* malaria have been studied by Seshadri *et al.* (1981).

The two experimental primate malaria infections namely *P.knowlesi* and *P.cynomolgi* B simulate the patterns of acute and often fatal course of *P.falciparum* infection in non-immune subjects and children, and a chronic non-fatal relapsing course of *P.vivax* infection respectively. Srivastava *et al.* (1982) carried out a sequential study on haematology and liver function tests from early to acute *P.knowlesi* infection in rhesus monkey. The major changes observed in *P.knowlesi* infection included a significant decrease in Hb, RBC and PCV and a corresponding highly significant increase in polymorph. The increase in total absolute lymphocyte count (TALC) during peak infection was marginal. Among the liver function tests serum bilirubin increased during acute infection, though changes in SGOT, SGPT and LDH were not significant.

The present study reports the sequential changes in haematological and liver function tests during *P. cynomolgi* B malaria infection in rhesus monkeys.

MATERIAL AND METHODS

Six normal healthy female rhesus monkeys weighing about 4-5 Kg. were acclimatized to the laboratory conditions and maintained on standard diet in primate house of the Institute. These monkeys were free of tuberculosis as shown by negative tuberculin test and chest X-ray.

The haematological parameters analysed were haemoglobin (Hb-g%), RBC count x 10^6/mm^3, packed cell volume (PCV, %), differential leucocyte count (DLC) % of polymorphs, lymphocytes, monocytes and eosinophils, total leucocyte count (TLC)/mm^3, total absolute polymorph count (TAPC)/mm^3 and total absolute

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lymphocyte count (TALC)/mm³. Tests were carried out according to the techniques of Dacie and Lewis (1968) and Eastham (1977).

The biochemical parameters (Liver function tests) viz. serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvate transaminase (SGPT), and lactic dehydrogenase (LDH) were done according to the methods described by Oser (1976). Serum bilirubin was estimated by the technique of Malloy and Evelyn (1957).

These parameters were studied before (day 0) and after i/v infection in rhesus monkeys with $x10^4$ *P.cynomolgi* B infected RBC on day 5, 10, 15, 20, 25, 30 and 40. Parasitaemia in blood smears was checked microscopically by staining the slides with Giemsa.

RESULTS

Haematological parameters were the first to show alteration by 10th day of *P.cynomolgi* B infection as evidenced by the decrease in Hb, RBC count and PCV % together with increase in total leucocyte count (TLC) and total absolute polymorph count (TAPC). Between day 15-20, the changes in these parameters were highly significant. With the appearance of primary peak parasitaemia by day 15, the total absolute lymphocyte count (TALC) registered a non-significant increase ($P > 0.05$) and by 25th day the number of circulating lymphocytes had shown a highly significant increase ($P < 0.001$). Together with the increase in number of circulating lymphocytes, there was a sharp decline of parasitaemia and infection became chronic beyond 25th day as indicated by a low level of parasitaemia which persisted beyond 40 days.

On day 40, these parameters returned almost to normal status except the Hb level which showed slower recovery (Table 1). Though RBC count was also lowered, but the decrease was not significant as the normal RBC range was 3.8 - 5.6 x 10^6/mm³. No significant changes in monocytes, eosinophils and TAPC were recorded.

No significant alterations in liver function tests (SGOT, SGPT, LDH and serum bilirubin) were recorded during entire course of *P.cynomolgi* infection.

DISCUSSION

Taliaferro and Kluver (1940) have studied the detail haematological findings during acute infection of *Plasmodium brasilianum* in panamanian monkeys and reported an increase in TLC and polymorph % and a decrease in lymphocyte percentage. When infection became chronic these haematological changes revealed a slight decrease in TLC and polymorph % and there was fluctuation in lymphocyte % during chronic phase of infection. In the present study of *P.cynomolgi* B also TLC and TAPC showed initial rise during early course of infection, but the increase in circulating absolute lymphocyte count (TALC) was highly significant and their increase coincided with the depression of parasitaemia by the host immune response. A comparison of haematological and liver function tests (LFT) in acute and fatal *P.knowlesi* and chronic non-fatal *P.cynomolgi* infections can be made. The acute *P.knowlesi* infection showed a high degree of leucocytosis at the height of parasitaemia which was followed by death, but in *P.cynomolgi* a non-significant increase in circulating lymphocytes was noticed at peak parasitaemia. During the declining phase of parasitaemia, the circulating lymphocytes showed further proliferation as shown by a highly significant increase in their number and this was closely related to the development of effective immune response. Following primary peak parasitaemia, the IFA antibody titres of rhesus monkeys infected with *P.cynomolgi* are known to increase from day 15 onwards, suggesting a correlation between lymphocyte proliferation and the stimulation of effective immune response. During chronic phase of *P.cynomolgi* B, the lymphocytes showed fluctuations but they were maintained at higher level than the pre-infection levels. All other parameters returned to normal by day 40, excepting Hb which showed slow recovery. Among the liver function tests, serum bilirubin which is adversely affected by *P.knowlesi* infection, was not significantly altered by the chronic *P.cynomolgi* infection. Our findings are in conformity with the observations of Seshadri *et al.*

Table 1 Effect of *Plasmodium cynomolgi* B infection on haematology in *Macaca mulatta*

