INDIAN JOURNAL OF MALARIOLOGY

Volume 25 **Number 1**

June 1988

MALARIA RESEARCH CENTRE
Indian Council of Medical Research
22-Sham Nath Marg
Delhi-110054

INDIAN J. MALARIOL.

Half Yearly

Year of revival: 1981

SUBSCRIPTION RATE

Annual	India	Rs. 75.00*
	Other countries (including airmail postage)	$ 20.00

*25% discount would be admissible to the individual subscriber on annual basis.

Payments in respect of subscription may be sent by money order/bank draft/postal order/cheque crossed and marked payable to Malaria Research Centre, 22-Sham Nath Marg, Delhi—110054.

The 'Indian Journal of Malariology' is indexed by 'BIOSIS', 'Drugs and Pharmaceuticals Current Indian Titles', 'Index Medicus', 'Indian Science Abstracts', 'Review of Applied Entomology', 'Protozoological Abstracts', 'Quarterly Bibliography of Major Tropical Diseases' and it is selectively abstracted by 'Tropical Diseases Bulletin'. This journal is also accessible on the CAB Computer Database and MEDLINE.

INDIAN JOURNAL OF MALARIOLOGY

INDIAN JOURNAL OF MALARIOLOGY

Volume 25
Number 1
June 1988

CONTENTS

Selection of *Plasmodium yoelii nigeriensis* Resistant to Triple Drug combination (sulphadoxine/pyrimethamine/mefloquine) and its Response to other Antimalarials 1
Binduja Shukla, Renu Bajpai and *G.P. Dutta*

Isolation of different Erythrocytic Stages of Plasmodium falciparum and Synchronization in Culture 7
S. Biswas, Q.B. Saxena, A. Roy and *V.P. Sharma*

Status of Natural Killer Activity in the Peripheral Blood of *P. vivax* and *P. falciparum* Malaria Patients 11
Q.B. Saxena, S. Biswas and *V.P. Sharma*

The Value of Spraying Cattlesheds in a Control Programme 17
M.A. Ansari, V.P. Sharma, R.K. Razdan, C.P. Batra and *P.K. Mittal*

Cryopreserved Human Erythrocytes for *in vitro* Cultivation of *Plasmodium falciparum* 23
H. Srinivasa, Sam Jayanth Samuel and *M.J. Sebastian*

Stabilization of *Plasmodium knowlesi* Antigen for Seroepidemiology of malaria 27
S.K. Saxena and *G.P. Dutta*

Fractionation of *Plasmodium knowlesi* Antigen for Seroepidemiology of Human Malaria by IHA test 33
S.K. Saxena, V.C. Pandey and *G.P. Dutta*

Malaria Outbreak in Kundam block District Jabalpur (M.P.) 41
Neeru Singh, V.P. Sharma, M.M. Shukla and *Gyan Chand*

Some Components of Kinin System in Human Malaria 51
S. Roy, U. Bhattacharya, P.K. Kar and *S.C. Lahiri*

Short Note

Natural Parasitic Infections in Anopheline Larvae of Mandya district, Karnataka state 59
C. Achuthan

Obituary

C.P. Pant 65

Indian Journal of Malariology
Vol. 25, June 1988, pp. 1-6.

Selection of *Plasmodium yoelii nigeriensis* Resistant to Triple Drug combination (sulphadoxine/pyrimethamine/mefloquine) and its Response to other Antimalarials

BINDUJA SHUKLA[1], RENU BAJPAI[1] and G.P. DUTTA[1]

A strain of *P. yoelii nigeriensis* was exposed to sub-curative doses of sulphadoxine + pyrimethamine + mefloquine in Swiss Mice for 31 serial passages over a period of 464 days. The strain finally developed 16-fold resistance to this triple combination. The normal parasite was sensitive to a drug combination comprising of sulphadoxine 0.156 mg/kg + pyrimethamine 0.0156 mg/kg + mefloquine 0.0625 mg/kg (× 4 day schedule) while the sensitivity of resistant strain decreased substantially and it was susceptible to a combination of sulphadoxine 2.5 mg/kg + pyrimethamine 0.25 mg/kg + mefloquine 1.0 mg/kg (× 4 day)

The resistant line also developed high level of cross-resistance to chloroquine, quinine and mefloquine, while the sensitivity of the strain to pyrimethamine and sulphadoxine decreased slightly.

INTRODUCTION

Multiple drug resistant strains of *P. falciparum* have appeared in several countries of the South-East Asia and Western Pacific region, while chloroquine resistance is becoming widespread in several countries including India, Africa and Southern America. The hope of achieving eradication of *P. falciparum* in these countries has received a setback, because of the emergence of drug resistant strains (Puri and Dutta, 1982; Sharma, 1984). The major advance in chemotherapy of malaria during the last 25 years, was the development of mefloquine as a powerful blood schizontocide effective against multiple drug-resistant strains of *P. falciparum*, and the use of Fansidar (synergistic combination of sulphadoxine and pyrimethamine) against chloroquine resistant strains of *P. falciparum* (Doberstyn *et al.*, 1976). Efficacy of this combination to control resistant *P. falciparum* is gradually decreasing as indicated by reports of Fansidar resistance from Thailand Burma, Vietnam, Colombia and Brazil (Hurwitz *et al.*, 1981; Pinichpongse *et al.*, 1982; Tin *et al.*, 1982; Dixon *et al.*, 1982; Chongsuphajaisiddhi and Sabchare'on, 1981; Eugene *et al.*, 1979; Eichenloub *et al.*, 1983; Timmermanns *et al.*, 1982; Larry *et al.*, 1979). A combination of tetracycline and quinine was found effective in the control of Fansidar resistant cases (Puri and Dutta, 1982).

Subsequent studies showed that the cure rate of quinine-tetracycline combination decreased to 57% in Kampuchean cases, suggesting a possibility of emergence of quinine-tetracycline resistance also. Today, the promising drug available for control of Fansidar as well as quinine-

Accepted for publication: 29 February 1988.
[1]Central Drug Research Institute
Lucknow-226001, India.

tetracycline resistant strains of *P. falciparum* is mefloquine. Resistance of this drug has been reported in a few clinical cases (Boudreau *et al.*, 1982; Bygbjerg *et al.*, 1983). Resistance against mefloquine can be easily accomplished by experimental manipulation among strains of rodent malaria (Kazim *et al.*, 1979). The present study reports triple drug resistance (sulphadoxine + pyrimethamine + mefloquine) in a strain of *P. yoelii nigeriensis*.

MATERIAL AND METHODS

Swiss mice (20 ± 1 gm) of either sex were infected with *P. yoelii nigeriensis* using an inoculum of 1×10^7 parasitized red cells given by i/p route. The parasitaemia was recorded daily after Giemsa staining of the blood films.

The strain of *P. yoelii nigeriensis* was repeatedly exposed to sub-curative doses of triple combination of sulphadoxine + pyrimethamine + mefloquine in increasing doses in 31 serial passages over a period of 464 days. The methodology for selection of resistant line was the same as used earlier for the selection of mefloquine, pyrimethamine and mefloquine-pyrimethamine combined resistant lines of *P. berghei* in Swiss mice (Kazim *et al.*, 1979; Puri and Dutta, 1982; Agarwal *et al.*, 1979). The strain was tested for level of resistance by exposing the resistant line to various doses of the triple combination (sulphadoxine, pyrimethamine and mefloquine). The parasitaemia and survival of mice was recorded upto 21 days. Sensitivity of the resistant strain to other antimalarials such as chloroquine, quinine, mefloquine, pyrimethamine and sulphadoxine was also investigated.

RESULTS

The normal strain of *P. yoelii nigeriensis* used in this study has a low level of innate (natural) resistance to chloroquine and mefloquine, their MED in 4 days test, being 32 and 8 mg/kg respectively. The MED of this strain for other drugs is as follows: quinine 450 mg/kg, amodiaquine 16 mg/kg and pyrimethamine, 1 mg/kg (Pande and Dutta, 1982).

The results of the development of resistance in *P. yoelii nigeriensis* to triple drug combination are presented in Table 1. The sensitivity of the strain exposed to triple combination for 31 passages to standard drugs was evaluated and the results presented in Table 2 show that this strain

Table 1. Attempts to induce resistance in *P. yoelii nigeriensis* to triple combination of drugs (sulphadoxine + pyrimethamine + mefloquine) through 31 serial passages of strain in Swiss mice

S. No.	Single dose drug exposure*	Serial passages	Duration of passage (Days)	Parasitaemia in last passage		
				Day 4	Day 7	Day 10
1.	Sulphadoxine 0.1562 mg/kg Pyrimethamine 0.0156 mg/kg Mefloquine 0.0625 mg/kg	1–12	1–147	8.166 ± 13.93	1.6 ± 1.673	8.82 ± 8.32
2.	Sulphadoxine 0.3125 mg/kg Pyrimethamine 0.0312 mg/kg Mefloquine 0.125 mg/kg	13–19	148–309	2.33 ± 2.875	2.833 ± 3.656	19 ± 17.572
3.	Sulphadoxine 0.625 mg/kg Pyrimethamine 0.0625 mg/kg Mefloquine 0.25 mg/kg	20	310–315	12.25 ± 8.035	– ve	– ve
4.	Sulphadoxine 2.5 mg/kg Pyrimethamine 0.25 mg/kg Mefloquine 1 mg/kg	21–31	316–429	5.33 ± 15.47	– ve	– ve

*Oral administration was single dose between day 3–7.

Table 2. Chemotherapeutic response of *P. yoelii nigeriensis* exposed to triple combination (sulphadoxine + pyrimethamine + mefloquine) in Swiss mice

S No.	Drug	Treatment schedule dose/day mg/kg	No of Drug doses	Total dose admtd. mg/kg	Inoculum	No of mice	Parasitaemia (Mean ± S.D.) No. of mice (Surviving) 4	7	14	21	M.S.T.† ± S.D. (Days)
1	Chloroquine*	16	17	272	1×10^7	20	1.575 ± 2.165 (20)	2.42 ± 2.750 (19)	5.25 ± 11.350 (13)	-ve (7)	16.05
		32	8	256	1×10^7	4	4 ± 1.612 (6)	14.37 ± 14.661 (4)	7.5 ± 2.828 (2)	-ve (1)	11.83
		64	8	512	1×10^7	7	4.57 ± 2.280 (7)	8.21 ± 4.07 (7)	9.87 ± 2.86 (4)	-ve (1)	11.85
2.	Quinine*	300	7	2100	1×10^7	7	14 ± 5.422 (6)	18.5 ± 3.605 (3)	—	—	6.71
3.	Mefloquine*	32	3	96	1×10^7	6	1 ± 1.06 (5)	19 ± 1.47 (5)	—	—	10.5
		64	4	256	1×10^7	6	0.033 ± 0.05 (6)	0.1 ± 0.22 (5)	1.25 ± 1.84 (4)	42 ± 25.45 (4)	16
		128	4	512	1×10^7	6	-ve (6)	-ve (5)	-ve (3)	-ve (1)	17
4.	Pyrimethamine**	2	4	8	1×10^7	6	-ve (6)	-ve (6)	0.4 ± 0.89 (4)	3.0 ± 2.58 (3)	16.83
5.	Sulphadoxine**	0.5	4	2	1×10^7	6	-ve (6)	4.25 ± 4.86 (6)	21.65 ± 13.033 (5)	—	18.33
		1	4	4	1×10^7	6	-ve (6)	-ve (6)	19.08 ± 17.53 (6)	-ve (4)	20.3

*Drug administration started after patency.
**Drug administration started on Day 0.
†Mean survival time.

Table 3. Chemotherapeutic response of resistant strain of *P.y. nigeriensis* exposed to triple combination (sulphadoxine + pyrimethamine + mefloquine) for 32 passages in Swiss mice

Strain of *P. yoelii nigeriensis*	Treatment schedule dose mg/kg	No. of Drug doses	Total drug administered mg/kg	Inoculum	Parasitaemia (Mean +S.D.)(Survival of mice)				M.S.T.† ± S.D. (Days)
					Day 4	Day 7	Day 14	Day 21	
1. Normal (W_1) strain of *P.y. nigeriensis*	(i) *SD* 0.078 + *Py* 0.0078 + *Mfq.* 0.031	4	*SD* 0.312 + *Py* 0.0312 + *Mfq.* 0.125	1×10^7	-ve (6)	-ve (6)	3.2 ± 7.15 (6)	4 ± 894 (5)	21.3
	(ii) *SD* 0.156 + *Py* 0.0156 + *Mfq.* 0.0625	4	*SD* 0.624 + *Py* 0.0624 + *Mfq.* 0.25	1×10^7	-ve (6)	-ve (6)	-ve (6)	-ve (5)	21.3
2. Derived strain of *P.y. nigeriensis*	(i) *SD* 0.625 + *Py* 0.0625 + *Mfq.* 0.25	4	*SD* 2.5 + *Py* 0.25 + *Mfq.* 1	1×10^7	-ve (6)	0.0066 (6)	24.75 ± 2.06 (4)	25 (1)	18.5
	(ii) *SD* 1.25 + *Py* 0.125 + *Mfq.* 0.5	4	*SD* 5 + *Py* 0.5 + *Mfq.* 2	1×10^7	-ve (6)	-ve (6)	4.16 ± 6.99 (6)	3 ± 5 (3)	21
	(iii) *SD* 2.5 + *Py* 0.25 + *Mfq.* 1.0	4	*SD* 10 + *Py* 1 + *Mfq.* 4	1×10^7	-ve (6)	-ve (6)	-ve (6)	-ve (5)	21.8

SD = Sulphadoxine; Py = Pyrimethamine; Mfq = Mefloquine; † Mean survival time

had acquired resistance to chloroquine upto 16 mg/kg × 17 days or 64 mg/kg × 8 days, quinine 300 mg/kg × 7 days, mefloquine 128 mg/kg × 4 days. However, pyrimethamine 2 mg/kg × 4 days and sulphadoxine 1 mg/kg × 4 days showed partial suppression of parasitaemia.

The response of original (sensitive) strain as well as the strain exposed to triple combination (sulphadoxine + pyrimethamine + mefloquine) for 31 passages was also studied in 4 day test (Table 3). The data shows that the original strain was sensitive to a combination of 0.156 mg/kg sulphadoxine +0.0156 mg/kg pyrimethamine + 0.0625 mg/kg mefloquine, while the derived strain acquired 16-fold resistance to triple combination.

DISCUSSION

During last 15 years two powerful drug combinations have been used for the control of multiple resistant *P. falciparum* cases which include the Fansidar and quinine-tetracycline combination. Field reports show that large-scale resistance to Fansidar was emerging in the field and the quinine-tetracycline combination was also becoming less effective in some areas (Hurwitz *et al.*, 1981; Pinichpongse *et al.*, 1982; Tin *et al.*, 1982; Dixon *et al.*, 1982; Bygbjerg *et al.*, 1983; Black *et al.*, 1981; Chongsuphajaisiddhi and Sabchareon, 1981; Eugene *et al.*, 1979; Eichenloub *et al.*, 1983; Timmermanns *et al.*, 1982; Larry *et al.*, 1979).

It is suspected that mefloquine, initially developed as blood schizontocide for control of drug resistant falciparum strains, might not maintain its efficacy in the field and resistance to mefloquine may eventually develop if it is used alone for the control of drug resistant strains of falciparum. WHO (1984) has advised the use of mefloquine in combination with Fansidar which could possibly either prevent the emergence of mefloquine resistance or significanly delay the development of this resistance.

Present study reveals that Fansidar can not protect the emergence of mefloquine resistance if both the drugs are used in sub-curative doses and there is a need for caution to use mefloquine-fansidar combination in field on a large-scale against multiresistant strains of *P. falciparum*.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. M.M. Dhar for encouragement and support for the present investigations. Our thanks are also due to the Director-General, Indian Council of Medical Research for the award of Research Fellowships to Miss Binduja Shukla and Miss Renu Bajpai.

REFERENCES

1. Agarwal, K., S.K. Puri and G.P. Dutta (1979). Selection of pyrimethamine resistant strain of *P. berghei*. *Indian J. Med. Res.*, **69**: 577–582.
2. Black, F., I. Bygbjerg, P. Effersoe, Gomme Crethe, S. Jepsen and G. Axelgarrd Jepsen (1981). Fansidar resistant falciparum malaria acquired in South East Asia. *Trans. R. Soc. Trop. Med. Hyg.*, **75**: 715–716.
3. Boudreau, E.F., H.K. Webster, K. Paranand and L. Thosingha (1982). Type II mefloquine resistance in Thailand. *Lancet*, **II**: 1335.
4. Bygbjerg, I.C., A. Schapira, H. Flachs, G. Gomme and S. Jepsen (1983). Mefloquine resistance of falciparum malaria from Tanzania enhanced by treatment. *Lancet*, **1**: 774–775.
5. Chongsuphajaisiddhi, T. and A. Sabchareon (1981). Sulphadoxine-pyrimethamine resistant falciparum malaria in Thai children. *Southeast Asian J. Trop. Med. Pub. Hlth.*, **12**: 418–421.
6. Dixon, K.E., R.G. Williams, T. Pongsupat, V. Pitaktong and P. Phintuyothin (1982). A comparative trial of mefloquine and fansidar in the treatment of falciparum malaria. Failure of fansidar. *Trans. R. Soc. Trop. Med. Hyg.*, **76**: 664–667.
7. Doberstyn, E.B., A.P. Hall, K. Vetvutanapibul and P. Sonkom (1976). Single dose therapy of falciparum malaria using pyrimethamine in combination with diformyl dapsone or sulphadoxine. *American J. Trop. Med. Hyg.*, **25**: 14–19.
8. Eichenloub, D., H.G. Hoffman, G. Rogler and H.J. Weise (1983). Falciparum malaria in East African tourist in spite of fansidar prevention. A contribution on increased chloroquine and pyrimethamine-sulphadoxine resistance in areas of East Africa. *Dtsch. Med. Wochensch.*, **108**: 338–343.