Day	Parasitaemia (%)	Haemoglobin	RBC Count X 10⁶ /mm³	Packed Cell Volume (%)	Differential Leucocyte Count (DLC)% Polymorph	Lymphocyte	Monocyte	Eosinophil	Total Leucocyte Count (TLC) /mm³	Total Absolute Count Poly (TAPC) /mm³	Lymphocyte (TALC) /mm³
0 (Before infection)	0	13.0±.16	5.3±.16	39±.43	60±1.47	36±1.76	2±.56	2±.6	8633±289	5325±215	3196±25*
5	0	12.2±.49*	5.1±.45*	36±1.56*	58±1.76*	37±2.45*	3±.49*	2±.48	9450±646*	5481±297*	3566±534*
10	2	9.2±.19 P<0.001	3.4±.89 P<0.001	29±8.9 P<0.001	63±3.29*	31±2.62*	3±.85*	3±.65*	10200±673* P>0.1	6426±472* P>0.1	3091±414*
15	5	8.5±.11 P<0.001	2.8±.03 P<0.001	28±.52 P<0.001	55±.67*	39±.45*	4±.72*	2±.17*	10066±1156* P>0.05	5546±.59	3914±40.2* P>0.05
20	5	8.3±.18 P<0.001	2.8±.07 P<0.001	28±1.05 P<0.001	55±3.9*	39±2.95*	4±1.12*	2±.24*	11225±1429 P<0.005	6146±6.1 P>0.01	4480±562 P<0.005
25	0.4	9.6±.42 P<0.005	3.3±.12 P<0.005	30±1.78 P<0.005	52±8.1 P<0.01	44±3.38 P<0.01	2±.29*	2±.25*	11125±1971 P<0.05	5632±771*	5199±1256 P<0.001
30	6.5	9.5±.79 P<0.005	3.2±.28 P<0.001	29±2.27 P<0.01	56±1.63*	41±1.63*	2±0.0*	1±.29	8750±598*	4918±322*	3530±303*
40	0	10.1±1.18 P<0.1	3.8±.36*	33±2.34*	57±.48*	40±.29*	2±0.0*	1±0.29*	9800±1517*	5620±598*	3880±623*

* Non-significant

Note: Mean±SE calculated. Statistical comparison was made with day 0 to obtain P values

(1981), who reported no change in liver function tests in *P.vivax* malaria in south Madras area.

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New Antimalarials : Synthesis and Activity of 4,6-Diamino-1, 2-dihydro-1-(*P*-(4'-(2',5'-di-substituted)thiazolyl)phenylene)-2,2-dimethyl-*s*-triazines-II

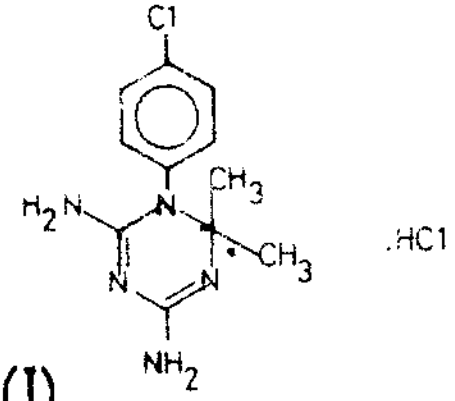
P.P. GUDADHE[1], D.T. CHAUDHARI[1]* and D.M. RENAPURKAR[2]

A variety of the title compounds were prepared from various 4-(4'-aminophenyl)thiazoles. The antimalarial action of these synthetic derivatives was evaluated by Rane's blood schizonticidal test. In all, 25 dihydrotriazines were synthesized and the activity of nine candidate compounds was determined vs. *P. berghei yoelii*.SAR is discussed.

INTRODUCTION

Compounds containing the thiazole ring system are found very useful in medicinal chemistry. Drugs like thiabendazole, tetramisole, niridazole, aminitrozole, promizole, fenclosic acid, penicillin, sulphathiazole and vitamin B_1 contain thiazole ring in one form or the other.

It is presumed that the molecule of cycloguanil (I) hydrochloride has *p*-chlorophenyl group which is acting as a transport or carrier molecule for the active moiety of *s*-triazine. Other groups such as phenoxyalkoxy, benzyloxy (WHO 1973; Sweeney and Strube, 1979; Canfield and Rozman, 1974), alkyl or arylthio-phenyl, naphthyl (Schalit and Cutler, 1959; Capps *et al.*, 1968), attached to active *s*-triazine molecule are also reported and their antiparasitic activity disclosed. However, there are no reports of a thiazole ring attached to dihydro-*s*-triazine molecule for investigating its possible antimalarial activity.



(I)

A number of compounds derived from 4-(4'-aminophenyl)thiazoles (II) have shown anticancer (Modi *et al.*, 1970), antituberculosis (Chaudhari *et al.*, 1976) and antiparasitic (Khadse *et al.*, 1977) activity

During the past two decades, several 1-aryl-4, 6-diamino-1,2-dihydro-*s*-triazines have been synthesized and evaluated for biological activity. Inhibitory effects against *Lactobacillus casei* in the dihydrotriazine series (Roth *et al.*, 1963) were generally thought to be a measure of antifolic

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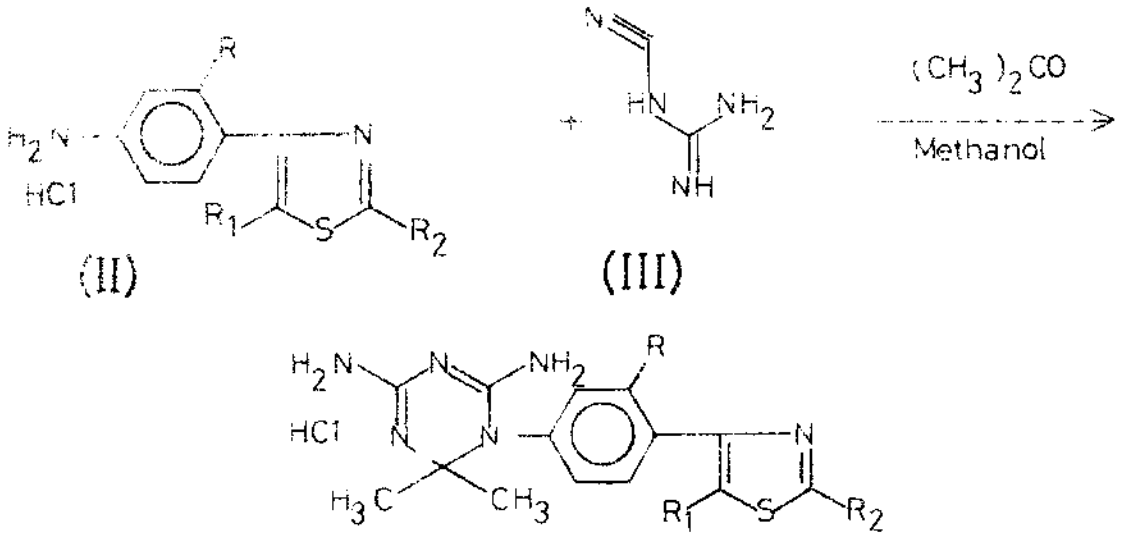
Chemistry



where, R = H or Cl;

R_1 = H or Cl;

R_2 = H, aryl, aralkyl, haloaryloxyaryl.

activity and this is presumed to be the basis for the antimalarial activity of cycloguanil (Capps *et al.*, 1968).