9. Eugene, S., E.S. Hurwitz, D. Johnson and C. Campbell (1979). Resistance of *P. falciparum* malaria to sulphadoxine-pyrimethamine (Fansidar) in a refugee Camp in Thailand. ***Bull. WHO*, 52:** 275–279.
10. Harinasuta, T., D. Bunnag, R. Lasserre, R. Leimer, S. Vinijanont (1985). Trial of mefloquine in vivax and of mefloquine +Fansidar in falciparum malaria. ***Lancet*, I:** 885–888.
11. Hurwitz, E.S., D. Johnson and C.C. Campbell (1981). Resistance of *P. falciparum* malaria to sulphadoxine + pyrimethamine Fansidar in a refugee camp in Thailand. ***Lancet*, I:** 1068.
12. Kazim, M., S.K. Puri and G.P. Dutta (1979). Chemotherapeutic studies with mefloquine and selection of a mefloquine resistant strain of *P. berghei*. ***Indian J. Med. Res.*, 70** (Suppl): 95–102.
13. Larry, W., Rumans, T. David and Dannis soeroto Atmosoedjono (1979). Fansidar resistant falciparum malaria in Indonesia. ***Lancet*, II:** 580–581.
14. Pande, A. and G.P. Dutta (1982). Chemotherapeutic response of *P.y. nigeriensis* to antimalarial drugs. ***Indian J. Malariol.*, 19:** 121–124.
15. Pinichpongse, S., E.B. Doberstyn, J.I. Cullen, L. Yisusri, Thongsombun and K. Thimasarn (1982). An evaluation of five regimens for the outpatient therapy of falciparum malaria in Thailand, 1980–81. ***Bull. WHO*, 60:** 907–912.
16. Puri, S.K. and G.P. Dutta (1982). Induction of pyrimethamine resistance in a mefloquine resistant strain of *P. berghei*. ***Indian J. Malariol.*, 19:** 91–96.
17. Sharma, G.K. (1984). Review of malaria and its control in India. In *Indo-UK workshop on malaria, Nov. 14–19, 1983*, edited by V.P. Sharma. (Malaria Research Centre, ICMR, Delhi): 13–40.
18. Timmermanns, P.M., V. Hess and M.E. Iones (1982). Pyrimethamine-sulphadoxine resistant falciparum malaria in East Africa. ***Lancet*, I:** 1181.
19. Tin, F., N. Hlaing and R. Lanserre (1982). Single dose treatment of falciparum malaria with mefloquine: field studies with different doses in semi-immune adults and children in Burma. ***Bull. WHO*, 60:** 913–917.
20. WHO (1984). Advances in malaria chemotherapy Report of a WHO Scientific Group. ***Tech. Rep. Ser.*,** 711.

Indian Journal of Malariology
Vol. 25, June 1988, pp. 7–10.

Isolation of different Erythrocytic Stages of *Plasmodium falciparum* and Synchronization in Culture

S. BISWAS[1], Q.B. SAXENA[1], A. ROY[1] and V.P. SHARMA[1]

Plasmodium falciparum in culture has a heterogeneous population of different stages. Erythrocytes which harbour parasites have different densities; isolation of the various stages has been done by taking advantage of this difference. From healthy culture different stages were isolated by stepwise Percoll density gradient method as 30, 45, 50 and 65 per cent. Purity of parasite preparations was checked by microscopic examination. Parasitized erythrocytes having homogeneous stages were put for synchronous culture with 45 per cent Percoll top layer (45T) and pellet after 65 per cent (65B) gradient. Parasitaemia of the culture was monitored after every 24 hours. Synchronicity was maintained in both the sets upto 48 hours. Gradient was repeated after 48 hours to 'fine tune' the synchronization. Two sets of culture with 45T and 65B give more yield of homogeneous stages. No alteration of parasite viability at various stages could be detected.

INTRODUCTION

Extensive studies on the development of a malaria vaccine culminated by Prof. William Trager's success in producing a continuous *in vitro* culture of *P. falciparum* (Trager and Jensen, 1976). Growth of *P. falciparum* in culture is asynchronous and generally parasitaemia greater than 10% is difficult to achieve. Extensive studies have been done on the methods for enrichment of the parasite stages, but none of these methods totally purifies a particular intraerythrocytic stage (Jensen, 1978; Mrema *et al.*, 1979). Free merozoites can be isolated from culture supernatant (David *et al.*, 1978; Tharavanij and Prasartsiriroj, 1981) but they are of limited value in reintroducing into culture because of their very brief extraerythrocytic viability. Synchronization of *P. falciparum* erythrocytic stages in culture has successfully been done by using 5% D-sorbitol in culture medium (Lambros and Vanderberg, 1979) by which only ring form parasites could be retained due to the varying degree of lysis of infected RBCs containing mature stages.

In the present study, an attempt has been made to standardise a stepwise gradient of Percoll with increasing concentraion for isolation of different stages of parasites. Concentrations were taken as 30, 45, 50 and 65 per cent. Homogeneous stages isolated by gradient method were taken for synchronous culture. The utility of synchronous cultures of *P. falciparum* includes quantitative studies on the immunological, biochemical and physiological differences among the various stages of development.

Accepted for publication: 24 March 1988.
[1]Malaria Research Centre (ICMR)
22-Sham Nath Marg
Delhi-110054, India.

MATERIAL AND METHODS

A single *P. falciparum* isolate FSJ-M (F-falciparum, SJ-Shahjahanpur, M-MRC) from Shahjahanpur, U.P., India was adapted *in vitro* and put for continuous culture as described by Trager and Jensen (1976). Healthy culture having parasitaemia above 5 per cent was taken for Percoll density gradient. Ten per cent suspension of RBCs was prepared with RPMI containing 15 per cent AB + serum.

Percoll was adjusted to iso-osmolarity with 1.5 M PBS, pH 7.2 (9 parts Percoll: 1 part PBS). Different concentrations as 30, 45, 50 and 65 per cent were prepared for diluting the iso-osmolar Percoll with 15 per cent serum containing medium. Cell suspension was overlayed on to 30 per cent Percoll and centrifuged at 2000 rpm for 15 mins. in cold. Top layer was aspirated and washed twice with complete medium. Pellet was washed and resuspended in medium to make 10% suspension. In this way the suspension was subjected to 45, 50 and 65 per cent gradient. Top Percoll layer and bottom layer were collected and checked for purity by microscopic examination. Parasitized erythrocytes having homogeneous preparation were put for synchronous culture.

Synchronous culture was established with 45% Percoll top layer (45T) and pellet after 65% gradient (65B) by addition of normal A + erythrocytes with a starting inoculum of 0.5% by the candle jar technique. Medium RPMI containing 15% AB + serum was supplemented with 0.1% glucose and 2 mM L-glutamine. Parasitaemia of the culture was monitored after every 24 hours. Synchronicity was maintained in both the sets upto 48 hours. At this stage, culture from 45T set was repeated for 45% Percoll gradient. New culture was established with top layer which maintained synchronicity upto 96 hours. Synchronization was repeated with the 65B set also, but no significant change has been observed.

RESULTS

Culture having healthy stages with parasitaemia more than 5 per cent was considered for Percoll gradient. Purity of the cell preparations after each gradient was checked by microscopic examination (Table 1). After removal of free merozoites and haemozoin pigments by 30% gradient a pure population of schizonts was collected by 45% (45T). Observations on culture samples initiated

Table 1. The purity of the various preparations

Percoll gradient %	Source of cells T/B	Stages
30	T	Free merozoites and haemozoin pigments
45	T	Schizonts
50	T	Schizonts and late trophozoites
65	T	Early and late trophozoites
65	B	Rings, early trophozoites

T—Top layer; B –Pellet or Bottom layer

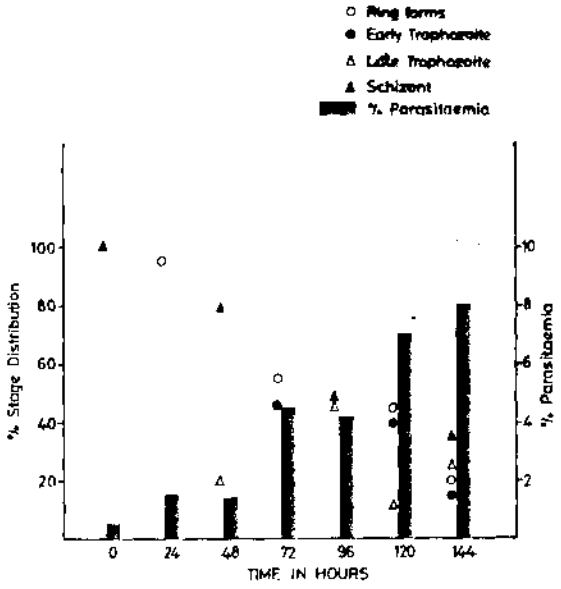


Fig. 1. Development of *P. falciparum* in culture introducing 45 per cent Percoll top layer (45T).

with 45T taken after every 24 hours are shown in Fig. 1. Another culture set with 65 per cent parasitized RBC pellet is shown in Fig. 2. In both the sets synchronicity was observed upto 48 hours. About 80% schizonts of 45T set at 48 hours were harvested by 45% Percoll and reintroduced in a fresh set of culture. This repetition of the density gradient at 45 per cent results in almost uniform aged schizonts which in the fresh culture showed synchronicity upto 96 hours (Fig. 3).

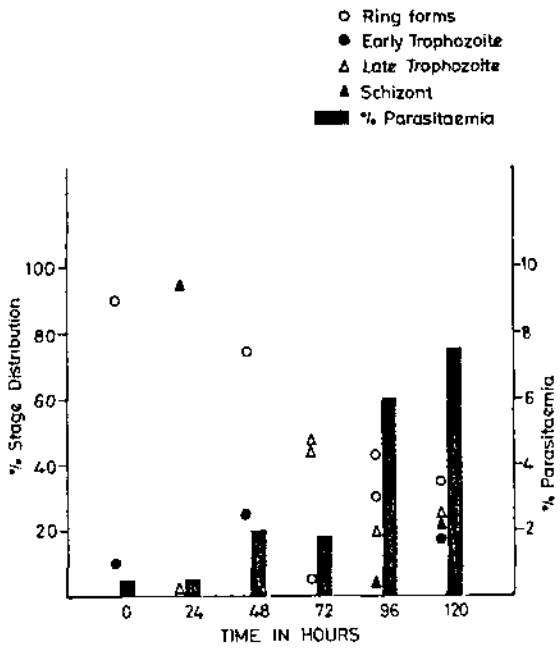


Fig. 2: Development of *P. falciparum* in culture introducing 65 per cent bottom layer (65B)

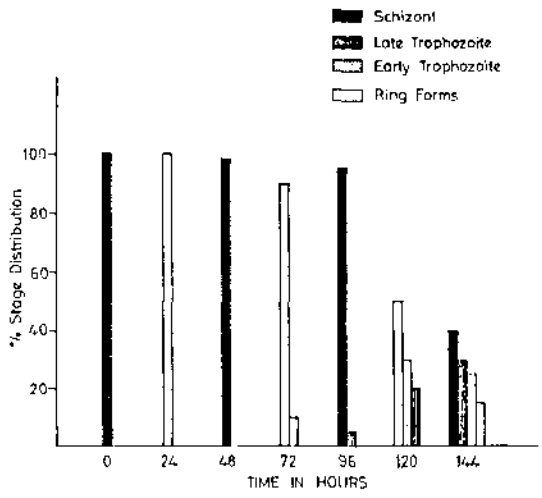


Fig. 3: Relative per cent distribution of *P. falciparum* stages after repeating synchronization with 45 per cent top layer.

DISCUSSION

Isolation of infected erythrocytes from the blood of experimental animals or from cultures has been studied extensively by colloidal silica gradient centrifugation (Reese *et al.*, 1979). Methods for the enrichment of the parasite stages have been developed but none of these methods totally purifies a particular intraerythrocytic stage (Jensen, 1978; Mrema *et al.*, 1979). Colloidal cushions of Percoll and Ficoll were used to separate RBCs infected with *P. yoelii* and *P. berghei* from uninfected RBCs. The best yield of parasitized erythrocytes was achieved using 55% Percoll when about 95% of the RBCs infected by the late stages (late trophozoites and schizonts) were recovered (Tosta *et al.*, 1980). Still this enrichment was not of a homogeneous nature. Recently, discontinuous gradients of Percoll as 30, 35, 45 and 50 per cent were used to enrich the various stages of *P. vivax* and *P. ovale* according to their densities (Andrysiak *et al.*, 1986) which showed that by manipulation of the concentrations of colloidal cushion, stages of various densities could be achieved. Present study with the stepwise gradient from lower to higher concentrations has a number of advantages. Firstly, removal of free merozoites, extracellular late stage parasites and haemozoin pigments give a clarity in the culture and this particular layer can be used as a source of antigen for serological purposes. Secondly, slight increase in Percoll concentration helps to isolate a healthy population of schizonts which shows better synchronous development. Thirdly,conventional method for synchronization by treating the culture with 5 per cent D-sorbitol has a lytic effect towards the late stages, whereas by Percoll

gradient all the stages can be kept viable and they can grow normally. Lastly, two sets of culture give more yield of synchronous stages which permit quantitative studies on the immunological, biochemical and physiological differences among the various stages of development.

ACKNOWLEDGEMENTS

Thanks are due to Mrs. N.K. Ammini and other laboratory colleagues for their cooperation, to Mrs. Meenu Talwar for preparing the illustrations, to Miss Renu Bansal for word processing and Miss Rani Dutta for secretarial assistance.

REFERENCES

1. Andrysiak, P.M., W.E. Collins and G.H. Campbell (1986). Concentration of *Plasmodium ovale* and *Plasmodium vivax* infected erythrocytes from non-human primate blood using Percoll gradients. *American J. Trop. Med. Hyg.*, **35**(2): 251–254.
2. David, P.H., M. Hommel, J.C. Benichou, H.A. Eisen and L.H. Pereira da Silva (1978). Isolation of malaria merozoites: Release of *Plasmodium chabaudi* merozoites from schizonts bound to immobilized Con. A. *Proc. Natl. Acad. Sci. (USA)*, **75**: 5081–5084.
3. Jensen, J.B. (1978). Concentrations from continuous culture of erythrocytes infected with trophozoites and schizonts of *Plasmodium falciparum*. *American J. Trop. Med. Hyg.*, **27**: 1274–1276.
4. Lambros, C. and J.P. Vanderberg (1979). Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol.*, **65**(3): 418–420.
5. Mrema, J.E., G.H. Campbell, R. Miranda, A.L. Jaramillo and K.H. Rieckmann (1979). Concentration and separation of erythrocytes infected with *Plasmodium falciparum* by gradient centrifugation. *Bull. WHO*, **57**: 133–138.
6. Reese, R.T., S.G. Langreth and W. Trager (1979) Isolation of stages of the human parasite *Plasmodium falciparum* from culture and from animal blood. *Bull. WHO*, **57**: 53–61.
7. Tharavanij, S and Prasartsiriroj (1981). Simultaneous preparations of merozoites and concentrated schizonts of *Plasmodium falciparum*. *Southeast Asian J. Trop. Med. Pub. Hlth.*, **12**(4): 518.
8. Tosta, C.E., M. Sedegah, D.C. Henderson and N. Wedderburn (1980). *P. yoelii* and *P. berghei*: Isolation of infected erythrocytes from blood by colloidal silica gradient centrifugation. *Exp. Parasitol.*, **50**: 7–15.
9. Trager, W. and J.B. Jensen (1976). Human malaria parasites in continuous culture. *Science*, **193**: 673–675.

Indian Journal of Malariology
Vol. 25, June 1988, pp. 11–15.

Status of Natural Killer Activity in the Peripheral Blood of *P.vivax* and *P. falciparum* Malaria Patients

Q.B. SAXENA[1], S. BISWAS[1] and V.P. SHARMA[1]

Status of natural killer activity (NK) was studied in peripheral blood lymphocytes (PBL) of patients suffering from *Plasmodium falciparum* and *Plasmodium vivax* malaria. The levels of NK activity in patients of *P. falciparum* malaria were not significantly different from the NK levels in healthy controls. However, there was a significant decrease in the levels of NK activity in patients suffering from *P. vivax* malaria. Further the decrease in NK activity in *P. vivax* cases could not be correlated with the age, sex and the degree of parasitaemia of the blood donors.

INTRODUCTION

Natural killer (NK) cells constitute one of the several naturally occurring defence mechanisms in the immune response. NK cells have been known to play an important role in protection against parasitic infections (Eugui and Allison, 1980) besides their role in spontaneously arising tumors (Haller *et al.*, 1977). NK cells may play an important role in conferring early resistance to malaria in beige mutant mouse strain (Solomon *et al.*, 1985). Previous studies in human have shown that the level of NK activity increases in children with malaria (Emmanuel *et al.*, 1981). In adults no significant change in the NK activity between normal individuals and individuals suffering from acute malaria was observed. However, a significant decline in the NK activity was noticed during convalescent period in the same study (Chaicumpa *et al.*, 1982). Status of NK activity in *P. vivax* malaria patients is not clearly known. In the present study we have examined the levels of NK activity in both *P. vivax* and *P. falciparum* malarial cases and compared these with the corresponding levels in healthy donors.

MATERIAL AND METHODS

Present study was carried out in blood specimen obtained from Distt. Shahjahanpur, U.P. which is a highly endemic area with a slide parasite rate and slide falciparum rate of 75.8% and 11.4% respectively during the study year. About 5 ml samples of peripheral blood were collected aseptically from either normal healthy individuals or active malaria cases and brought in ice to the Centre. Only those malaria patients were included in the study who had no apparent symptoms of any other disease. The diagnosis of malaria cases was based upon elevated body temperature and the presence of parasites in peripheral blood. PBL were isolated from the heparinized blood samples within 24 hrs of collection by Ficoll-Hypaque

Accepted for publication: 25 March 1988.
[1]Malaria Research Centre (ICMR)
22-Sham Nath Marg
Delhi-110054, India.

discontinuous gradient centrifugation, washed three times and suspended in complete medium (RPMI 1640 with 10% FCS, 40 μg/ml of gentamycin and 20 mM HEPES) and used as the effector cell preparation in NK assays. The K_{562} human erythromyeloid leukemia target cell line was maintained in complete RPMI medium and were labelled with ^{51}Cr as described before (Saxena *et al.*, 1980).

Natural killer assay

Natural killer (NK) assays were carried out at different effector: target ratios using round bottom microtitre plates as previously described (Saxena *et al.*, 1980). In brief 100 μl of effector cell suspension and 100 μl of suspension of Chromium-51 labelled target cells (10^5/ml) were placed in the round bottom wells of 96 well costar plates. Plates were centrifuged at 500 rpm for 3 mins. and incubated for 4 hours at 37°C in humidified atmosphere of 5% CO_2. At the end of the experiment 100 μl of supernatants were removed from each well without disturbing the pellets and the released Chromium-51 was counted in LKB Gamma counter. Base line release (SR) of ^{51}Cr was studied by incubating an equal volume of medium instead of effector cells with the labelled target cells. For estimating maximum release (MR) of ^{51}Cr, 1×10^4 labelled target cells were suspended in 1.0 ml of distilled water and after four hours incubation the radioactivity in 0.5 ml of supernatant was determined. Percentage specific lysis of target cells was calculated by the following formula:

$$\text{Per cent lysis} = \frac{\text{Test CPM} - \text{SR CPM}}{\text{MR CPM} - \text{SR CPM}} \times 100$$

RESULTS

NK activity of PBL derived from *P. falciparum* and *P. vivax* infected patients

Peripheral blood lymphocytes (PBL) derived from blood samples were tested for NK activity in a standard 4 hour chromium release assay using K_{562} target cells. Blood samples from a total of 12 controls, 13 *P. falciparum* and 41 *P. vivax* cases were examined over a period of 4 months between July to October 1986. Detailed results of these assays are shown in Table 1. Values of target lysis at effector: target ratios of 50:1 and 25:1 have been given. In some cases, due to the lack of sufficient effector cells, the assays could only be conducted at a single effector: target ratio. Values of per cent parasitaemia in the blood samples are also given. Mean NK activity ± SD at each E:T ratio for each group of blood donors has been shown in Fig. 1. These results show that there is a 31.37% (E:T, 50:1) and 44.5% (E:T, 25:1) decline in the cytotoxic activity in PBL derived from *P. vivax* patients as compared to control values. These declines are statistically significant by non paired student's t-test of significance ($p < 0.01$). Mean value for cytotoxic activity in *P. falciparum* cases is 18.78% (E:T, 50:1) and 30.0% (E:T, 25:1) lower as compared to control levels but these do not represent significant decline in the NK activity ($p > 0.05$).

We checked for a possible relationship between the per cent parasitaemia and the levels of NK activity in different blood samples from malarial patients but no correlation could be found between these two parameters ($r = 0.265$ and 0.317 for *P. vivax* and *P. falciparum* samples respectively). Significant difference could also not be found between the levels of NK activity in blood samples from male and female donors.

DISCUSSION

In the present study we have assessed the levels of NK activity in the peripheral blood lymphocytes derived from malarial patients. Our results indicate that the mean NK activity in *P. falciparum* blood samples was lower than control but the decline was not statistically significant. A significant decline in the NK activity could however be demonstrated in *P. vivax* cases of malaria. It should be noted that we tested a larger number of

Table 1. Natural killer activity in PBL of normal subjects and patients suffering from malaria

Donor code No.	Sex	Status	Parasitaemia	Per cent lysis of K_{562}	
				E:T, 50:1	25:1
118	M	Control	—	18.5	12.5
119	M	"	—	—	30.5
126	M	"	—	54.0	38.5
128	M	"	—	28.9	27.0
132	M	"	—	30.0	19.5
136	M	"	—	52.0	41.0
140	M	"	—	50.0	31.0
143	M	"	—	22.0	15.0
144	F	"	—	20.0	13.0
150	M	"	—	21.0	—
160	F	"	—	22.0	27.5
164	M	"	—	52.0	40.5
173	F	"	—	71.0	60.0
110	M	*P. falciparum*	0.2% (R)	29.0	20.5
111	F	"	3.5% (R)	—	11.0
112	M	"	0.4% (R)	58.0	39.0
113	M	"	2.0% (R)	12.0	8.6
114	M	"	0.05% (R)	27.8	18.8
116	M	"	0.5% (R)	45.5	28.0
138	F	"	0.05% (R)	35.5	24.0
139	M	"	1.0% (R)	23.0	14.5
141	M	"	0.005% (R)	9.8	8.8
145	M	"	ND	30.0	14.5
169	M	"	0.01% (R)	33.5	20.0
172	F	"	2.5% (R)	24.5	18.0
175	F	"	0.01% (R)	—	44.0
97	F	*P. vivax*	1.5% (TSGR)	—	14.0
98	F	"	0.5% (ND)	—	4.0
99	M	"	0.3% (RTG)	17.0	14.0
100	M	"	2.0% (ND)	22.0	18.0
102	F	"	1.0% (RTG)	36.0	27.0
103	F	"	0.005% (G)	21.0	15.0
104	F	"	1.0% (RTSG)	14.0	7.0
105	M	"	0.2% (RG)	16.3	97.0
106	F	"	0.5% (RGS)	23.5	16.0
107	F	"	0.5% (RT)	41.0	27.5
109	F	"	0.01% (RG)	28.0	20.1
115	F	"	1.0% (RTSG)	6.0	2.0
117	M	"	ND	31.0	20.0
120	M	"	0.1% (RT)	12.5	4.5
121	M	"	2.0% (RTS)	22.5	16.8
122	M	"	ND	10.0	4.5
123	F	"	0.2% (RT)	5.0	3.0
124	F	"	0.001% (R)	39.6	30.0
127	NK	"	ND	37.5	2.0
130	F	"	0.05% (R)	30.0	17.5
134		"	0.1% (RT)	27.5	15.0
142	F	"	0.02% (RS)	21.5	15.5

Contd.

Table 1. *(Contd.)*

Donor code No.	Sex	Status	Parasitaemia	Per cent lysis of K_{562}	
				E:T, 50:1	25:1
145	M	"	ND	12.5	7.5
147	F	"	0.8% (RTG)	29.5	20.0
149	F	"	0.005% (RG)	29.5	12.0
151	F	"	0.002% (R)	26.0	10.0
152	M	"	0.5% (RTG)	18.0	4.0
153	M	"	0.1% (RG)	33.5	18.0
154		"	0.2% (TSR)	—	22.0
155		"	2.0% (R)	—	37.0
156	NK	"	0.01% (R)	32.0	24.0
158	M	"	ND	33.0	18.5
161	M	"	0.001% (R)	43.2	31.0
162	M	"	ND	—	21.5
163	M	"	ND	—	19.0
165	M	"	0.01% (RG)	38.5	30.0
167	F	"	0.4% (RT)		
168	F	"	ND	—	36.0
176	F	"	ND	41.5	26.0
177	M	"	ND	24.0	14.5

R = Ring form; T = Trophozoite; S = Schizont; G = Gametocyte; ND = Not done

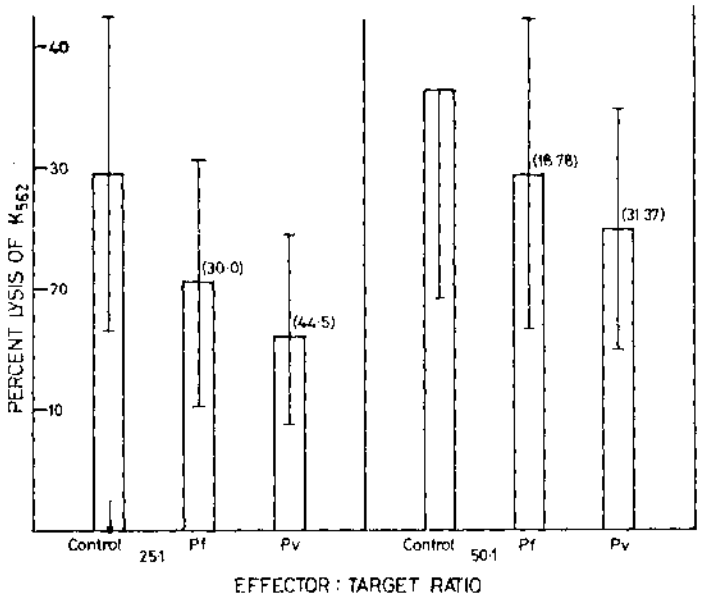


Fig. 1: Combined results of levels of natural killer activity in the peripheral blood lymphocytes of malarial patients and healthy donors. Experimental protocol and individual values are given in Table 1. Values in parentheses denote the per cent inhibition as compared to control values * $p < 0.05$; ** $p < 0.01$.

P. vivax samples (41 total) as compared to *P. falciparum* samples (13 cases). It is not unlikely that the decline in NK activity in Pf cases may also turn out to be significant if a larger number of Pf cases are examined. There are reports in literature showing high (Emmanuel *et al.*, 1981) or significant decline (Chaicumpa *et al.*, 1982) in levels of NK activity in Pf and Pv cases. It appears, therefore, that even in the levels of NK activity decline in Pf cases, quantitatively the decline is not likely to be marked.

To our knowledge, this is the first report where decline in the peripheral blood NK cell activity in *P. vivax* infection has been demonstrated. Sample to sample variations in NK cell activities were rather high in all groups of donors but this is expected in human studies. Our own previous results about the NK levels in human alcoholics had shown similar variations (Saxena *et al.*, 1980). In view of large variations, it is difficult to assess whether the NK levels in a given PV blood sample represent a significant decline. In order to delineate the effect of malarial infection on the status of NK cells in an individual one possible way could be to follow the levels of blood NK activity in a given subject during the course of the disease. We have initiated such studies in monkeys and our initial results indicate that if monkeys are infected with *P. cynomolgi*, an initial decline in NK levels is followed by a return to normal levels in later parts of the infection (unpublished data). In view of the monkey results a part of the variability we have seen in the NK levels in human malarial cases could be due to the fact that the blood samples were collected at different time points during the course of infection.

The significance for the decline in blood NK levels in malarial subjects is not clear. On the efferent side of this phenomenon, a drop in NK activity could be a pointer to the role of NK cells in resistance to malarial parasite. For instance it is possible that the NK cells may have been recruited to the infection sites in liver or spleen resulting in an apparent fall in blood levels of NK cells. This possibility arises in view of our recent observation indicating that the human NK cells can interact with malarial parasites (manuscript in preparation). Decline in NK levels could also result from non-specific stress associated with fever. These possibilities would be further investigated in animal models. In the efferent side low levels of NK activity in malaria may predispose an individual to those infections in which NK cells have a protective role.

ACKNOWLEDGEMENTS

We thank Mrs. Anandi Sharma and Mrs. N.K. Ammini Sivaraman for technical assistance. We also thank Dr. A. Srivastava for statistical analysis.

REFERENCES

1. Chaicumpa, Wanper, Nobpawan Atthasishtha, Somchai Looareesuwan and Savanat Tharavanij (1982). Natural killer cells in peripheral blood of healthy individuals and patients with malaria. *Southeast Asian J. Trop. Med. Pub. Hlth.*, **13**(1): 61–68.
2. Emmanuel, A., O.J.O. Amaize, Ekan S. Salimonu, I.O. Akintunde Williams, Olaclele A.O. Akinwolere, Raad Shabo, Gunnar V. Alm and Hans Wigzell (1981). Positive correlation between degree of parasitaemia, Interferon titres and natural killer cell activity in *Plasmodium falciparum* infected children. *J. Immunol.*, **125**(6): 2296–2300.
3. Eugui, E.M. and A.C. Allison (1980). Difference in susceptibility of various mouse strains to haemoprotozoan infections. Possible correlation with natural killer activity. *Parasite Immunol.*, **2**: 277.
4. Haller, O., M. Hansson, R. Kiessling and H. Wigzell (1977). Role of non-conventional natural killer cells in resistance against syngeneic tumor cells *in vivo*. *Nature*, **270**: 609.
5. Saxena, Q.B., E. Mezey and W.H. Adler (1980). Regulation of natural killer activity *in vivo*. II. The effect of alcohol consumption on human peripheral blood natural killer activity. *Intnatl. J. Cancer*, **26**: 413–417.
6. Solomon, J.B., M.G. Forbes and G.R.A. Solomon (1985). A possible role for natural killer cells in providing protection against *Plasmodium berghei* in early stages of infection. *Immunol. Letters*, **7**: 350–352.

Indian Journal of Malariology
Vol. 25, June 1988, pp. 17–22.

The Value of Spraying Cattlesheds in a Control Programme

M.A. ANSARI[1], V.P. SHARMA[1], R.K. RAZDAN[1], C.P. BATRA[1] and P.K. MITTAL[1]

Spraying of DDT was carried out in (i) houses and (ii) houses and cattlesheds in Muradnagar PHC, Distt. Ghaziabad (U.P.). The main vector *A. culicifacies* is resistant to DDT and HCH in this region. The entire PHC was divided into 4 zones of about 30 thousand population each. Villages of zone I were held as control and sprayed by NMEP with HCH @ 200 mg/sq.m while in zone II and IV only houses were sprayed with DDT @ 1 and 2 gm/sq.m respectively. In zone III DDT was sprayed @ 1 gm/sq.m in both cattlesheds and houses. Results of the comparative studies revealed that spraying the houses alone or houses and cattlesheds did not produce any significant difference in either the reduction in vector density or the transmission of malaria. The impact of spraying DDT @ 1 gm or 2 gm/sq.m also produced non-significant results. The study brought out the importance of spraying the houses alone and that spraying of cattlesheds is not necessary in a control programme.

INTRODUCTION

DDT is still the insecticide of choice to interrupt malaria transmission in the country, although *A. culicifacies*, the vector responsible for most rural and peri-urban malaria transmission has developed various levels of resistance to it. At present DDT is sprayed in about 210 million population and in about 110 million population where *A. culicifacies* has become highly resistant to DDT, HCH is sprayed, and in about 22 million population with high degree of DDT and HCH resistance malathion is sprayed. Change of insecticide from DDT to any other suitable replacement insecticide is very expensive and mosquitoes have the inherent characteristic to develop multiple resistance. Use of replacement insecticide should, therefore, be avoided in favour of DDT as long as it interrupts transmission to acceptable levels.

Under the modified plan of operation (MPO) of the National Malaria Eradication Programme (NMEP) all sections (about 10,000 population) showing 2 or more API are sprayed to interrupt transmission. Spraying is targeted to achieve maximum coverage, but in the field it has been observed that the spraying coverage achieved is far below the expected levels of $> 90\%$. Further most refusals to spraying are for houses and not the cattlesheds. The spraying of cattlesheds may result in natural deviation of mosquito populations towards houses which may in fact enhance man-mosquito contact. Since MPO is a control and not eradication programme,

Accepted for publication: 21 April 1988.
[1]Malaria Research Centre (ICMR)
22-Sham Nath Marg
Delhi-110054, India.

spraying of houses alone and not the cattlesheds may be a more suitable strategy, provided the level of malaria control achieved remains at the same levels as would normally be achieved by spraying all the structures i.e., houses and cattlesheds. This strategy would not only save insecticides and labour but may also repel mosquitoes from houses towards the cattlesheds thus reducing man-mosquito contact. This may be particularly suitable for *A. culicifacies* which is basically a zooophilic species.