In order to develop new chemotherapeutic agents, we thought that the linking of 4-(*p*-aminophenyl)thiazoles to dihydrotriazine may result in compounds possessing good antimalarial activity. The compounds that may be formed are 1-/ *p*-/4'-(disubstituted)thiazolyl/phenylene/ -4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochlorides (IV). This paper describes the preparation and antimalarial activity of (IV) against *Plasmodium berghei yoelii*, a rodent malarial parasite.

MATERIAL AND METHODS

The dihydro-*s*-triazines (IV) were prepared in 60-65% yield by condensing 2,5-disubstituted-4-(4'-aminophenyl)thiazole hydrochlorides (II) with cyanoguanidine (III) and acetone in the presence of methanol at reflux temperature by the method of Modest (1956). 4-(*p*-Aminophenyl)- and 4-(4'-amino-2'-chlorophenyl)thiazoles were prepared according to known methods (Modi *et al.*, 1970).

All the *p*-(4-thiazolyl)phenyl-*s*-triazine hydrochlorides (IV) revealed IR and UV spectral features in agreement with their assigned structure (Modest, 1956; Roth *et al.*, 1963; Capps *et al.*, 1968). Most of the dihydro-*s*-triazines (IV) described (Table 1) were checked for appropriate spectral changes upon heating with 0.5N sodium hydroxide to confirm that the indicated isomers were obtained

Experimental:— 2-(*p*-Chlorophenoxymethyl)-4-(2'-chloro-4'-aminophenyl) thiazole monohydrochloride (II, R=2-Cl; R_1=H and

R_2=*p*-$ClC_6H_4OCH_2$).

A mixture of *p*-chlorophenoxythioacetamide (0.404 g; 2mmole) and 2-chloro-4-amino-2'-chloroaceto-phenone (0.408 g; 2 mmole) in 5 ml of absolute ethanol was refluxed for 4 hours, when a crystalline product was obtained. It was filtered, washed with ethanol and dried in vacuum over $CaCl_2$.

Yield 0.61 g (79 %), m.p. 208-210°C.

Elemental analysis for C 16 H 12 Cl 2 N 2 OS. HCl :

Calculated C, 49.55; H, 3.35; N, 7.22%.

Found C, 49.22; H, 3.61; N, 7.59 %.

4,6-Diamino-1,2-dihydro-1-[*p*-[2'-(3-methoxy-4-ethoxyphenyl)-4-thiazolyl] phenylene] -2,2-dimethyl-*s*-triazine monohydrochloride (No. 8, Table 1):

A mixture of 0.56 g (1.5 mmole) of 2-(3-methoxy-4-ethoxyphenyl)-4-(*p*-aminophenyl)thiazole hydrochloride, 0.146 g of cyanoguanidine (17 mmole), 0.5 ml of dry methanol and 5 ml of dry acetone was stirred and heated under reflux for 20 hr. The reaction mixture was homogeneous within five minutes and soon the compound began to separate in crystalline form. After the heating was over, the product was collected by filtration and washed with 10 ml portions of acetone and ether. This gave brown prisms of the title compound as monohydrate of analytical purity.

Yield 0.55 g (70 %), m.p. 236-237°C

Elemental analysis for C 23 H 26 N 6 O 2 S.HCl.H 2 O :

Calculated C, 54.71; H, 5,74; N, 16.65%

Table 1 — 4,6-Diamino-1,2-dihydro-1-[*p*-[4'-(2',5'-disubstituted)thiazolyl]-phenylene]-2,2-dimethyl-*s*-triazine hydrochlorides (IV).

Compound	*R*	R_1	R_2	Yield %	M.p. °C* (uncorr).	Mol. formula**
1.	H	CH_3	CH_3	50	195-98	$C_{16}H_{20}N_6S.HCl$
2.	H	CH_3	3-BrC_6H_4	52	221-22	$C_{21}H_{21}BrN_6S.HCl.H_2O$
3.	H	CH_3	4-ClC_6H_4	85	228-31	$C_{21}H_{21}ClN_6S.HCl.H_2O$
4.	H	CH_3	2,4-$Cl_2C_6H_3$	52	235-40	$C_{21}H_{20}Cl_2N_6S.HCl$
5.	H	CH_3	4-$EtOC_6H_4$	80	214-15	$C_{23}H_{28}N_6OS.HCl.H_2O$
6.	H	CH_3	4-$PrOC_6H_4$	75	228-29	$C_{24}H_{28}N_6OS.HCl.H_2O$
7.	H	H	3,4-$(MeO)_2C_6H_3$	62	216-19	$C_{22}H_{24}N_6O_2S.HCl.H_2O$
8.	H	H	3,4-MeO(EtO)C_6H_3	70	236-37	$C_{23}H_{26}N_6O_2S.HCl.H_2O$
9.	H	H	3,4-$(EtO)_2C_6H_3$	50	197-98	$C_{24}H_{28}N_6O_2S.HCl$
10.	H	CH_3	3,4-EtO(PrO)C_6H_3	58	223-24	$C_{26}H_{32}N_6O_2S.HCl.H_2O$
11.	H	CH_3	3,4-MeO($PhCH_2O$)C_6H_3	87	218-20	$C_{29}H_{30}N_6O_2S.HCl.H_2O$
12.	H	CH_3	3,4,5-$(MeO)_3C_6H_2$	90	254-55	$C_{24}H_{28}N_6O_3S.HCl$
13.	H	H	$C_6H_5CH_2$	68	236-38	$C_{21}H_{22}N_6S.HCl.H_2O$
14.	H	CH_3	4-$ClC_6H_4CH_2$	62	196-98	$C_{22}H_{23}ClN_6S.HCl.H_2O$
15.	H	CH_3	4-$MeOC_6H_4CH_2$	59	203-04	$C_{23}H_{26}N_6OS.HCl.2H_2O$
16.	H	H	4-$ClC_6H_4OCH_2$	72	234-35	$C_{21}H_{21}ClN_6OS.HCl.H_2O$
17.	H	H	2,4-$Cl_2C_6H_3OCH_2$	69	235-36	$C_{21}H_{20}Cl_2N_6OS.HCl$
18.	Cl	H	C_6H_5	59	213-15	$C_{20}H_{19}ClN_6S.HCl$
19.	Cl	H	4-ClC_6H_4	68	227-29	$C_{20}H_{18}Cl_2N_6S.HCl.H_2O$
20.	Cl	H	4-$PrOC_6H_4$	87	215-16	$C_{23}H_{25}ClN_6OS.HCl.H_2O$
21.	Cl	H	3,4-$(EtO)_2C_6H_3$	58	168-70	$C_{24}H_{27}ClN_6O_2S.HCl.H_2O$
22.	Cl	H	3,4-EtO(PrO)C_6H_3	62	210-12	$C_{25}H_{29}ClN_6O_2S.HCl$
23.	Cl	H	3,4-EtO($PhCH_2O$)C_6H_3	68	212-13	$C_{29}H_{29}ClN_6O_2S.HCl.H_2O$
24.	Cl	H	3,4-MeO($PhCH_2O$)C_6H_3	53	208-11	$C_{28}H_{27}ClN_6O_2S.HCl.H_2O$
25.	Cl	H	4-$ClC_6H_4OCH_2$	66	212-15	$C_{21}H_{20}Cl_2N_6OS.HCl.H_2O$