MATERIAL AND METHODS

Muradnagar primary health centre (PHC) of Ghaziabad distt., (U.P.) was selected for the study. In this PHC about 1.14 lakh people live in 57 villages and the remaining 0.02 lakh in one town. The area of PHC is about 220 sq. kms of which about 16 hectares is irrigated through canals, tubewells and wells. The main crops are wheat, maize, rice and millets. Spraying of DDT was discontinued in 1977 and since then HCH was being sprayed during the transmission season (June–October). This PHC was taken up for field study for a period of one year from April 1985 to May 1986.

The PHC was divided into four zones, each comprising of about 28000 population. Zone I was held as control. For ethical considerations it was not possible to leave the control area unsprayed and therefore in this area HCH (3 rounds @ 200 mg/m^2) was sprayed by the NMEP. Villages of zones II, III and IV were sprayed with 2 rounds of DDT (50% WP) supplied by the NMEP but sprayed under the supervision of MRC staff. In zone II DDT was sprayed @ 1 gm/m^2 in houses only. In zone III houses and cattlesheds were sprayed with DDT @ 1 gm/m^2. In zone IV DDT was sprayed in houses only @ 2 gm/m^2. While spraying the discharge rate was maintained at >750 ml/40 stroke per min. using the stirrup pumps. In each zone, 5 villages, roughly equidistant from each other were selected for monitoring the entomological and epidemiological indices. Indoor vector density was monitored using a suction tube during dawn in houses and cattlesheds. Data of all villages in each zone was pooled and averaged for each month to calculate per man hour density. Door to door active surveillance was maintained by the project staff on weekly basis throughout the study period. All fever cases were given presumptive treatment (600 mg chloroquine adult dose) and radical treatment was administered to malaria cases within 48 hours which comprised of 600 mg chloroquine. In addition, for *P. vivax* cases 15 mg primaquine daily for 5 days and for *P. falciparum* cases 45 mg primaquine single dose were given. Children were given proportionately low dosages and pregnant women were not given primaquine.

Insecticide susceptibility tests were carried out by exposing field collected gravid *A. culicifacies* to

Table 1. Results of insecticide susceptibility tests of *A. culicifacies* before spraying

Muradnagar PHC villages	DDT 4% (1 hour)		Dieldrin 4% (1 hour)		Malathion 5% (1 hour)	
	No. exposed	% corrected mortality	No. exposed	% corrected mortality	No. exposed	% corrected mortality
Suthari	20	50	—	—	—	—
Kaithwari	20	27.8	20	38.9	—	—
Manoli	20	50.0	20	0	20	100
Milak Rawli	10	50	10	10	—	—
Asalat Nagar	20	27.8	—	—	20	100
Nabipur	30	39.3	20	3.6	—	—
Total	120	40.2	70	13.1	40	100

DDT (4%) dieldrin (4%) and malathion (5%) impregnated papers for one hour with concurrent controls. Mortality was recorded 24 hrs post exposure.

RESULTS AND DISCUSSION

Table 1 gives the result of insecticide susceptibility tests before starting the experiments. Exposure of *A. culicifacies* to DDT (4%) and dieldrin (4%) papers produced 40.2% (range 27.8–50%) and 13.1% (0–38.9%) mortality respectively. There was 100% mortality in adults exposed to malathion (5%) *A. culicifaces* was fully susceptible to malathion but resistant to DDT and dieldrin. The level of resistance to dieldrin was higher than DDT which may be the result of several years of HCH spraying in these villages.

The spraying coverage is given in Table 2. In control area (zone I) coverage of human dwelling and cattlesheds varied from 76–87% and room coverage in houses was between 62–68%. In contrast spraying coverage in human dwellings was better in the experimental zones and in both the rounds it was >90%, the room coverage was also better i.e., 70–86% whereas coverage in the cattleshed in zone III was about the same as in the control zone.

Table 3 gives monthly per man hour density of *A. culicifacies*. Pre-spray density of *A. culicifacies* in April was comparable in all the 4 zones. The first round of HCH spraying was started in May (zone I) while spraying commenced in June in the remaining 3 zones. The density of *A. culicifacies* remained at about the same level upto June and started to increase afterwards in zone I attaining peak in August despite HCH spraying. *A. culicifacies* densities did not increase to such high levels in any of the DDT sprayed zones, although *A. culicifacies* densities were considerably high in cattlesheds as compared to houses in all the zones. Statistical analysis (t-test) for the months June to October i.e., transmission period showed that the differences in MHD in zone II was significant as compared to zone I even at 1% level while it was significant at 5 and 10% level with zone III and IV respectively, showing thereby the DDT spraying in zone II, III and IV was superior to zone I held as HCH sprayed control. *A. culicifacies* densities in zone II and III i.e., where houses alone and houses and cattlesheds were sprayed showed non-significant results even at 10% level. It was, therefore, clear that DDT spraying in houses @ 1 gm/m^2 produced comparable results in terms of the reduction of *A. culicifacies* populations when compared to spraying the houses and cattlesheds. There was however significant difference in zone II and IV at 5% level i.e., 1 gm DDT/m^2 spraying was slightly superior to 2 gm/m^2 dosage. This may be due to normal variation in the vector populations in the two zones or due to low coverage of rooms in zone IV (Table 2).

Results of parasitological monitoring of the incidence of malaria are given in Table 4. The annual blood examination rate (ABER) in zones I to IV was 15.0, 10.3, 9.7 and 11.9 respectively. In comparison to control there was significant reduction in SPR in all the 3 DDT sprayed zones but the differences within the DDT sprayed zones were not significant. Similar results were obtained, when slide falciparum rate (SfR) or total malaria cases or *P. falciparum* cases per thousand population in the experimental areas were compared with the control area. There was no difference in either spraying the houses and cattleshed or the houses alone with 1 or 2 gm/sq. m DDT. Statistical validation (t-test) was done at 5% level of significance during transmission months, for SPR and cases/1000 from (June–October) and for SfR and Pf/1000 from (September–November) it was done at 1% and 10% level respectively. It may be pointed out that our comparison area zone I was sprayed with HCH by the NMEP and, therefore, it would have interrupted some transmission but still SPR, SfR and cases per 1000 population were very high. If the control area was held as unsprayed, the impact of DDT spraying possibly would have become very much pronounced.

Table 2. Spraying coverage (%) in different zones of Muradnagar PHC

Spray Round	Zone I HCH @ 0.2gm/sq.m				Zone II DDT @ 1 gm/sq.m (Houses only)			Zone III DDT 1 gm/sq.m (Houses + CS)				Zone IV DDT @ 2 gm/sq.m (Houses only)		
		HD	R	CS		HD	R		HD	R	CS		HD	R
I	7–5–85	83.9	63.8	82.5	30–5–85	95.7	78.5	30–5–85	93.5	79.7	87.9	30–5–85	95.3	74.0
II	3–7–85	89.5	68.1	76.4	14–8–85	98.9	86.7	14–8–85	95.2	82.6	90.9	15–8–85	95.2	71.2
III	9–8–85	91.9	62.1	87.3	—	—	—	—	—	—	—	—	—	—

HD = Human Dwelling including temporary sheds; R = Rooms in houses; CS = Cattlesheds

Table 3. *Anopheles culicifacies* man hour densities in each zone

Months 1985–86	Zone I HCH 200 mg/sq.m (NMEP spray)		Zone II DDT 1 gm/sq.m (Houses only)		Zone III DDT 1 gm/sq.m (Houses + Cattlesheds)		Zone IV DDT 2 gm/sq.m (Houses only)	
	Houses	Cattlesheds	Houses	Cattlesheds	Houses	Cattlesheds	Houses	Cattlesheds
Apr	4.75	3.55	3.40	3.55	3.50	2.10	4.25	4.10
May	7.95	7.30	3.50	3.90	5.95	5.70	8.35	10.65
Jun	7.75	5.80	1.90	4.70	6.25	5.95	5.30	9.60
Jul	7.55	19.50	1.95	4.30	1.90	5.65	4.35	10.35
Aug	18.75	46.10	4.00	10.25	4.55	12.10	8.70	23.90
Sep	14.75	34.00	0.90	3.40	0.70	2.55	2.90	10.00
Oct	2.70	7.25	0.05	0.80	0.05	0.55	0.80	2.35
Nov	1.00	3.50	0.10	0.50	0.00	0.05	0.20	0.70
Dec	0.55	1.65	0.00	0.05	0.00	0.10	0.00	0.30
Jan	0.25	1.20	0.00	0.00	0.00	0.00	0.00	0.00
Feb	0.40	0.65	0.00	0.05	0.00	0.00	0.00	0.00
Mar	0.15	1.00	0.00	0.00	0.00	0.00	0.00	0.05

Zone wise t-test results of vector densities (June-October) analysed for statistical validation

Houses	I–II (3.55)	I–III (2.76)	I–IV (2.09)*	II–III (1.39 NS)	II–IV (2.66)	III–IV (1.09 NS)
Cattlesheds	I–II (2.51)	I–III (2.39)	I–IV (1.48 NS)	II–III (0.14 NS)	II–IV (1.91 NS)	III–IV (1.64 NS)

Note : All values are significant at 5% except I–IV* which is at 10% level; Non-significant values have been shown as NS

Table 4. Results of parasitological survey in Muradnagar PHC

Month	Zone I HCH 200 gm/sq. m (NMEP spray)					Zone II DDT 1 gm/sq. m (Houses only)					Zone III DDT 1 gm/sq. m (Houses + Cattlesheds)					Zone IV DDT 2 gm/sq. m (Houses only)				
	BSC	SPR	SFR	Cases /000	Pf /000	BSC	SPR	SFR	Cases /000	Pf /000	BSC	SPR	SFR	Cases /000	Pf /000	BSC	SPR	SFR	Cases /000	Pf /000
1985-86																				
Apr	500	15.2	2.2	9.8	1.4	195	11.8	1.0	2.9	0.3	333	13.5	3.3	5.4	1.3	432	15.9	2.5	7.7	1.2
May	404	27.2	1.3	14.2	0.7	371	20.5	0.8	9.7	0.4	355	20.0	0.0	8.5	0.0	426	20.4	1.4	9.8	0.7
Jun	272	32.4	1.8	11.4	0.7	281	27.8	0.7	9.9	0.3	221	18.1	0.0	4.8	0.0	237	20.7	0.8	5.5	0.2
Jul	418	27.8	1.4	14.9	0.5	335	24.8	0.6	10.6	0.3	202	21.8	0.9	5.3	0.2	326	19.9	0.6	7.3	0.2
Aug	862	40.3	1.8	44.9	24.0	577	25.3	10.2	18.7	7.6	417	26.9	14.9	13.5	7.6	551	30.7	16.5	18.9	10.2
Sep	792	52.1	41.7	53.4	42.7	467	33.4	22.3	19.9	13.3	397	31.9	24.4	15.3	11.7	438	32.4	23.7	15.9	11.6
Oct	399	50.4	49.1	25.9	25.3	278	22.7	16.9	8.1	6.0	295	22.4	17.8	7.9	6.3	342	18.1	14.3	6.9	5.5
Nov	189	40.7	39.2	9.9	9.6	177	11.1	10.3	1.7	1.5	163	15.3	10.4	3.0	2.0	186	15.6	10.8	3.3	2.2
Dec	127	26.7	20.5	4.4	3.4	74	12.2	8.1	1.2	0.8	89	6.7	5.6	0.7	0.6	113	9.7	7.9	1.2	1.0
Jan	102	25.5	22.5	3.4	2.9	97	5.2	3.1	0.6	0.4	84	4.8	2.4	0.5	0.2	103	2.9	1.9	0.3	0.2
Feb	75	9.3	6.7	0.9	0.7	47	8.5	6.4	0.5	0.3	77	1.3	1.3	0.1	0.1	100	4.0	4.0	0.5	0.5
Mar	85	4.7	4.7	0.5	0.5	43	2.3	0.0	0.1	0.0	92	0.0	0.0	0.0	0.0	87	0.0	0.0	0.0	0.0
Total	4225	35.5	20.6	193.6	112.3	2882	22.8	8.4	84.0	31.1	2725	19.9	9.2	64.9	30.0	3341	20.7	8.9	77.3	33.6
(%reduction in comparison with control)							35.7	59.2	56.6	72.3		44.0	55.5	66.4	73.3		41.8	56.9	60.1	70.1

Zone wise t-test results of parasitological parameters analysed for statistical validation

SPR	I–II	(2.99)*	I–III	(3.40)*	I–IV	(3.20)*	II–III	(0.95)	II–IV	(0.77)	III–IV	(0.04)	(Jun-Oct)	(* – p = 5%)
SFR	I–II	(7.19)*	I–III	(6.31)*	I–IV	(6.81)*	II–III	(0.22)	II–IV	(0.05)	III–IV	0.26)	(Sep-Nov)	(* – p = 1%)
Cases/000	I–II	(2.56)*	I–III	(3.08)*	I–IV	(2.84)*	II–III	(1.41)	II–IV	(0.77)	III–IV	(0.50)	(Jun-Oct)	(* – p = 5%)
Pf/000	I–II	(2.28)*	I–III	(2.36)*	I–IV	(2.39)*	II–III	(0.07)	II–IV	(0.14)	III–IV	(0.07)	(Sep-Nov)	(* – p = 10%)

Note: Unstarred figures are non-significant

It has been observed in the field that refusals to spraying are mainly in regard to houses and not the cattlesheds and, therefore, if all cattlesheds and some houses are sprayed then *A. culicifacies* which is basically a zoophilic vector would be repelled from cattlesheds towards houses which may in turn increase man mosquito contact. The field experiments for one year have clearly shown that spraying the cattlesheds does not produce any enhanced impact either on reduction in disease transmission or on the vector densities, and in fact improved coverage of houses alone yielded better results in zone II which was sprayed with DDT @ 1 gm/m^2.

In malaria control programme spraying of DDT is preferred because it has the lowest cost per million coverage i.e., 34 lakhs against 37 with HCH and 199 with malathion (Sharma and Sharma, 1986). Sharma *et al.* (1982; 1986), demonstrated that spraying of DDT in areas where *A. culicifacies* was resistant to DDT and HCH, reduces the vector densities and malaria transmission. It was later discovered that one reason for this phenomenon was that among the sympatric populations of sibling species A and B, species A was more susceptible to DDT than B, and species A was an efficient vector of malaria in Loni PHC of Distt. Ghaziabad, U.P. (Subbarao *et al.*, 1988a; 1988b). It would, therefore, make economic sense to spray DDT in houses alone as long as it produces desired epidemiological impact on transmission. This would also result in savings of insecticides, operational cost and may also produce some zooprophylaxis effect on transmission.

ACKNOWLEDGEMENTS

Authors wish to thank Dr. (Mrs.) Aruna Srivastava for statistical analysis of data. Authors are also grateful to Director NMEP for supplying DDT and to DMO, Ghaziabad for supplying spraying squad and equipments. Thanks are also due to Mr. Alok Srivastava for data processing and to Mr. Intezar Ahmed, Mr. Pooran Singh, Mr. S.N.S. Kuchwaha, Mr. K.C. Pushap, Mr. C.S. Sahota and other staff for their technical assistance in the field work.

REFERENCES

1. Sharma, V.P., H.C. Uprety, Nutan Nanda, V.K. Raina, S.K. Parida and V.K. Gupta (1982). Impact of DDT spraying on malaria transmission in villages with resistant *Anopheles culicifacies*. *Indian J. Malariol.*, **19**: 5–12.
2. Sharma, V.P., R.K. Chandrahas, M. A. Ansari, P.K. Srivastava, R.K. Razdan, C.P. Batra, K. Raghuvendra, B.N. Nagpal, S.C. Bhalla and G.K. Sharma (1986). Impact of DDT and HCH spraying on Malaria Transmission in villages with DDT and HCH resistant *Anopheles culicifacies*. *Indian J. Malariol.*, **23**(1) 27–38.
3. Sharma, V.P. and R.C. Sharma (1986). Cost effectiveness of the bio-environmental control of malaria in Kheda district, Gujarat. *Indian J. Malariol.*, **23**(2): 141–145.
4. Subbarao, S.K., K. Vasantha and V.P. Sharma (1988a). Responses of *Anopheles culicifacies* sibling species A and B to DDT and HCH in India; implications in malaria control. *Med. Vet. Entomol.*, **2**: ..
5. Subbarao, S.K., T. Adak, K. Vasantha, H. Joshi, K. Raghuvendra, A.H. Cochrane and V.P. Sharma (1988b). Susceptibility of *Anopheles culicifacies* species A and B to *Plasmodium vivax* and *Plasmodium falciparum* as detected by immunoradiometric assay. *Trans. R. Soc. Trop. Med. Hyg.*, **82**: 394–397.

Indian Journal of Malariology
Vol. 25, June 1988, pp 23-25.