* Capillary melting points.

** Analysed correctly for C, H and N within ± 0.45%.

Found C, 54.91; H, 5.53; N, 17.03 %

Other *p*-(4-thiazolyl)phenyl dihydrotriazines (IV) prepared by the above procedure are listed in Table 1.

Biological activity:—*Primary screening:* The antimalarial activity of compounds was evaluated using *P.b. yoelii*-mouse model by Rane's blood schizonticidal test as described by Osdene *et al.* (1967).

Albino mice of H.I. strain weighing approx. 20 g each received standard i.p. inoculum of 10^7 infected donor red cells. This dose killed untreated controls in 8 to 9 days. Test drugs were dissolved in sterile distilled water or suspended in Tween-80 and injected once subcutaneously 72 hr. after infection. A drug is considered to be active (A) if treated animals survive at least twice as long as controls, i.e., the animals not receiving any drugs (this is MED). The drug is considered curative (C) if the treated mice survived for 60 days. If death occurred within 48 hr. of treatment, the mortality in mice was classified as death due to toxicity (T). All mice receiving drugs and showing a survival time of 14-18 days were followed up for presence of parasites by regular examination of blood smears. There was no parasitaemia in mice surviving 18 days period. A group of infected animals treated with pyrimethamine at 4.5 mg/kg dose levels producing definite increase in survival time was included as a positive control in every experiment for comparison.

RESULTS AND DISCUSSION

In all, nine dihydrotriazines (IV) were examined against *P. b. yoelii* for their antimalarial action in mice. Eight of them (Table 2) Nos. 6, 7, 14, 15, 17, 19, 20 and 23 showed activity at dosages varying from 20 to 160 mg/kg. In all these cases no parasitaemia developed even after 18 days.

Compound No. 15 exhibited promising activity and cured four out of five treated mice at 160 mg/kg dose level. The corresponding chloro derivative (No. 14) though active and nontoxic at 40 and 80 mg/kg was not found curative. Compounds 6 and 7, with propoxy and dimethoxy grouping in phenyl ring at 2-position of the thiazole moiety, were found to have suppressive activity and were nontoxic at 40 mg/kg and 160 mg/kg. However, the replacement of aryl (compound No. 6) and aralkyl group (No. 14, 15) with haloaryloxymethyl group resulted in an active compound (No. 17). This compound was active at the lowest dose of 20 mg/kg but it was found toxic at a dose level of 160 mg/kg. Compounds 19, 20 and 23 with *p*-(4-thiazolyl)-*m*-

Table 2 - Antimalarial activity against *P. b. yoelii* in mice by Rane's Method.

Compound*	(D + 3) Single s.c. dose; Δ MST, T or C dosages after mg/kg				
	10	20	40	80	160
6.	—	—	5.7	12.2 (A)	13.2 (A)
7.	5.7	—	12.5 (A)	—	6.8
13.	—	—	8.9	—	8.9
14.	—	—	6.7	11.0 (A)	11.0 (A)
15.	7.9	—	7.9	—	4.0 (C)
17.	—	2.0 (C)	0.4	—	2.0 (T)
19.	6.7	—	18.0 (A)	4.0 (T)	3.0 (T)
20.	—	8.3	—	6.3	—
23.	2.0	—	14.0 (A)	—	8.7

*Compounds refer to those from Table 1. D = Day of infection; (D+3) = 72 hr;
Δ MST = MSTT - MSTC (see Gudadhe *et al.* Dec. 1981). *Ind. J. Malariol.*, **18**: 73-79;
T = Death in mice due to toxicity; C = Animals classed as cured;
(—) represents no test being done. In each run pyrimethamine and cycloguanil hydrochloride were included as standard drugs for comparison.

chlorophenyl grouping at 1-position of the triazine ring displayed good activity at dose levels ranging from 10 to 40 mg/kg. However, compound No. 19 proved to be highly toxic at 80 & 160 mg/kg.

This preliminary study shows that compounds 6, 7, 14 and 15 had considerable antimalarial activity. Further studies are, however, needed to evaluate these compounds as potential antimalarials for use in clinical practice.

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SHORT NOTES

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Observations on the Incidence of Malaria in India

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Malaria incidence figures are collected through the active and passive agencies throughout the country. These agencies prepare blood smears from the fever cases. In some areas mass blood surveys are also done. All these blood smears are ained and examined under the microscope for the presence of malaria parasite. Slides showing the presence of malaria parasite represent malaria positive cases which becomes the basis of all planning and future projections. Thus Pattanayak and Roy (1980) reported malaria incidence in India from 1971-1978. NMEP's data have been used in many researches on the stratification, diffusion models and other interpretations. This data are also the basis for calculating various epidemiological indices viz., annual blood examination rate (ABER), slide positivity rate (SPR), slide falciparum rate (SFR) and annual parasite index (API).

Table 1 — Malaria positive cases in India (1971-80).

Year	Blood slides collected	Blood slides examined	Malaria positive cases reported by NMEP on blood smear examination	Probability of malaria cases in the unexamined slides	Corrected incidence of malaria
1971	41565296	40453164	1322398	36355	1358753
1972	41420000	39200000	1428649	80908	1509557
1973	44140000	42450000	1930273	76847	2007120
1974	48140000	45450000	3167658	187481	3355139
1975	53269227	51818351	5166142	144648	5310790
1976	58081609	55978173	6467215	243012	6710227
1977	59002187	57010347	4740900	165638	4906538
1978	62276054	60462306	4144385	124323	4268708
1979	62172471	61415178	3064697	37790	3102487
1980	67627506	65920583	2844815	73662	2918477

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We feel that blood slides collected from the fever cases and not examined for various reasons should also be included in calculating the incidence and the API. Since microscopically examined slides constitute bulk of the slides, it would be correct to add a proportionate number of unexamined slides and therefore the malaria cases (based on SPR) to the total incidence of malaria. The revised figures of malaria incidence in India have been shown in table 1 using this criteria. To achieve greater accuracy, calculations of the unexamined slides may be done on the basis of stratification or sectionwise.

This criteria should be adopted in calculating API which is the sheet anchor of decision making. The method would also dispel any tendency of showing better performance on the basis of low slide examination rate

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Insecticide Susceptibility Status of Malaria Vectors in Maharashtra

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Anopheles culicifacies is the principal vector of malaria in rural areas of Maharashtra. The vector was highly susceptible to DDT from 1953 to 1958 (Bhatia *et al.*,1958). In subsequent years physiological resistance to DDT resulted in control failures so that in 1962 HCH was substituted in certain areas to interrupt transmission. By 1968 resistance to DDT became widespread (Rao *et al.*, 1971) and to HCH also in most areas. Resistance to HCH in DDT resistant *A. culicifacies* was noted after 4 cycles of HCH spraying, and to malathion in DDT and HCH resistant populations after 13 cycles of spraying (State Government unpublished records). It may be mentioned that in Maharashtra DDT was sprayed since 1953 @ 1 gm/sq. m. HCH since 1962 @ 0.25 gm/sq. m. and malathion since 1969 @ 2 gm/sq. m. The first report of resistance to malathion was received in 1973 in Dahanu tehsil, Thane district. In subsequent years it was observed in several other districts and in neighbouring Gujarat State (Rajagopal 1977). To day *A. culicifacies* is resistant to DDT, HCH and malathion so that very effective interruption of malaria transmission is not achievable in certain areas.

We carried out insecticide susceptibility tests on the commonly found anopheline vector species using the standard WHO procedures (WHO 1970). Adult females were collected from the field. These females were held in Barraud cages and kept in room maintained with optimum humidity and temperature. Care was taken to select uninjured and healthy mosquitoes. In each tube, 15-25 blood fed females were exposed to insecticide treated papers for a fixed period and mortalities recorded after 24 hours. Where necessary, mortalities were corrected using the Abbott's formula. Results of this study are given in Table 1.

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Table 1 — Results of insecticide susceptibility tests

Insecticide & exposure period.	Year	*Areas under DDT spray		**Areas under HCH spray		***Areas under Malathion spray	
		Total test	Corrected mortality (%) with range.	Total test	Corrected mortality (%) with range.	Total test	Corrected mortality (%) with range.
1. *A. culicifacies*							
(i) DDT 4% (4 hr)	1978	17	27.3 (0-59)	20	25.5 (6-39)	13	21.4 (5-44)
	1979	3	19.0 (14-23)	8	27.4 (8-62)	4	39.0 (10-58)
	1980	16	23.2 (8-34)	14	23.9 (9-45)	5	23.4 (16-40)
(ii) Dieldrin 4% (4 hr)	1978	17	36.2 (8-66)	19	18.1 (0-45)	11	18.6 (4-30)
	1979	—	—	2	23.1 (20-26)	2	18.3 (11-24)
	1980	2	61.3 (41-74)	7	27.8 (7-35)	3	28.7 (21-31)
(iii) Malathion 5% (1 hr)	1978	11	65.6 (52-100)	15	54.9 (40-81)	10	47.4 (15-70)
	1979	5	71.3 (51-98)	10	50.2 (25-73)	8	52.2 (4-90)
	1980	11	88.7 (51-100)	8	65.4 (23-98)	5	52.9 (18-93)
(iv) Fenitrothion 1% (1 hr)	1979	—	—	3	96.9 (96-97)	6	92.9 (83-100)
	1980	1	100	2	98.1 (97-100)	—	—
(v) Fenthion 2.5% (1 hr)	1979	—		4	100	2	100
	1980	1	100	2	100	1	100
(vi) propoxur 0.1% (1 hr)	1978	—	—	1	93.0	—	—
	1979	—	—	4	100	5	99.6 (98-100)
	1980	1	100	5	97.4 (93-100)	2	100
2. *A.fluviatilis*							
(i) DDT 4% (1 hr)	1978	—	—	—	—	2	90.8 (86-94)
	1980	1	93.4	2	86.5 (75-97)	—	—
(ii) Dieldrin 4% (1 hr)	1978	—	—	—	—	1	93.5
	1981	1	96.8	1	91.5	—	
3. *A.annularis*							
(i) DDT 4% (1 hr)	1979	—	—	—	—	1	2.2
	1980	—	—	1	3.2	—	—
(ii) Dieldrin 4% (1 hr)	1979	—	—	—	—	1	30.9
	1980	—	—	1	5.7	—	—

Include districts of *: 1. Amravati, 2. Akola, 3. Bhandara, 4. Buldhana, 5. Chandrapur, 6. Kolhapur, 7. Nagpur, 8. Nanded, 9. Parbhani, 10. Ratnagiri, 11. Wardha, 12. Yawatmal. **: 1. Ahmednagar (Part), 2. Deed, 3. Osmanabad, 4. Pune, 5. Sangli, 6. Satara, 7. Solapur(part). ***: Ahmednagar (Part), 2. Aurangabad, 3. Dhule, 4. Jalgaon, 5. Nasik, 6. Raigad, 7. Solapur (Part). 8. Thane.

Results revealed that *A. culicifacies* was highly resistant to DDT and dieldrin in all the areas tested. High degree of resistance to malathion was observed in areas under HCH and malathion spray, whereas some tests showed susceptibility to malathion in DDT sprayed areas. *A. annularis* was also resistant to DDT and dieldrin. *A. fluviatilis* has developed some degree of tolerance to DDT and dieldrin.