Cryopreserved Human Erythrocytes for *in vitro* Cultivation of *Plasmodium falciparum*

H. SRINIVASA[2], SAM JAYANTH SAMUEL[1] and M.J. SEBASTIAN[1]

Fresh human erythrocytes are the best medium for *in vitro* culture of malaria parasites. However, fresh healthy blood is not always readily available, so cryopreserved erythrocytes were used for cultivation of *P. falciparum in vitro*. Cryopreserved RBCs compared well with refrigerated RBCs as regards the parasite count at peak parasitaemia. Refrigerated cells haemolysed rapidly after 28 days of culture whereas cryopreserved RBCs continued to support good parasite growth.

The only disadvantage noted was fragility and increased lysis of the cryopreserved cells which can be overcome by washing.

INTRODUCTION

The *in vitro* cultivation of *P. falciparum* by the petri dish-candle jar technique of Trager and Jensen (1976) opened a new dimension in malaria research. Studies on parasite metabolism, immunology, biochemistry and pharmacology hitherto not feasible have been made possible by this. The candle jar method, though simple, still relies on a continuous supply of human serum and erythrocytes from healthy voluntary blood donors and this is a limitation. In the last decade many attempts have been made to substitute human with animal sera or replace it with defined constituents. These have been partially successful (Trigg, 1985). However, not much emphasis has been given to improving the erythrocyte component. Erythrocytes stored in Alsever's at 4°C support *in vitro* culture of *P. falciparum* upto three weeks only (Trager and Jensen, 1976). Since a continuous supply of fresh erythrocytes of the desired blood group which also supports good parasite growth is not always possible, the present study was carried out to determine if cryopreserved erythrocytes could serve as an alternative to refrigerated erythrocytes for *in vitro* cultivation of *P. falciparum*.

Accepted for publication: 4 May 1988.
[1]Department of Microbiology
St. John's Medical College
Bangalore-560034, India.

MATERIAL AND METHODS

Isolate

FCK-2 a local isolate of *P. falciparum* being maintained by continuous *in vitro* culture (Trager and Jensen, 1976) using human 'O' erythrocytes and human 'O' serum, was used.

Erythrocytes

Erythrocytes from a voluntary human donor of the blood group 'O' was collected in Alsever's

solution. Aliquots were made and after sterility tests which lasted for seven days, a few were stored at 4°C (refrigerated erythrocytes) and the remaining were cryopreserved in liquid nitrogen employing the rapid freezing technique (Bhat and Srinivasa, 1985). These erythrocytes were labelled as cryopreserved erythrocytes. When required the aliquots were thawed for use by a modification of Trager's method (Bhat and Gupta, 1981).

In vitro culture of *P. falciparum* in refrigerated erythrocytes

Experiment 1

The *P. falciparum* infected erythrocytes from continuous culture at the height of parasitaemia were subcultured to reduce the parasitaemia to around 1% using seven day old refrigerated erythrocytes. Medium was changed daily till day 4 after which the experiment was terminated. In order to determine parasitaemia, smear preparations of infected erythrocytes was done daily and stained by JSB method (Jaswant Singh, 1956).

Experiments 2–6

Five more experiments were done using *P. falciparum* infected erythrocytes derived from continuous culture. The protocol followed was same as described above, but the ages of refrigerated erythrocytes used for subculture were different in each experiment. The ages of erythrocytes used for the successive experiments were respectively 7, 14, 21, 28, 42 and 57 days.

In vitro culture of *P. falciparum* in cryopreserved erythrocytes

A total of six experiments were conducted with *P. falciparum* derived from continuous *in vitro* culture as the initial source of inoculum. The protocols were similar to the ones using refrigerated erythrocytes except that cryopreserved erythrocytes were used for subculture instead of refrigerated erythrocytes.

RESULTS AND DISCUSSION

In the initial phase of the study, the cryopreserved erythrocytes could be readily differentiated from refrigerated erythrocytes by their smaller size and crenated appearance in the smear preparations. These types of erythrocytes contained healthy parasites in different stages thereby demonstrating that parasites indeed invaded these erythrocytes. The mean parasitaemia obtained on the third and fourth day of each experiment with cryopreserved erythrocytes generally correlated well with that obtained using refrigerated eryth-

Table 1. ***In vitro*** **culture of** ***P. falciparum*****: Mean parasitaemias obtained using refrigerated and cryopreserved human 'O' erythrocytes**

Experiment No.	Erythrocytes					
	Refrigerated			Cryopreserved		
	Age (Days)	Mean parasitaemia		Age (Days)	Mean parasitaemia	
		Day 3*	Day 4*		Day 3*	Day 4*
1	7	5.9	3.2	0**	3.5	3.2
2	14	12.2	3.6	7	5.3	9.1
3	21	3.4	5.9	14	3.6	6.6
4	28	3.0	3.7	21	2.5	5.5
5	42	3.9	6.4	35	7.4	10.3
6	57	5.3	6.5	50	9.8	16.7

* Refers to day after each subculture—see text for further details.

** Seven day old refrigerated erythrocytes were cryopreserved, thawed and used on the same day—day '0'.

rocytes. In experiment No. 2 using refrigerated erythrocytes the peak parasitaemia was reached as early as day 3 whereas in the case of cryopreserved erythrocytes, the peak count was observed on the fourth day. Among the six experiments the maximum parasitaemia obtained with refrigerated erythrocytes was 12.2% and with cryopreserved erythrocytes it was 16.7% (Table 1).

Although the refrigerated erythrocytes which were older than 28 days supported parasite growth, they progressively haemolysed—a feature which was marked in the sixth experiment. In contrast the cryopreserved erythrocytes continued to support parasite growth throughout with good yield of parasites. The parasitaemia obtained in the last two experiments namely experiment No. 5 and 6 were better with cryopreserved erythrocytes when compared with refrigerated erythrocytes.

Recent research highlights the importance of erythrocytes in successful *in vitro* cultivation of *P. falciparum*. All types of human erythrocytes are not uniformly amenable for *P. falciparum* invasion and growth. Certain factors associated with haemoglobin type, presence of vital enzymes and membrane characteristics are crucial in this regard (Trigg, 1985). Human erythrocytes En (a⁻) that lack glycophorin, Tn and Cad erythrocytes, Thalassemic erythrocytes in homozygous and heterozygous states and G-6-PD deficient erythrocytes are relatively resistant to parasite growth (WHO, 1987; Brockelman *et al.*, 1987; Trigg, 1985). Hence, fresh human erythrocytes which support parasite growth can be cryopreserved in aliquots for use at a later date for short term/continuous cultivation of *P. falciparum*. This procedure would be an effective alternative to erythrocytes stored by the conventional method. It will be interesting to extend this method of cryopreservation of blood rich in young erythrocytes/reticulocytes for short term culture of *P. vivax*.

A minor disadvantage that was noted was the increased fragility of thawed erythrocytes with resultant lysis of quite a few cells, but this can be overcome by several washes before using for culture.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support received from the Indian Council of Medical Research. They thank Dr. Prema Bhat, former Professor and Head, Department of Microbiology, St. John's Medical College for her encouragement in doing this study. Thanks are also due to the staff, Department of Microbiology and the staff, Central Laboratory, St. John's Medical College and Hospital, Bangalore for their assistance.

REFERENCES

1. Bhat, P. and M.M. Gupta (1981). *In vitro*-culture of *P. falciparum* and serodiagnosis of malaria: A laboratory manual. (Dept. of Microbiology, St. John's Medical College, Bangalore).
2. Bhat, P. and H. Srinivasa (1985). Isolation, maintenance under *in vitro* culture system, preservation and preparation of antigens from the local strains of human plasmodium prevalent in Karnataka state. *Final Report.* (Submitted to the Indian Council of Medical Research).
3. Brockelman, C.R., B. Wongsattayanont, P. Tan-Ariya and S. Fucharoen (1987). Thalassemic erythrocytes inhibit *in vitro* growth of *Plasmodium falciparum*. *J. Clin. Microbiol.*, **25**: 56–60.
4. Jaswant Singh (1956). JSB Stain–a review. *Indian J. Malariol.*, **10**: 117–129.
5. Trager, W. and J.B. Jensen 1976). Human malaria parasites in continuous culture. *Science*, **193**: 673–675.
6. Trigg, P.I. (1985). Recent advances in malaria parasite cultivation and their application to studies on host-parasite relationships: a review. *Bull. WHO*, **63**: 387–398.
7. WHO (1987). The biology of malaria parasites. Report of a WHO Scientific Group. *Tech. Rep. Ser.*, 743.

Indian Journal of Malariology
Vol. 25, June 1988, pp. 27–32.

Stabilization of *Plasmodium knowlesi* Antigen for Seroepidemiology of malaria

S.K. SAXENA[1] and G.P. DUTTA[1]

Frozen malaria antigens used by earlier workers for seroepidemiology of human malaria, were not stable because of the autolysis of the antigenic proteins during storage, and they could not be stored in cell-free conditions at 4° or −10°C. High level of proteases have been reported in the parasite which could be responsible for deterioration of the antigenic proteins. Therefore, cell-free *P. knowlesi* schizont antigen was stored at 4°C in the presence of different protease inhibitors and the antigenicity was monitored at different intervals using indirect haemagglutination test. Pepstatin and phenyl methyl sulphonyl fluoride (PMSF) were able to stabilize the antigenic reactivity of the extract for a period upto 30 days as compared to soybean trypsin inhibitor which failed to stabilize the antigen. *P. knowlesi* antigen thus could be stabilized by the addition of specific protease inhibitors.

INTRODUCTION

The serological evaluation of malaria field samples by indirect haemagglutination (IHA) test requires stable malaria antigen which should have long shelf-life. The deterioration of antigenic proteins on storage can adversely affect the IHA test and it is impossible to use the same batch of antigen for screening large number of sera samples. The major drawback of frozen antigen used by earlier workers, was the lability of malaria antigen and the loss of antigenicity, if stored at room temperature or even at −10°C.

Rogers *et al.* (1968) reported the labile nature of *P. knowlesi* antigen when stored even at −70°C and its antigenicity was reported to gradually diminish over a period of three months, while the cell-free extract of this antigen was reported to lose its antigenicity within two weeks of storage at −70°C. At 4°C, this preparation deteriorated within three days due to autolysis. Meuwissen *et al.* (1972) have also reported the deterioration of frozen antigen at −70°C.

The stability of the complement fixing antigens over a longer period of time was achieved by D'Antonio *et al.* (1966). These workers incorporated purified polyvinyl pyrollodone (PVP) in the antigenic preparation and then lyophilized it. Kagan (1972), however, failed to reproduce the results using PVP.

The unstability of antigen might be due to the autolytic degradation of antigenic proteins. The presence of proteases and peptidases in the malarial parasites has already been documented (Banyal *et al.*, 1981; Dutta and Banyal, 1981; Gyang *et al.*, 1982; Kaushal *et al.*, 1983; Hempel-

Accepted for publication: 6 May 1988.
[1]Division of Microbiology
Central Drug Research Institute
Lucknow-226001, India.

mann and Wilson, 1980; Sherman and Tanigoshi, 1983; Vanderjagt *et al.*, 1986). Endogenous proteases and other hydrolases might also be the cause of deterioration of antigens. In the present study, protease inhibitors were incorporated in the cell-free *P. knowlesi* antigen and its antigenicity as shown by IHA test was monitored at intervals upto 30 days.

MATERIAL AND METHODS

Preparation of cell-free extract

P. knowlesi schizont antigen was purified as reported by Dutta and Nath (1986) using Ficoll-Hypaque gradient followed by saponin lysis. The parasite preparation was homogenized by sonication and the homogenate was centrifuged at 8000g for 15 mins. in refrigerated centrifuge and the supernatant (cell-free extract) was aspirated and used as antigen. Protein was monitored by the method of Lowry *et al.* (1951) and the volume of the extract was adjusted in such a way that 100 μl of the extract contained 296 μg of protein (0.2 ml of extract of lyophilized antigen also contains 296 μg of protein in 100 μl).

Storage of cell-free extract (antigen)

Cell-free extract was dispensed in 35 sterile vials (1 ml in each vial). Five vials were used as controls in which no inhibitor was added. In another 30 vials different protease inhibitors were incorporated. For each inhibitor, a set of 10 vials was used, 5 containing 25 μg and five with 50 μg of inhibitors. Before incubation one vial of control sample and six with inhibitors (25 μg and 50 μg respectively of three inhibitors) were taken out and IHA test for the optimum dilution was carried out. Rest of the vials were stored at 4°C.

Day schedules for storage

Each set of vials containing controls and inhibitors which were stored for 4,8,15 and 30 days respectively, were tested for antigenicity by the IHA test.

IHA Test

IHA test was carried out essentially as described by Dutta *et al.* (1986). For determining the optimum antigen concentration 0,50,100 and 200 μl of cell-free extract were used to sensitize 2 ml of fixed and tanned human 'O' group erythrocytes. The test was performed using a battery of two reference malaria positive sera and one negative serum.

RESULTS

Optimum concentration of cell-free *P. knowlesi* antigen (without inhibitors) for IHA test was found to be 100 μl. Antibody assay results showed decrease of titre from 32 and 64 on day 0 to nil and 4 on day 30, when the antigen was stored at 4°C (Table 1).

Tables 2,3 and 4 show the results of IHA test with cell-free antigen stored in presence of protease inhibitors [pepstatin, phenyl methyl sulphonyl fluoride (PMSF) and soybean trypsin inhibitor (STI)] upto 30 days. Two of the inhibitors (pepstatin and PMSF) have been found to prevent the deterioration of the antigen at 4°C. Fifty μg/ml of pepstatin was able to stabilise the antigen to a large extent upto 30 days (Table 2). Similar results were obtained in presence of PMSF except that this inhibitor proved to be more effective and it stabilized the cell-free antigen even at 25 μg concentration.

Soybean trypsin inhibitor (STI) failed to stabilize the antigen at both 25 μg and 50 μg per ml concentrations. The antigenicity was reduced to half on day 4 and to one fourth on day 15 (Table 4).

DISCUSSION

The indirect haemagglutination test with frozen antigens, has been extensively applied for the seroepidemiology of malaria (Mahajan *et al.*, 1981; Agarwal *et al.*, 1981; 1982). The sensitivity of the test depends upon several factors such as

Table 1. Optimum antigen concentration determination of *P. knowlesi* cell-free extract without inhibitor

Inhibitor	Antigen concentration*	Reference sera	Period of storage without inhibitor				
			Day 0	Day 4	Day 8	Day 15	Day 30
Nil	0	Ref +ve	Nil	Nil	Nil	Nil	Nil
		Ref +ve	Nil	Nil	Nil	Nil	Nil
		Ref − ve	Nil	Nil	Nil	Nil	Nil
Control	50	Ref +ve	32	8	4	Nil	Nil
		Ref +ve	32	8	4	Nil	Nil
		Ref − ve	Nil	Nil	Nil	Nil	Nil
	100	Ref +ve	64	16	8	4	4
		Ref +ve	64	16	8	4	4
		Ref − ve	Nil	Nil	Nil	Nil	Nil
	200	Ref +ve	64	16	8	4	4
		Ref +ve	64	16	8	4	4
		Ref − ve	Nil	Nil	Nil	Nil	Nil

* μl of cell-free extract used to sensitise 2 ml of fixed and tanned HRBC.

Table 2. Optimum antigen concentration determination of *P. knowlesi* cell-free extract in presence of inhibitor

Inhibitor	Antigen concentration*	Reference sera	Period of storage with pepstatin (conc. 25μg and 50μg respectively)									
			Day 0		Day 4		Day 8		Day 15		Day 30	
			25 μg	50 μg	25 μg	50 μg	25 μg	50 μg	25 μg	50 μg	25 μg	50 μg
Pepstatin	0	Ref +ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
		Ref +ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
		Ref − ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	50	Ref +ve	32	32	16	32	16	32	16	16	16	16
		Ref +ve	32	32	32	32	32	32	16	16	16	16
		Ref − ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	100	Ref +ve	64	64	64	64	32	64	32	32	32	32
		Ref +ve	64	64	64	64	32	64	32	32	32	32
		Ref − ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	200	Ref +ve	64	64	64	64	64	64	32	32	32	32
		Ref +ve	64	64	64	64	64	64	32	32	32	32
		Ref − ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

* μl of cell-free extract used to sensitise 2 ml of fixed and tanned HRBC.

Table 3. Optimum antigen concentration determination of *P. knowlesi* cell-free extract in presence of inhibitor

Inhibitor	Antigen concentration*	Reference sera	Period of storage with PMSF (conc. 25 μg and 50 μg respectively)									
			Day 0		Day 4		Day 8		Day 15		Day 30	
			25 μg	50 μg	25 μg	50 μg	25 μg	50 μg	25 μg	50 μg	25 μg	50 μg
Phenyl Methyl Sulphonyl Fluoride (PMSF)	0	Ref +ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
		Ref +ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
		Ref – ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	50	Ref +ve	32	32	32	32	32	32	16	16	16	16
		Ref +ve	32	32	32	16	32	32	16	16	16	16
		Ref – ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	100	Ref +ve	64	64	64	64	64	64	32	32	32	32
		Ref +ve	64	64	64	64	64	64	32	32	32	32
		Ref – ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	200	Ref +ve	64	64	64	64	64	64	32	32	32	32
		Ref +ve	64	64	64	64	64	64	32	32	32	32
		Ref – ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

*μl of cell-free extract used to sensitise 2 ml of fixed and tanned HRBC.