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A Note on the Feeding Behaviour of Mosquitoes in Maharashtra Villages.

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The feeding preference of mosquitoes was observed in 4 villages of Maharashtra State situated in the riverine area i.e. Sendurwafa and Kumbhli in Bhandara district and Ganeshpur and Chikhalgaon in Yavatmal district. These studies were initiated with the objective to see the biting and feeding site of mosquitoes when the entire human body was exposed.

Observations were made fortnightly, indoors and outdoors, between 6.30 to 8.30 p.m., from 1966 to 1973 in two selected mixed dwellings. A bare man with a bathing trunk sat on a stool.

Table-I — Mosquitoes collected on human bait in Maharashtra villages (1966-73)

Species	Numbers collected from							Total Captured
	Head	Shoulder	Chest	Belly	Back	Hand	Legs	
1. *Anopheles* (A.) *hyrcanus*	0	0	0	0	0	4	78	82
A. (C.) annularis	0	0	0	1	0	2	85	88
A. (C.) culicifacies	0	0	0	0	0	1	18	19
A. (C.) fluviatilis	0	1	1	0	1	2	47	52
A. (C.) Jamesi	0	0	0	0	0	0	4	4
A. (C.) Jeyporiensis	0	0	0	0	0	0	2	2
A. (C.) maculatus	0	0	0	0	0	0	1	1
A. (C.) pallidus	0	0	0	0	0	0	65	65
A. (C.) splendidus	0	0	0	0	0	0	1	1
A. (C.) subpictus	0	0	0	2	3	4	63	72
A. (C.) tessallatus	1	0	0	0	0	0	9	10
A. (C.) theobaldi	0	0	0	0	1	1	34	36
A. (C.) turkhudi	0	0	0	0	0	0	1	1
A. (C.) vagus	0	0	0	0	0	0	2	2
A. (C.) varuna	0	0	0	0	0	0	3	3
2. *Culex spp.*	2	5	0	20	36	53	954	1070
3. *Aedes aegypti*	1	0	0	2	28	2	8	41

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Table 2 - *A. culicifacies* collected from the living rooms showing the numbers with fresh blood meal (1968-70).

Time (30 mt. collection)	Total collected	Freshly fed mosquitoes collected with percentage
6 p.m.	747	18 (2.4)
8 p.m.	420	53 (12.6)
10 p.m.	334	81 (24.3)
12 p.m.	267	73 (27.3)
2 a.m.	195	37 (19.0)
4 a.m.	521	66 (12.7)
6 a.m.	3831	68 (1.8)

Mosquitoes found alighting or feeding were collected with the help of a suction tube and a flash light. In one study in October 1967, during the focal outbreak of malaria, daily collections were made for one week between 5.30 p.m. to 12.30 p.m. with particular emphasis on *A. fluviatilis*. Results of this study are given in Table 1.

Results revealed that the most preferred site of feeding of the anophelines was legs. A few mosquitoes were also found feeding on hands. It was interesting to find some zoophilic species viz., *A. hyrcanus* (total 82) and *A. tessallatus* (total 10) feeding on man during one week focal outbreak study. The *Culex* spp. mosquitoes also preferred legs but small numbers were also found feeding on belly, back and hands etc. In contrast *Aedes aegypti* preferred back, but specimens were also collected from legs and other body parts. The study clearly brings out importance of providing adequate protection to the legs to prevent/reduce the chances of an infective bite.

In another study, resting *A. culicifacies* were collected from the living rooms at 2 hourly intervals from 6 p.m. to 6 a.m. for a 30 mt. period. All collections were examined with the help of a hand lens. Females showing fresh irridescent red blood in their abdomens were recorded as against the total females collected. Data from 1968-70 are given in Table 2. Results revealed that biting of *A. culicifacies* continues all night with a peak activity between 10 p.m. to 2 a.m. as observed by Viswanathan *et al.* (1955).

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Ecology of Anophelines in Basantpur Village Situated on the Bank of Jumna

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Observations in the recent past have shown that *Anopheles culicifacies* is the major vector of malaria in rural areas and maintains high endemicity in riverine villages. Therefore studies on the ecology of anophelines along Jumna with particular emphasis on *A. culicifacies* were indicated. These studies were carried out in Basantpur which was selected because of its location on the bank of Jumna, small size and isolation from other villages. It is situated on western bank of Jumna, 20-km south east of Delhi in district Faridabad (Haryana State). The village has about 100 structures. There are about 400 people and 1200 cattles living in close proximity. Eastern outskirts of the village are swampy. There are 3 wells within 1-km and a drain flows at a distance of about 1-km which falls in Jumna. The climate is dry tropical with an average rainfall of 1000 mm. per year. There were occasionally long spells of heavy rains. Every year floods inundate the village and fields, and the receding waters leave several pools for mosquito breeding. Studies started in May 1978 and continued till August 1979. The results of the observations made during this period are reported in this paper.

Entomological observations were made on weekly basis. Mosquitoes were collected between 6.30 a.m. to 10.30 a.m. from houses and cattlesheds using suction tubes. Density of adults was recorded as per man hour collection of anophelines. Vector incrimination was done by dissecting gut/glands of the field collected females. Blood from the freshly engorged females was collected on filter paper for precipitin test. Any sample that gave cross reaction was rejected. For immatures, a dipper (10x5x5cm) was used to estimate density in their breeding habitat, similarly for wells a bucket (30x10cm) was used. Larvae thus sampled were counted and reared in the laboratory for identification at the adult stage. Susceptibility of larvae to insecticides was tested using the WHO larval test kit. a record of fever was maintained and cases found positive for malaria were given antimalarials.

Adult collections revealed the presence of six anophelines viz., *A. annularis*, *A. culicifacies* *A. hyrcanus*, *A. pulcherimus*, *A. stephensi* and *A. subpictus*. Bhatia *et al.* (1958) studied the seasonal prevalence of anophelines in Delhi-Ghaziabad border villages near Hindon river and found the presence of additional 4 species viz., *A. barbirostris*, *A. fluviatilis*, *A. pallidus*

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and *A. splendidus*. The first 2 species were considered as an accidental introduction from upstreams and the later 2 species were considered rare. *A. annularis* was the most dominant species of Delhi-Ghaziabad villages. Of the anophelines found in Basantpur, *A. culicifacies* and *A. stephensi* are the primary vectors of malaria and *A. annularis*, a secondary vector. Among the vector species *A. culicifacies* were found in good numbers.