Table 4. Optimum antigen concentration determination of *P. knowlesi* cell-free extract in presence of inhibitor

Inhibitor	Antigen concentration*	Reference sera	Period of storage with STI (conc. 25 μg and 50 μg respectively)									
			Day 0		Day 4		Day 8		Day 15		Day 30	
			25 μg	50 μg	25 μg	50 μg	25 μg	50 μg	25 μg	50 μg	25 μg	50 μg
Soybean Trypsin Inhibitor (STI)	0	Ref +ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
		Ref +ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
		Ref – ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	50	Ref +ve	32	32	16	16	16	16	8	8	8	8
		Ref +ve	32	32	16	16	16	16	8	8	8	8
		Ref – ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	100	Ref +ve	64	64	32	32	32	32	16	16	16	16
		Ref +ve	64	64	32	32	32	32	16	16	16	16
		Ref – ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	200	Ref +ve	64	64	64	64	32	32	16	16	16	16
		Ref +ve	64	64	64	64	32	32	16	16	16	16
		Ref – ve	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

*μl of cell-free extract used to sensitise 2 ml of fixed and tanned HRBC.

the type of carrier cells, stabilization of carrier red cells as well as the stability of antigen used for sensitizing the cells. Rogers *et al.* (1968) have reported the unstable nature of *P. knowlesi* antigen and its antigenic reactivity deteriorated after three months of storage at −70°C while the cell-free extract of the antigen was found stable only upto three days.

Proteases are the protein degrading enzymes and antigenic deterioration might be due to the autolytic digestion of antigenic protein. Different protease inhibitors have been selectively used in this study to block the proteolytic enzyme activity which could probably prevent the antigenic decay. In order to prove the hypothesis, pepstatin an inhibitor of cathepsin D (Umezawa *et al.*, 1970), phenyl methyl sulphonyl fluoride and soybean trypsin inhibitor for alkaline proteases, were added to the cell-free antigen (*P. knowlesi* extract) and stored at 4°C.

The optimum antigen concentration determination was performed in IHA test to see whether inhibitors had any stabilizing effect on the antigen as shown by optimum concentration of antigen required for sensitization of red cells, as well as antibody assay with reference positive sera. It was observed that when the cell-free antigen was stabilized by inhibitors, optimum antigen concentration remained the same even after storage at 4°C for 30 days (100 μl or 296 μg). Results suggested that PMSF was most active of the three inhibitors used, while soybean trypsin inhibitor was least effective.

Inhibitor studies show that both acid and alkaline proteases seem to be responsible for autolysis as indicated by the stabilizing effects of pepstatin—an inhibitor of acid proteases and PMSF—an inhibitor of alkaline proteases. This study shows that proteolytic enzymes are the major cause of autolysis of antigen which may be stopped by the addition of combination of protease inhibitors of both acid and alkaline range.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. M.M. Dhar, Director, CDRI for continued support and encouragement. This investigation was carried out during the tenure of ICMR sponsored project "Bulk production of malaria antigen for seroepidemiology of human malaria" under Dr. G.P. Dutta and one of the authors (SKS) was supported by a Research Fellowship under the same. The research support received from the Director-General, Indian Council of Medical Research is gratefully acknowledged.

REFERENCES

1. Agarwal, S.S., P. Sharma, A. Nath, I.K. Srivastava, J. Rai and G.P. Dutta (1981) Indirect haemagglutination and indirect fluorescent antibody test for human malaria using *P. knowlesi* antigen. *Indian J. Malariol.*, **18**: 67–72.
2. Agarwal, S.S., A. Nath, P. Sharma, I.K. Srivastava, S.R. Dwivedi, R.L. Yadava and G.P. Dutta (1982). Seroepidemiology of human malaria. Indirect haemagglutination test using *P. knowlesi* antigen. *Indian J. Malariol.*, **19**: 21–25.
3. Banyal, H.S., V.C. Pandey and G.P. Dutta (1981) Acid hydrolases of merozoites and other erythrocytic stages of *P. knowlesi*. *Indian J. Med. Res.*, **73** (Suppl.): 55–62.
4. D'Antonio, L.E., A.L. Von Doenhoff Jr. and E.H. Fife Jr. (1966) A new method for isolation and fractionation of complement fixing antigens from *P. knowlesi*. *Proc. Soc. Exp. Biol. Med.*, **123**: 30–34.
5. Dutta, G.P. and H.S. Banyal (1981). *In vitro* susceptibility of erythrocytes of *Presbytis entellus* to *P. knowlesi* and the blocking of merozoite invasion process by certain protease inhibitors. *Indian J. Exp. Biol.*, **19**: 9–11.
6. Dutta, G.P. and A. Nath (1986). Preparation of malaria antigens: I. Protocol for bulk preparation of lyophilized *P. knowlesi* antigen. In *Technology for seroepidemiology of human malaria.* (CDRI Publication): 4–14.
7. Dutta, G.P., Q.Z. Hussain, R.C. Mahajan and S.S. Agarwal (1986). Protocols for IHA test: ICMR Multicentric studies on seroepidemiology of human malaria: Protocol for indirect haemagglutination test. In *Technology for seroepidemiology of human malaria.* (CDRI Publication): 27–33.
8. Gyang, F.N., B. Poole and W. Trager (1982). Peptidases from *P. falciparum* cultured *in vitro*. *Mol. Biochem. Parasitol.*, **5**: 263–273.
9. Hempelmann, E. and R.J.M. Wilson (1980). Endopeptidases for *P. knowlesi*. *Parasitol.*, **80**: 323–330.

10. Kagan, I.G. (1972). Malaria: Seroepidemiology and serologic diagnosis. *Exp. Parasitol.*, **31:** 126–135.
11. Kaushal, D.C., R. Carter, R.J. Howard and F.M. McAuliffe (1983). Characterization of antigens of mosquito midgut stages of *P. gallinaceum*. I. Zygote surface antigens. *Mol. Biochem. Parasitol.*, **8:** 53–69
12. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951). Protein measurement with Folin-Phenol reagent. *J. Biol. Chem.*, **193:** 265–275.
13. Mahajan, R.C., N.K. Ganguly, M. Thadani, N. Gill and R.E. Chandnani (1981). Comparison of ELISA with IHA and IFA tests in malaria. *Indian J. Med. Res.*, **73:** 874–879.
14. Meuwissen, J.H.E. Th., A.D.E.M. Leeuwenberg and G.E. Molenkamp (1972). Studies on the various aspects of indirect haemagglutination test for malaria. *Bull. WHO*, **46:** 771.
15. Rogers, W.A. Jr., J.A. Fried and I.G. Kagan (1968). A modified indirect micro-haemagglutination test for malaria. *American J. Trop. Med. Hyg.*, **17:** 804–809.
16. Sherman, I.W. and L. Tanigoshi (1983). Purification of *P. lophurae* cathepsin D and its effects on erythrocyte membrane protein. *Mol. Biochem. Parasitol.*, **8:** 207–226.
17. Umezawa, H., T. Aoyagi, M. Hamada and T. Takeuchi (1970). Chymostatin, a new chymotrypsin inhibitor produced by actinomycetes. *J. Antibiotics*, **23:** 425–427.
18. Vanderjagt, D.L., L.A. Hunsaker and N.M. Campos (1986). Characterization of a haemoglobin-degrading low molecular weight protease from *P. falciparum*. *Mol. Biochem. Parasitol.*, **18:** 389–400.

Indian Journal of Malariology
Vol. 25, June 1988, pp. 33–39.

Fractionation of *Plasmodium knowlesi* Antigen for Seroepidemiology of Human Malaria by IHA test

S.K. SAXENA[1], V.C. PANDEY[1] and G.P. DUTTA[1]

Practically very little work has been done on the fractionation of erythrocyte stage antigens of malaria parasite for the serology of human malaria. In order to improve sensitivity, crude *P. knowlesi* antigen was fractionated by the conventional method of purification, by ammonium sulphate saturation followed by ion exchange chromatography. Seven fractions obtained by this procedure were compared for their antigenic reactivity in IHA test using reference positive serum. The purified antigen fraction (V) gave optimum sensitization of glutaraldehyde fixed sheep RBC with one twenty fourth the sensitizing dose of antigen as compared to crude antigen.

Human 'O' RBC sensitized with the fraction V antigen also gave very good performance in IHA test with field sera.

INTRODUCTION

Various antigens have been tried for seroepidemiological studies on human malaria by indirect haemagglutination test which include *P. vivax*, *P. falciparum*, *P. knowlesi*, *P. cynomolgi*, *P. fieldi*, *P. innui* and *P. coatneyi*. Out of these *P. vivax* and *P. falciparum* antigens are considered superior for serology of malaria because of their higher sensitivity (WHO, 1974). Though simian antigens have been used for seroepidemiology by several investigators, their sensitivity is considered to be lower than the homologous antigens. Since *P. knowlesi* schizont antigen can be produced in bulk in lyophilized form under existing conditions, Indian investigators have preferred to use this simian malaria antigen for multicentric studies (Dutta and Nath, 1986). There is still a need to improve the sensitivity and specificity of *P. knowlesi* antigen for IHA test, which can probably be achieved by the fractionation of crude antigen.

In order to improve the sensitivity and specificity of the IHA system various attempts have been made by earlier investigators to partially purify the antigenic material from the parasitized erythrocyte lysate. Mahoney *et al.* (1966) and Rogers *et al.* (1968) fractionated crude *P. knowlesi* and *P. falciparum* antigen and reported an increased sensitivity and specificity of the fractionated antigen for the IHA test. Sadun *et al.* (1969) and McAlister (1972) carried out fractionation of the lysate of *P. falciparum* parasitised erythrocytes using DEAE-Sephadex column and found that the active antigenic fraction could work at higher dilution and gave better specificity than the

Accepted for publication: 7 May 1988.
[1]Division of Microbiology and Biochemistry
Central Drug Research Institute
Lucknow-226001, India.

crude antigen in both IHA and soluble antigen fluorescent antibody test (SAFA). Wellde *et al.* (1969) made a detailed fractionation study on *P. berghei* and *P. falciparum* antigens from the lysates of infected erythrocytes by gel filtration and DEAE-Sephadex ion exchange chromatography and reported improved sensitivity of one of the fractions.

In the present study the partially purified *P. knowlesi* antigen was fractionated by conventional methods of purification and results of its efficacy in the IHA test are described in this study.

MATERIAL AND METHODS

Preparation of antigen

The schizont antigen of *P. knowlesi* was prepared according to the protocol reported by Dutta and Nath (1986).

Fractionation of antigen

The cell-free parasite preparation was homogenized in prechilled triple distilled water using Potter-Elvejham homogenizer at 4 C and centrifuged in the cold to remove cellular debris. The supernatant thus obtained was subjected to 0–30% ammonium sulphate $[(NH_4)_2SO_4]$ fractionation. The cell-free antigen and ammonium sulphate solutions were mixed slowly in cold and kept overnight at 4 C. The $(NH_4)_2SO_4$ saturated preparation was centrifuged in cold and the supernatant was further subjected to 30–80% $(NH_4)_2SO_4$ saturation under the same conditions as above. The precipitate obtained was suspended in buffer (tris-HCl 0.1 M, pH 7.5) and dialysed against the buffer till free from $(NH_4)_2SO_4$.

Ion exchange chromatography

The dialysed preparation was applied on a DEAE-cellulose column (12 × 1.5 cm) pre-equilibrated with eluting buffer (tris-HCl 0.1 M, pH 7.5). The elution was carried out using different sodium chloride gradients in tris-HCl buffer. Ten tubes of each fraction of 5 ml volume were collected. All the operations were carried out in cold conditions. The protein concentration of the different fractions was monitored at 280 nm and colorimetrically at 620 nm using the method of Lowry *et al.* (1951).

IHA (indirect haemagglutination) test

The fractions with maximum protein contents were pooled, dialysed against eluting buffer and finally against triple distilled water and subsequently subjected to lyophilization. The different lyophilized antigenic fractions were tested for their suitability for IHA test using human 'O' + red blood cells as well as sheep red blood cells for sensitization, and reference malaria positive and negative sera were used for antigen titration according to the ICMR multicentric study protocol for IHA test (Dutta *et al.*, 1986).

Serum titres were expressed as the reciprocal of the highest serum dilution giving complete agglutinations and optimal antigen concentration was determined on the basis of 100% agglutination. The test was performed using microtitre plates.

RESULTS

Fractionation of *P. knowlesi* antigen revealed that mechanical homogenization is an effective procedure for maximum solubilization of the antigen. The nuclear fraction did not show

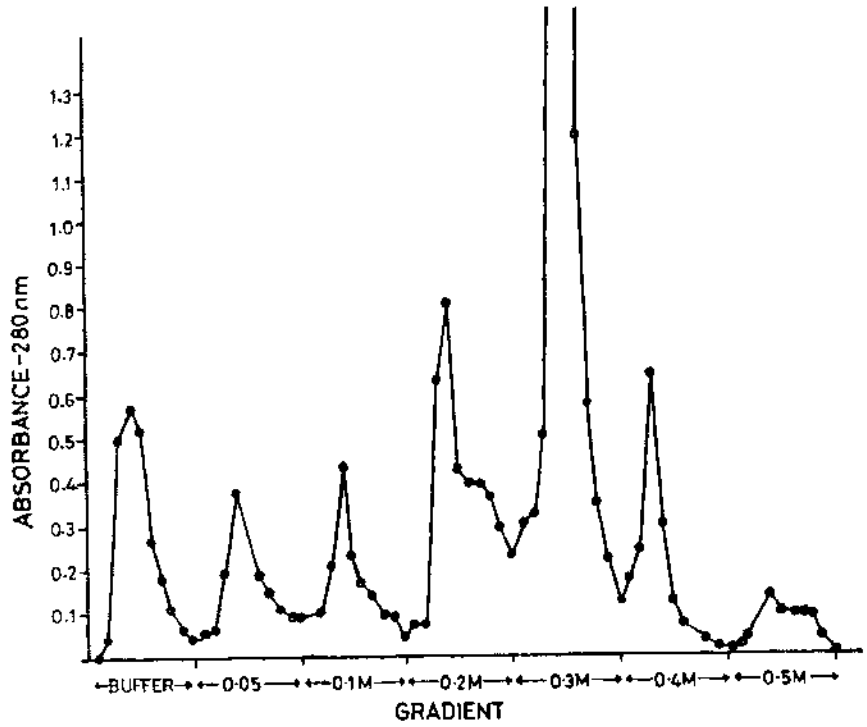


Fig. 1: Elution pattern of fractions obtained with DEAE-cellulose ion-exchange chromatography of *P. knowlesi* antigen.

antigenic reactivity in IHA test when the stabilized sheep RBC were sensitized with this fraction (Tables 1–2). The ammonium sulphate salt fractionation did not interfere with the antigenicity of the fractions and the maximum antigenicity was found in 30–80% ammonium sulphate precipitated protein using IHA test against reference positive serum (Table 2).

The DEAE-column chromatography (elution with NaCl stepwise gradient) revealed the presence of seven peaks. Fig. 1 shows the resolution of protein peaks with *P. knowlesi*. The protein eluted by 0.3M NaCl gradient in fraction V showed maximum protein content as well as maximum antigenicity when tested by IHA test. Although other fractions were also found to be antigenic by IHA test, their antigenic reactivity was of low order as compared to fraction V (Table 3).

Table 1. Protein recovery of malaria antigen from column

Total protein at the start of fractionation	146.159 mg
Protein recovered:	
In 30–80% Ammonium sulphate saturated fraction	27.60 mg
Protein loaded on DEAE-Cellulose column	20.670 mg
Protein recovered in different fractions	
Buffer	2.436 mg
0.05M NaCl	1.449 mg
0.1M NaCl	1.255 mg
0.2M NaCl	3.663 mg
0.3M NaCl	6.231 mg
0.4M NaCl	1.548 mg
0.5M NaCl	0.529 mg
Protein recovered:	17.111 mg
Recovery	82.78%

Table 2. Stepwise concentration of malaria antigen for IHA test

Preparation	IHA Titre*			
	Ref + ve sera		Ref – ve sera	
	(1)	(2)	(1)	(2)
Homogenate	256	512	2	4
Nuclear (Fraction)[1]	4	8	2	4
Nuclear (Supernatant)[2]	256	512	2	4
0–30% Salt (Pellet)	8	8	4	4
0–30% Salt (Supernatant)	256	512	4	4
30–80% Salt (Pellet)	256	512	4	4
30–80% Salt (Supernatant)	8	16	2	4

1. 1000 × g pellet; 2. 1000 × g supernatant; (All fractions were dialysed prior to IHA test).

*Glutaraldehyde fixed and tanned sheep RBC were sensitised with malaria antigen, its fractions were obtained after ammonium sulphate fractionation, and used for IHA test against two Ref +ve and two – ve sera.