Results of the relative densities of 3 most dominant anophelines found in Basantpur are given in Table 1. It was revealed that low densities of *A. annularis* were found during winter months. There was sudden peak in April followed by low densities in other months. The populations of *A. culicifacies* start building in April and reach peak densities in June and July followed by a drop to low numbers with second small rise in October. *A. subpictus* was the most dominant species of the village. Hodgson (1914) also observed that *A. subpictus* was the most dominant species of the new province of Delhi. The populations start building in May, peaking in July and August followed by decline with a second small rise in October. A sharp decline in anopheline densities was observed during monsoon which may be due to long spell of rain, and unprecedented floods. Both *A. stephensi* and *A. hyrcanus* were found in very low numbers throughout the study period.

A notable feature of the adult anopheline densities (resting indoors in houses and cattle sheds) was the presence of a highly distorted sex ratio in favour of females, which was more pronounced in houses than in the cattlesheds (see Table 1). Most of the anophelines were found resting on wall upto a height of 2 meters inside human and cattle dwellings. *A. subpictus* was found resting mainly on the hanging objects, and *A. culicifacies* mostly on the walls and ground.

All larval breeding sites were searched in and around the village on weekly basis. A dipper was used to estimate the density of immatures. Based on the collections of immatures from different breeding sites, breeding association of the anophelines was recorded (Table 2). The breeding of anophelines was observed in wheat fields and pools around the village, swampy areas of the riverbed, and wells. A flowing drain constituted permanent breeding site for the *A. culicifacies*. This drain had a discharge water (clean water) of the power station. *A. culicifacies* was found breeding in large numbers in association with *A. annularis, A. subpictus, A. stephensi* and *A. hyrcanus* and with or without culicines. There was no exclusive breeding habitat for any single species as was also observed by Russell and Rao (1940). Maximum breeding of *A. culicifacies* was observed in the month of May and June, whereas other months had low breeding. During monsoon bulk of the breeding was found in rain water pools and wells and *A. subpictus* was found breeding in all types of water collections and dominated the immature samples.

Insecticide susceptibility tests using the late 3rd and 4th instar larvae of *A. culicifacies* to discriminatory dosages revealed that the vector species was resistant to DDT (37.3% mortality), HCH (6% mortality) and susceptible to malathion and temephos.

A total of 1038 blood smears were collected from the field for the identification of the source of blood using the precipitin test. It was revealed that anthropophilic index for *A. culicifacies* was 26.3. Afridi *et al.* (1939) also recorded similar anthropophilic index for *A culicifacies* from Delhi i.e., 22.3%. Mosquitoes collected in the field were dissected for the vector incrimination. In 1978, out of a total of 38 *A. culicifacies* dissected, one was found positive for oocyst in December. Studies in other villages of Haryana bordering Delhi revealed 2% sporozoite rate in *A. culicifacies* (D.S. Choudhury, Personal communication). Therefore, high endemicity to malaria in riverine villages was being maintained by *A. culicifacies*. Parasitological investigations revealed that during transmission season, the slide positivity rate in this village was 47.5%.

Table 1 - Relative density of Anophelines resting indoors

1978	Houses				Cattlesheds			
	Total man hours	*A. annularis* PMH	*A.culicifacies* PMH	*A. subpictus* PMH	Total man hour	*A. annularis* PMH	*A.culicifacies* PMH	*A. subpictus* PMH
May	12	2.9 (1:34)	3.3 (1:7)	1.7 (1:9)	11	11.6 (1:6)	7.3 (1:5)	3.2 (1:4)
June	14	1.4 (0:20)	20.9 (1:2)	2.8 (1:5)	15	1.7 (1:7)	22.3 (1:6)	4.1 (1:4)
July	19	0.2 (0:4)	0.8 (1:3)	108.2 (1:5)	19	1.1 (1:7)	1.2 (1:22)	93.6 (1:8)
August	13	0.7 (1:1)	2.2 (1:5)	133.9 (1:2)	12	0.5 (0:6)	1.6 (1:5)	87.8 (1:4)
September	12	3.1 (1:0.6)	3.3 (1:5)	11.6 (1:3)	—*	—	—	—
October	20	13.6 (1:1.5)	4.9 (1:3)	25.3 (1:3)	—*	—	—	—
November	7	5.0 (1:1)	1.7 (1:0.7)	4.8 (1:5)	7	2.1 (1:3)	2.6 (1:4)	18.7 (1:3)
December	10	1.0 (1:10)	1.0 (1:2)	1.0 (1:9)	10	7.3 (1:23)	1.6 (1:7)	0.5 (0:8)
1979								
Jan.	16	1.7 (4:8)	0.2 (0:3)	0.0	16	9.4 (1:74)	1.7 (0:27)	0.06 (0:1)
Feb.	4	1.5 (0:6)	0.0	0.0	4	32.0 (0:128)	0.5 (0:2)	0.0
March	12	1.3 (1:4)	1.3 (1:1)	0.0	12	13.0 (1:11)	2.5 (1:5)	0.0
April	16	35.3 (1:64)	6.0 (1:46)	0.6 (1:4)	16	70.9 (1:11)	13.9 (1:10)	0.9 (1:4)
May	16	4.8 (1:29)	7.7 (1:72)	16.4 (1:43)	16	9.1 (1:5)	15.1 (1:6)	19.5 (1:5)
June	16	2.0 (1:7)	50.3 (1:40)	38.8 (1:25)	16	13.1 (1:40)	77.5 (1:5)	39.9 (1:3)
July	16	4.18 (1:45)	47.6 (1:35)	88.25 (1:33)	16	3.8 (1:9)	61.06 (1:7)	79.0 (1:3)
August	12	2.91 (1:28)	23.2 (1:38)	134.08 (1:3)	12	6.3 (1:7)	31.00 (1:5)	111.33 (1:3.0)

*Collection was not made as all cattles were removed from the village during floods.

P.M.H. = Per man hour

In parentheses male female ratio.

Table 2 — Breeding association of anophelines in Basantpur.