Table 3. IHA test with fractions of *P. knowlesi* antigen, using sheep RBC

Antigen	Conc.	Sensitising dose of antigen/2 ml of fixed and tanned sheep (RBC)							
		Ref + ve	Ref – ve	Ref + ve	Ref – ve	Ref + ve	Ref – ve	Ref + ve	Ref – ve
Crude	2.96	128	2		296 μg				
	mg/ml	64	2						
Fractions eluted with		6.25 μg		12.50 μg		25.0 μg		50.0 μg	
		Reciprocal titres							
1. (Buffer)	1 mg/ml	32	2	32	2	32	2	32	2
		32	2	32	2	32	2	32	2
2. (0.05M) NaCl	"	32	2	32	2	32	2	32	2
		16	2	16	16	16	2	16	2
3. (0.1M) NaCl	"	32	2	32	2	32	2	32	2
		16	2	16	2	16	2	16	2
4. (0.2M) NaCl	"	8	2	8	2	8	2	8	2
		8	2	8	2	8	2	8	2
5. (0.3M) NaCl	"	128	2	1024	2	1024	2	1024	2
		64	2	1024	2	1024	2	1024	2
6. (0.4M) NaCl	"	8	2	8	2	8	2	8	2
		8	2	8	2	8	2	8	2
7. (0.5M) NaCl	"	8	2	8	2	8	2	8	2
		8	2	8	2	8	2	8	2

Table 4. Reciprocal IHA titres with human 'O' cells using purified fraction V antigen of the *P. Knowlesi* and lyophilized whole antigen

Antigen	No. of cases	Reciprocal IHA titres								
		GMRT	512	≥ 256	≥ 128	≥ 64	≥ 32	≥ 16	≥ 8	8
					Results with human 'O' cells					
(1) Fraction V of *P. knowlesi*	287	11 66	—	—	11 (3 83)	12 (4.18)	29 (10.10)	104 (36 23)	287 (100%)	
	206	13.71	4 (1.94)	6 (2.91)	10 (4.85)	14 (6.79)	38 (18 44)	88 (42.72)	206 (100%)	
	274	12 02	—	1 (0.36)	4 (1.46)	6 (2.19)	38 (13.87)	112 (40.88)	274 (100%)	
	215	14 66	3 (1.39)	5 (2 33)	10 (4 65)	19 (8 84)	45 (20.93)	106 (49.30)	215 (100%)	
	982	13 01	7 (0.71)	12 (1 22)	35 (3.56)	51 (5 19)	150 (15.27)	410 (41.75)	982 (100%)	
(2) Lyophilized *P. knowlesi* *	3029	6 65	1 (0.031)	2 (0.062)	11 (0.36)	68 (2.24)	266 (8.78)	754 (24 89)	1124 (37.11)	1905 (62 89)

*Sera from Calcutta, post-monsoon collection by Dr. T N Ghosh, and screened at CDRI using glutaraldehyde fixed and tanned human 'O' cells.

In addition to being active antigenically, this fraction V also showed a very low optimum sensitizing dose for reactivity in the IHA test (12.50 μg per 2 ml) with fixed and tanned sheep erythrocytes (Table 3).

DISCUSSION

Intact infected erythrocyte lysate was used as a source of antigen for IHA test as well as for SAFA and complement fixation tests by earlier workers. Davis (1948) isolated complement fixing antigens of *P. knowlesi* infected erythrocytes while Mahoney *et al.* (1966), Wellde *et al.* (1969), Rogers *et al.* (1968) and McAlister (1972) utilized the lysates of infected erythrocytes of *P. knowlesi* or its fractions for IHA test. Sadun *et al.* (1969; 1970) employed the fractionated malaria antigen for IHA and SAFA tests. Antigen produced by the earlier workers was highly contaminated with the host haemoglobin. None of these antigens had been evaluated earlier for field studies on seroepidemiology of malaria. For the present study, *P. knowlesi* schizont antigen was first partially purified by Ficoll-Hypaque gradient and then subjected to saponin lysis to remove host cell haemoglobin followed by repeated washing.The purified schizont antigen thus obtained was subjected to ammonium sulphate (30–80%) precipitation and DEAE-column fractionation.

Further studies with DEAE-cellulose fractionation have shown that fraction V shows high antigenicity for IHA test. This fraction which was eluted by the 0.3M sodium chloride gradient, showed maximum IHA titres with the malaria positive sera. Moreover, the optimum sensitizing concentration of this antigen was found to be only 12.50 μg in comparison with the 296 μg of protein used in routine IHA test performed with the homogenized extract of the saponin lysed *P. knowlesi* crude antigen. This result indicates 24-fold purification of the crude antigen.

In order to assess the applicability of fraction V in field studies, a comparative evaluation was made using crude and fraction V antigens in IHA test. The samples screened were post-monsoon filter paper elutes from Calcutta (Table 4). The present study has established the high sensitivity of this antigen (fraction V) as indicated by higher GMRT in the random collection of the sera which were screened with human 'O' cells for IHA test. Results showed that titres in general were one or two steps lower with crude antigen. The study emphasizes that fractionated antigen of *P. knowlesi* seems to be superior for seroepidemiology.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. M.M. Dhar, Director, CDRI, for continued support and the Director-General, ICMR for financial support to our project "Bulk production of *P. knowlesi* antigen for seroepidemiology of malaria" under which the work was carried out. One of the authors (SKS) was supported by ICMR Research Fellowship.

REFERENCES

1. Davis, B.D. (1948). Complement fixation with soluble antigens of *Plasmodium knowlesi* and *Plasmodium lophurae*. *J. Immunol.*, **58**: 269–281.
2. Dutta, G.P. and A. Nath (1986). Preparation of malaria antigens I. Protocol for bulk preparation of lyophilized *P. knowlesi* antigen. In *Technology for seroepidemiology of human malaria.* (CDRI Publication): 4–14.
3. Dutta, G.P., Q.Z. Hussain, R.C. Mahajan and S.S. Agarwal (1986). Protocols for IHA test I. ICMR Multicentric studies on seroepidemiology of human malaria: Protocol for indirect haemagglutination test. In *Technology for seroepidemiology of human malaria.* (CDRI Publication): 27–33.
4. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall (1951). Protein measurement with Folin-phenol reagent. *J. Biol. Chem.*, **193**: 265–275.
5. Mahoney, D.F., D.C. Redington and M.J. Schoenbechler (1966). The preparation and serologic activity of plasmodial fractions. *Milit. Med.*, **131**(9) Suppl.: 1141–1151.

6. McAlister, R.O. (1972). Fractionation of the serologically reactive antigens of *P. falciparum*. *Proc. Helminth. Soc.*, **39:** 5:4.
7. Rogers, W.A. (Jr.), J.A. Fried and I.G. Kagan (1968). A modified indirect micro-haemagglutination test for malaria. *American J. Trop. Med. Hyg.*, **17:** 804–809.
8. Sadun, E.H R.W. Gore and B.T. Wellde (1970). Malaria serology using antigens from the lysate of parasitized erythrocytes. *J. Parasitol.*, **56:** 297.
9. Sadun, E.G., R.W. Gore, B.T. Wellde and D.F. Clyde (1969). Malarial antibodies in human malaria. A comparison of the soluble antigen fluorescent antibody (SAFA) and the indirect haemagglutination tests using an antigen *P. falciparum* parasitized erythrocytes as lysate. *Milit. Med.*, **134** (Suppl.): 1294.
10. Wellde, B.T., D.J. Stechschulte, M.J. Schoenbechler and W.A. Colgate (1969). An indirect haemagglutination test for malaria using an antigen from the lysate of parasitized erythrocytes. *Milit. Med.*, **134** (Suppl.): 1284–1293.
11. WHO Memorandum (1974). Serological testing in malaria. *Bull. WHO*, **50:** 577.

Indian Journal of Malariology
Vol. 25, June 1988, pp. 41–49.

Malaria Outbreak in Kundam block, District Jabalpur (M.P.)

NEERU SINGH[1], V.P. SHARMA[2], M.M. SHUKLA[1] and GYAN CHAND[1]

An outbreak of malaria was investigated in 1987 in Kundam block of Jabalpur district (M.P.). In these villages 4 rounds of HCH spraying followed by intensive surveillance and one round of fever radical treatment (FRT) and mass radical treatment (MRT) was not effective in suppressing *P. falciparum* epidemic. *A. culicifacies*, *A. fluviatilis* and *A. annularis* densities remained high and so did the SPR (70%) and pf% (60–100). The need to apply a suitable replacement insecticide in conjunction with environmental modifications as a long term control strategy is suggested.

INTRODUCTION

A field station of the science and technology project in mission mode on the integrated vector control of malaria is operating in Mandla district since 1986. The project is aimed at studying the feasibility of the integrated vector control of malaria in tribal villages situated in hilly and inaccessible terrain. During routine field work high fever rate and occasional deaths were reported from villages located outside the experimental areas. Preliminary enquiries revealed that malaria was rampant in this area and insecticidal spraying and drug distribution had been intensified by the state health authorities. Subsequently our Centre was requested by the Health Department, Government of Madhya Pradesh to investigate the epidemic. Systematic entomological and epidemiological investigations were taken up in two groups of villages i.e., sprayed with HCH and unsprayed. Results of this study are reported below.

MATERIAL AND METHODS

The study area included villages of Kundam block, District Jabalpur, M.P. The block has 193 villages with a population of about 84,000, 70% of which belong to Gond tribe. The study area has mostly rocky and undulating terrain with thick forests. Most of the villages are located near streams and remain cut off from other villages during the rainy season. Houses are made of bamboo walls mud plastered on both sides, with little or no ventilation and usually with an attached cattleshed. The villages have innumerable mosquito breeding sites such as streams, rocky pools, borrow pits, rice fields, wells, seepage and rain water collections etc. Besides this, during 1987 there was heavy rainfall in late August—September which created vast areas for mosquito breeding.

Accepted for publication: 26 May 1988.
[1]Malaria Research Centre (Field Station)
RMRC, Jabalpur Medical College
Jabalpur-482003, India.
[2]Malaria Research Centre (ICMR)
22-Sham Nath Marg
Delhi-110054, India.

Table 1. Malaria incidence in Kundam PHC

Year	Blood slide examined	Positive	Species			ABER	API
			Pv	Pf	Mix		
1984	6739	129	66	63	—	7.1	1.0
1985	6420	164	71	92	1	6.8	0.1
1986	7559	156	63	93	—	7.4	0.1
1987	21510	4680	1091	3535	54	21.71	47.23

Source: Health Department (NMEP), M.P.

Table 2. Sectionwise malaria indices of study areas in Kundam PHC

Sections	Population	ABER			API		
		1987	1986	1985	1987	1986	1985
Jhiriya	5665	29.5	2.1	1.6	128	0	0
Chaurai	5025	55.0	7.9	10.7	232	2.4	4.1
Jamgaon	5403	29.0	4.5	1.2	144	0.3	0.9
Piparia	5300	14.4	9.6	5.9	8.4	0	1.9

Source: Health Department (NMEP), M.P.

Table 3. History of spray in Kundam PHC

Year	Population Sprayed	Houses	Rooms	Cattle-sheds	Round	Period	
						From	To
1987	36777	8027	19088	4792	1st	1.5.87	15.6.87
		8008	20425	4792	2nd	16.6.87	31.7.87
		7848	23973	4872	3rd	1.8.87	15.9.87
	83619	19451	60232	9193	4th	15.10.87	10.12.87
1986	98237	18514	64418	11562	1st	16.6.86	7.8.86
		18862			2nd	8.8.86	20.9.86
1985	83369	16471	53137	9096	1st	16.6.85	31.7.85
		16456	47430	9096	2nd	1.8.85	15.9.85
1984	94000	15850	51227	12139	1st	16.6.84	31.7.84
		30167	49735	8759	2nd	1.8.84	30.9.84

Source: Health Department (NMEP), M.P.

A total of 21 villages (14 sprayed and 7 unsprayed villages separated by a distance of about 20–50 kms) were surveyed to study malaria prevalence. Survey was carried out in the third week of October 1987. Blood smears were collected from all fever cases and 600 mg chloroquine adult dose was given as presumptive treatment. Blood smears were stained with JSB and examined. All malaria positive cases identified by the project staff were administered radical treatment by NMEP (*P. vivax* 600 mg chloroquine and 15 mg primaquine daily for 5 days and for *P. falciparum* 600 mg chloroquine and 45 mg primaquine as single dose). Children were given

proportionately low dosages. Pregnant women were not given primaquine.

Mosquito collections were made using a suction tube between 0600 to 0800 hours from human and mixed dwellings and cattlesheds. Immatures were collected from their breeding habitats and identified at adult stage. Spleen enlargement was determined by Hackett's method (Christophers *et al.*, 1958) in children between 2–9 yrs age. The same 21 villages were resurveyed in the months of January, February, April and May 1988 to assess the impact of antimalaria measures implemented by the state Govt.

Systematic meteorological records were not available, but average annual rainfall of the area is about 1400 mm. The rainy season begins from June and lasts till the end of September. Total rainy days vary from 60 to 70. Minimum and maximum temperatures during winter and summer are in the range of 5–25°C and 25–42°C respectively. Before undertaking investigations, previous year's data for Kundam PHC was obtained from the District Malaria Officer (DMO) which is given in Tables 1–4. It was revealed that the block was sprayed with two rounds of HCH in 1984, 1985 and 1986. The ABER was about 7, and the API was 1.0 in 1984 and 0.1 in 1985 and 1986 and 47.23 in 1987 (Table 1). Sectionwise data of the villages surveyed by the project staff is given in Table 2. During 1987 three rounds of HCH had been sprayed; the rounds beginning on May 1, June 16 and August 1. (Table 3). The following additional measures were implemented by the state authorities to check malaria.

(i) One additional round of HCH was sprayed in 73 sections (706 villages) beginning from October 15 in all the villages. Spray coverage was about 90% and the spray quality was good as periodically checked by the project staff. Similar measures were carried out in district Mandla in the adjoining blocks where DDT was sprayed (Table 4).

(ii) Surveillance was improved by frequent inspections by the supervisory staff. Twelve additional surveillance workers and 6 supervisors were posted in the block. A team of medical specialists was also deputed. Five mobile teams

Table 4. HCH focal spray (special round) in district Jabalpur 1987*

No. of PHCs	No. of sections	Pop. sprayed approx.	No. of villages	% coverage		Duration
				HD	Rooms	
11	73	392981	706	97	91	15.10.87–31.12.87

*Total Pop.—1139810; Total PHCs—15; Total sections—283; Total villages—2880; Pop. above 2 API for spray in 1988—1139668.

DDT Focal spray (special round) in district Mandla 1987**

No. of PHCs	No. of sections	Pop. sprayed approx.	No. of villages	% coverage		Duration
				HD	Rooms	
3	39	57161	135	93	100	30.10.87–25.3.88

**Total Pop.—1035134; Total PHCs—16; Total sections—349; Total villages—2110; Pop. above 2 API for spray in 1988—5659462.

Source: Health Department (NMEP), M.P.

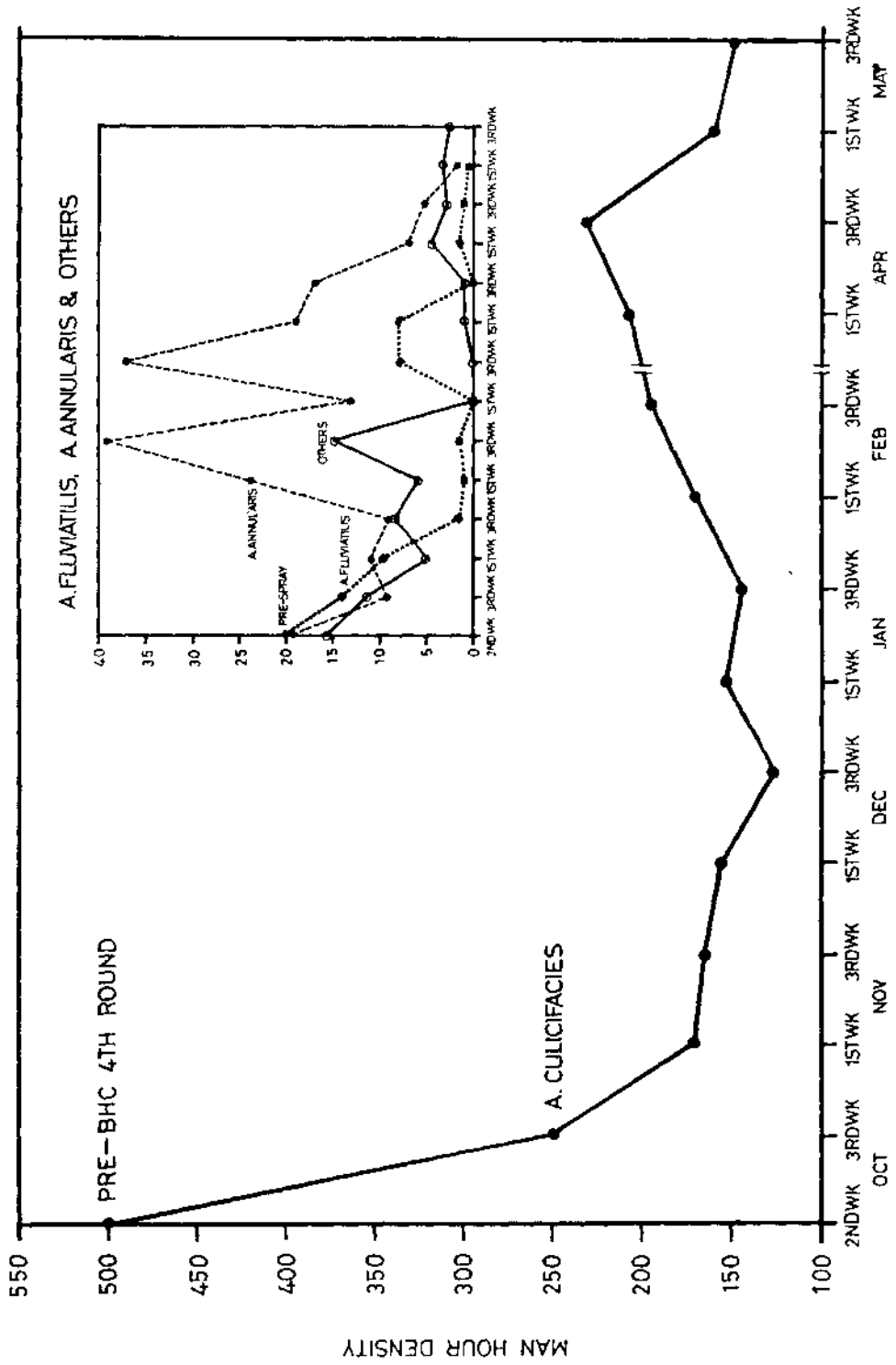


Fig. 1. Vector densities in Kundam block.

each consisting of a Medical Officer and four paramedicals were also deployed in the affected area.