Month* (1979) and breeding site	Density of immatures per dip	Results of mosquito identification with numbers collected					
		A.annularis	*A.culicifacies*	*A.hyrcanus*	*A.stephensi*	*A.subpictus*	*Culex*
March							
Wheat fields	(.4)	2	22	5	9	0	+
Pools	(.7)	0	19	2	7	0	+
April							
Riverbed pools	(2.8)	0	115	0	7	2	+
Borrow pits	(.1)	0	5	0	1	0	+
Drains	(2.5)	0	131	0	0	0	+
Wells	(.1)	0	1	0	0	0	+
May							
Riverbed pools	(7.3)	8	335	0	2	5	—
Drains	(5.6)	8	422	0	11	9	—
Wells	(.8)	0	3	0	1	3	+
June							
Riverbed pools	(3.6)	0	224	0	4	20	—
Drains	(6.6)	0	219	0	0	41	+
Wells	(.9)	0	18	0	0	0	+
July							
Riverbed pools	(1.4)	0	23	0	0	0	—
Drains	(5.3)	0	21	0	0	88	+
Drains	(9.2)	0	14	0	0	85	+
Pits & pools	(14.3)	1	12	0	0	63	+
August							
Riverbed pools	(1.2)	0	4	0	0	0	+
Drains	(.6)	0	18	0	0	0	—
Wells	(2.9)	0	6	0	0	33	—
Pits & pools	(5.9)	0	0	0	0	447	—

*No breeding was observed in Jan. & Feb..
+ present

Jumna villages are more prone to malaria because of the mosquitogenic conditions created by the swampy areas and yearly floods. The receding waters of Jumna leave innumerable breeding grounds which support heavy mosquito breeding. Therefore, in addition to the residual spraying of insecticides and drug distribution system, cleanliness of surroundings, i.e., cleaning of drains, wells, filling of pits etc. should receive primary emphasis.

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Plasmodium falciparum: Malaria in Haryana Villages and a Case Report of Aphasia

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Cerebral malaria is a complication of *P. falciparum* infection and is sometimes responsible for high mortality. Hyperpyrexia, severe headache, mental confusion, disorientation, hallucination, delirium, convulsion and coma are the usual manifestations of cerebral malaria. Disturbance of motor system such as monoplegia, hemiplegia, Jacksonian epilepsy and motor aphasia are sometimes associated with it. Some cases may present sensory changes of varying degrees and cranial nerve paralysis.

An outbreak of *P. falciparum* malaria was investigated in 15 villages of Sonepat distict bordering Narela PHC of Delhi during 1981. A total of 3434 blood smears was collected from febrile cases or persons having previous history of fever. Out of these, 1534 showed *P. falciparum* parasites giving *P. falciparum* positivity rate of 44.6 per cent. Few of these cases presented cerebral symptoms associated with hyperpyrexia, severe headache and mental confusion. These were treated successfully with chloroquine.

In one village, Thana Khurd under Kharkhoda PHC of this district, investigation on a *P. falciparum* outbreak was carried out during September to December 1981. A total of 226 blood slides was collected from febrile cases during this period. Out of these, 135 showed malarial parasites giving a slide positivity rate of 59.73 per cent. Out of the positives, 131 were *P. falciparum* and 4 *P. vivax*. Thus 97 per cent of the total positives were due to *P. falciparum*.

A total of 1862 *Anopheles culicifacies* was dissected from this village during this period. The result of the dissection is given below.

Period of collection	No. of mosquitoes dissected.	No. positive for sporozoites in glands.	Sporozoite rate.
September	153	3	1.96
October	165	3	1.82
November	1056	13*	1.23
December	488	0	0
Total	1862	19	1.02

*The last positive mosquito was collected on 27.11.81.

A girl aged 14 years suffering from aphasia was brought for treatment during one of the visits to this village in October. The patient had previous history of high fever for a week without proper treatment. Suddenly at the height of the febrile illness, she lost her speech. She was hospitalized and discharged after general treatment of the fever. After three days, she was again down with fever. When examined, she had high fever and was in a state of mental confusion. She could however recognise people but was not able to talk. A blood smear was prepared and examined. It showed fairly good numbers of *P. falciparum* rings (13% of the RBCs were infected). For the treatment of the febrile episode, the patient was advised oral administration of a course of 1.5 gm

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of chloroquine with an initial dose of 600 mgm followed by 300 mgm on the same day. In subsequent two days the patient was asked to take 300 mgm chloroquine each day. Due to logistic difficulties, intravenous administration of antimalarials was not advised. After the completion of the treatment, the patient became afebrile and gradually started regaining her speech within a week. Initially she could utter monosyllables only. Subsequently she could speak small sentences within one month of the treatment.

Aphasia results from a lesion of the cortex and the immediate subcortical white matter. The commonest cause is thrombosis or embolism of the cortical branches of left middle cerebral artery. The localised blockage of the capillaries by parasitized erythrocytes adhering to one another and to the capillary endothelium causing petechial haemorrhages is commonly seen in *P. falciparum* infection (Edington, 1954). Wright (1968) suggested that an autoantibody may play a role in the development of multiple petechial haemorrhages in the white matter of the brain. Aphasia is known to occur in association with cerebral malaria (Wilcocks and Manson-Bahr 1976). No published report of aphasia due to cerebral malaria is however available in this country. The recovery in the present case was unexpected as chloroquine was administered orally, late in the course of the disease.

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Serum Immunoglobulin Levels in Malaria

SATISH GUPTE, UMA SABHARWAL and I.D. CHUGH

Investigations were carried out on serum immunoglobulin in hundred patients suffering from *P. vivax* malaria. The level of IgG, IgM and IgA was compared with the immunoglobulin of 101 normal healthy matched controls. The estimation of immunoglobulins was carried out by radial immunodiffusion method described by Mancini *et al.* (1965).

The serum level of IgG and IgM showed significant elevation (P value <0.01 in both) in malaria cases. The IgG level was lound to be 2206 mg/100 ml±5.15. The IgM level was 168.55 mg/100 ml±49. The normal control showed a level of 1297 mg/100 ml±421 and 90.7 mg/100 ml±42 respectively. The IgA level in malaria cases and normal control was found to be 113.82 mg/100 ml±32 and 113.70 mg/100 ml±48 respectively. This confirms the earlier observations of Ghosh *et al.* (1977) and Butcher *et al.* (1976).

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