(iii) Fever and mass radical treatment (FRT and MRT) were given in October 1987. Fifty drug distribution centres (DDCs) were opened and chloroquine was distributed through the village guards (*Kotwar*).

(iv) Six field laboratories were established for prompt diagnosis and radical treatment.

(v) Health education material on malaria control was circulated and publicity was given through the local newspapers on the treatment of malaria and simple methods for the control of mosquito breeding.

(vi) The block was declared a restricted zone under the communicable diseases act 1987, section 2(3) to prevent further dissemination of the disease due to population movement.

(vii) The area was visited by state Health Minister in order to assess the magnitude of the problem.

RESULTS

Fig. 1 and Tables 5–7 give the results of surveys carried out by the project staff. It was revealed that the anti-vector and anti-parasitic measures implemented to control the epidemic did not result in any significant improvement of malaria situation. The densities of *A. culicifacies* and *A. fluviatilis* (primary vectors) and *A. annularis* (secondary vector) were extremely high before and after the 4th round of HCH spraying (Fig. 1). Although no incrimination studies were carried out but *A. culicifacies* and *A. fluviatilis* were incriminated as primary vectors of malaria by Kulkarni (1983) in Bastar, M.P. and Vaid *et al.* (1974) in northwestern parts of Madhya Pradesh.

The downward trend in anopheline densities was not the result of spraying but due to the onset of winters. This was substantiated by the fact that *A. annularis* densities showed an increasing trend after spraying which was the result of favourable weather conditions for *A. annularis* at that time of the year.

Results of parasitological surveys are given in Table 5. Two sets of villages were surveyed. The first group of 14 villages received 4 rounds of HCH spraying during 1987 whereas the second group of 7 villages was not sprayed. Certain sections of Kundam block were not sprayed for several years because of the reported <2 API and these seven villages belonged to those sections. After the outbreak, these 7 villages were sprayed with one round of HCH in October-November. As could be seen from both the groups of villages, the incidence of malaria was extremely high (SPR $>70\%$) and falciparum malaria was the main cause of high morbidity (Table 5). There was no difference in the two groups of villages with regard to malaria, and HCH spraying did not produce any tangible impact on transmission as evidenced by infant and child fever/spleen surveys (Tables 6 and 7).

Surveys were repeated in January, February, April and May 1988 with similar results with regard to malaria situation and vector densities. Surveys in April and May 1988 showed only very marginal reduction due to unfavourable weather conditions (Table 5). Spleen examination of children in the months of October 1987 and April 1988 in both groups of villages showed 48% children with enlarged spleen (Table 7). Most children with enlarged spleen had no fever at the time of survey. Blood smears from these children were invariably positive for the malarial parasite and the data has been included in Table 6. The population of this area was, therefore, experiencing intense transmission.

During the study period several cases of falciparum malaria which had been administered full adult dose of 600 mg chloroquine and 45 mg primaquine returned again with fever. On examination these cases were found harbouring *P.*

Table 5. Epidemiological situation of Kundam PHC (rapid fever survey)

Date of spray	Spray history	(HCH)	No. of villages	Population	BSE	+ve	Pv	Pf	Mix	SPR	SFR	Pf%
14–20 Oct 87	Sprayed	(3 rounds)	14	8419	944	696	155	528	13	73.7(51–96)	55.9(43–87)	75.9(61–91)
	Unsprayed		7	3748	362	255	101	151	3	70.4(61–81)	41.7(20–56)	59.2(33–78)
1–7 Jan 88	Sprayed	(4 rounds)*	14	8419	274	202	10	187	5	73.72(25–88)	68.25(33–84)	92.50(50–100)
	Sprayed	(1 round)**	7	3748	226	142	9	129	4	62.83(37–80)	57.08(37–80)	90.85(75–100)
10–20 Feb 88	Sprayed	(4 rounds)	14	8419	281	132	26	101	5	46.98(10–100)	35.94(11–66)	76.52(33–100)
	Sprayed	(1 round)	7	3748	96	35	2	32	1	36.46(18–50)	33.33(9–50)	91.43(50–100)
15–21 Apr 88	Sprayed	(4 rounds)	14	8419	458	197	115	80	2	43.01(13–68)	17.47(2–38)	40.61(8–62)
	Sprayed	(1 round)	7	3748	202	110	74	34	2	54.46(43–71)	16.83(6–29)	30.91(14–45)
9–15 May 88***	Sprayed	(5 rounds)	14	8419	553	242	149	84	9	43.76(14–51)	15.19(6–24)	34.71(12–50)
	Sprayed	(2 rounds)	7	3748	156	73	53	20	--	46.79(38–100)	12.82(15–100)	27.39(11–100)

*One special round of HCH was sprayed in October-November 1987.
**Only one round of HCH was sprayed in November 1987.
***Spray started in all the villages from 1st May 1988.

Table 6. Results of parasitological surveys

Month/ Year	Spray history (HCH)	No. of villages	BSE	+ve	Pv	Pf	SPR
Infants (<1yr)							
Oct 87	3 rounds	14	9	5	—	5	55.55
	No spray	7	10	4	3	1	40.00
Jan 88	4 rounds	14	4	1	—	1	25.00
	1 round	7	3	1	—	1	33.33
Apr 88	4 rounds	14	12	5	3	2	41.66
	1 round	7	10	5	5	—	50.00
May 88	4 rounds	14	27	7	6	1	25.92
	1 round	7	5	2	1	1	40.00
Month/year	Spray history	BSE	+ve	Pv	Pf	Mix	SPR
Children (>1–9 yrs)							
Oct 87	3 rounds	236	193	51	136	6	81.77
	No spray	183	129	51	73	5	70.49
Jan 88	4 rounds	65	34	2	31	1	52.30
	1 round	40	26	7	15	4	65.00
Feb 88	4 rounds	130	62	10	50	2	47.69
	1 round	28	17	1	16	—	60.71
Apr 88	4 rounds	221	105	61	43	1	47.51
	1 round	86	56	41	14	1	65.11
May 88	4 rounds	194	88	54	30	4	45.36
	1 round	43	28	24	4	—	65.11

Table 7. Result of spleen survey in Kundam PHC

Spray history	Date of surveys	No. of villages	Children examined	Children with enlarged spleen	Enlarged spleen rate (range)
Sprayed	Oct 14–21	7	674	314	46.59 (20–72)
Sprayed	Apr 10–20	5	277	131	47.3 (40–65)
Unsprayed	Oct 14–21	5	558	265	47.49 (21–67)
Sprayed	Apr 10–20	5	391	195	49.8 (31–73)

Note: Parasitological data included in Table 6.

falciparum infection. At first instance it was not clear whether this was the result of reinfection, recrudescence or incipient resistance but follow-up studies of such cases by both *in vivo* and *in vitro* methods confirmed that these cases were resistant to chloroquine. Micro *in vitro* test showed that out of 22 cases, 14 were resistant to chloroquine and *in vivo* tests revealed that 60% cases were resistant to chloroquine (unpublished data).

DISCUSSION

Malaria control in rural areas is carried out by spraying residual insecticides such as DDT, HCH or malathion. In areas with DDT resistant

vectors HCH is sprayed. At present about 210 million population is under DDT spray and about 100 million under HCH spray. In areas with DDT and HCH resistance malathion is sprayed. The cost of spraying 1 million population increases from Rs. 34 lakhs and Rs. 37 lakhs for DDT and HCH respectively to Rs. 199 lakhs for malathion (Sharma, 1987a). But in areas where the vector is highly resistant to HCH such as in Kundam block, it would be advisable to control the epidemic with a suitable replacement insecticide. At present malathion is sprayed in about 21 million population as replacement insecticide mainly in Gujarat and Maharashtra. Resistance to malathion had developed rapidly in Gujarat (Rajagopal, 1977) and now the residual spraying of malathion is only partially effective in the control of vector populations (Sharma, 1984). In this connection it is noteworthy to mention that malathion resistance in *A. culicifacies* developed in areas of Orissa and Andhra Pradesh where this insecticide was never used in public health. This development of resistance in *A. culicifacies* was the result of the use of organophosphate compounds in agriculture (Nagpal, 1986; Sharma, 1987b). Malathion is an expensive insecticide and resistance to it is likely to be selected rapidly in other areas as well.

Field studies by Ansari *et al.* (1986a) have shown that the spraying of deltamethrin in *A. culicifacies* populations resistant to DDT and HCH results in almost complete suppression of vector populations and other mosquito species. These insecticides are also relatively safe for the environment as very little quantity of the active ingredient is used i.e., about 1/10, 1/50 and 1/100 of DDT, HCH and malathion respectively. The added advantage of pyrethroids is their high social acceptability as against malathion which has high refusal rate partly due to bad odour. The economics of spraying synthetic pyrethroids could not be worked out but discussions with the representatives of the manufacturers revealed that it would be cheaper than malathion in terms of population coverage.

The Centre has investigated similar epidemics in other parts of the country, e.g., Meerut (Ansari *et al.*, 1986b), Bareilly (Ansari *et al.*, 1984) and Shahjahanpur (Chandrahas and Sharma, 1983; Sharma *et al.*, 1985). At Shahjahanpur the bio-environmental control of malaria strategy was implemented in 1986. Results so far achieved have shown major reduction in vector densities and the SPR was reduced from 80–90% to 20–30%. It would, therefore, be advisable to control malaria epidemic in Kundam PHC with a suitable residual insecticide followed by bio-environmental methods as a long-term strategy (Sharma, 1987b).

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the encouragement and guidance given by Dr. B.N. Saxena, Senior Deputy Director-General, ICMR, New Delhi. The authors are also grateful to Late Dr. S.D. Verma, Project Director, RMRC, Jabalpur for the help and facilities provided for this study. Authors are thankful to Shri M.P. Singh, Chandan Karforma, R.K. Minocha and Miss Kiran for their technical help.

REFERENCES

1. Ansari, M.A., V.P. Sharma, C.P. Batra, R.K. Razdan and P.K. Mittal (1986a). Village scale trial of the impact of deltamethrin (K-othrine) spraying in areas with DDT and HCH resistant *Anopheles culicifacies*. *Indian J. Malariol.*, **23**: 127–131.
2. Ansari, M.A., V.P. Sharma, R.K. Razdan and C.P. Batra (1986b). Malaria situation in Meerut district villages (U.P). *Indian J. Malariol.*, **23**: 147–150.
3. Ansari, M.A., C.P. Batra and V.P. Sharma (1984). Outbreak of malaria in villages of Bareilly, district U.P. *Indian J. Malariol.*, **21**: 121–123.
4. Chandrahas, R.K. and V.P. Sharma (1983). Malaria epidemic in Shahjahanpur. *Indian J. Malariol.*, **20**: 163–166.
5. Christophers, S.R., J.A. Sinton and G. Covell (1958). *How to do a malaria Survey*. Hlth. Bull. No. 14, Malaria Bureau No. 6. (Government of India Press).
6. Kulkarni, S.M. (1983). Detection of spotozoites in *Anopheles subpictus* in Bastar district, M.P. *Indian J. Malariol.*, **20**: 159–160.
7. Nagpal, B.N. (1986). A contribution to the knowledge

of family Culicidae (Diptera) from Orissa. *Ph. D. Thesis*, (Berhampur University, Berhampur, Orissa): 1–166.

8. Rajagopal, R. (1977). Malathion resistance in *Anopheles culicifacies* in Gujarat. *Indian J. Med. Res.*, **66**: 27–28.
9. Sharma, G.K. (1984). Review of malaria and its control in Delhi. In *Proceedings of the Indo-UK Workshop on Malaria, New Delhi, November 14–19, 1983.* (Malaria Research Centre, Delhi): 13–40.
10. Sharma, V.P., R.K. Chandrahas, B.N. Nagpal and P.K. Srivastava (1985). Follow-up studies of malaria epidemic in villages of Shahjahanpur district (U.P.). *Indian J. Malariol.*, **22**: 119–121.
11. Sharma, V.P. (1987a). Community based malaria control in India. *Parasitology Today*, **3**(7): 222–226.
12. Sharma, V.P. (1987b). The green revolution in India and ecological succession of malaria vectors. In *Effect of Agricultural development on vector-borne diseases.* (FAO publication)AGL/Misc/12/87: 116–125.
13. Vaid, B.K., S. Nagendra and P.K. Paithane (1974). Spring transmission of malaria due to *Anopheles culicifacies* in North-Western Madhya Pradesh. *J. Com. Dis.*, **6**: 270.

Indian Journal of Malariology
Vol. 25, June 1988, pp. 51–55.

Some Components of Kinin System in Human Malaria

S. ROY[1], U. BHATTACHARYA[1], P.K. KAR[1] and S.C. LAHIRI[1]

The kinin system is comprised of diverse constituents such as prekallikrein-kallikrein/kininogenase-kinin-kininase. Blood kinin reflects a dynamic equilibrium between formation and destruction of kinins. In *Plasmodium vivax* infected man, plasma prekallikrein level is lowered. Compared to healthy subjects, the plasma total kininogen and high mol. wt. kininogen level decreased significantly in *P. vivax* infected patients. Plasma kininase activity rose in infected patients.

INTRODUCTION

Vasodilation, circulatory stasis and increased capillary permeability are important components of the pathophysiology of malaria. Certain endogenous substances such as histamine and kinin, have these actions and may possibly be relevant. Injection of kallikrein was found to produce vascular changes similar to that in the brain of *Plasmodium knowlesi* infected monkey by Onabanjo and Maegraith (1970). However, these workers found kallikrein activity in the blood of a plasmodium infected rhesus monkey but not in the blood of a healthy (control) monkey. It was also shown that plasma kininogen level in the infected monkeys is markedly reduced (Tella and Maegraith, 1966). Enhanced blood kinin levels in rodent malaria was reported by Ohtomo and Katori (1972) and later by Onabanjo (1974). There are no reports concerning prekallikrein levels in malaria and no reports concerning changes in the individual components of the kinin system in *P. vivax* infected humans.

Accepted for publication: 2 June 1988.
[1]Department of Pharmacology
School of Tropical Medicine
Calcutta-700073, India.

MATERIAL AND METHODS

Blood collection

Human venous blood was obtained from malaria infected patients with demonstrable *Plasmodium vivax* in blood smears (blood films were stained with Leishman stain); *P. vivax* is the common infection in Calcutta area. For control, samples of venous blood were obtained from healthy volunteers of matching age and sex.

The collected blood was transferred to plastic/siliconized tubes containing 1 vol. of 3.8% sodium citrate for 9 vol. of blood. After centrifugation the plasma was used for the experiments described below.

Estimation of kallikrein inhibitor

Kallikrein inhibitor content was estimated after Colman and co-workers (1969) in human pla-

Table 1. Comparison of plasma prekallikrein, kallikrein-inhibitor, total kininogen, HMW-kininogen and kininase level (Mean ± SE) between *P. vivax* infected and normal subjects

Components of kinin system	Control	Infected	P-value	Remarks
Prekallikrein (μ Kat/litre)	8450.16 ± 281.5	6204.6 ± 224.45	$P < 0.001$	S
Kallikrein inhibitor (Unit)	0.94 ± 0.05	1.01 ± 0.1	$P > 0.05$	NS
Total Kininogen (Bradykinin released ng/mg plasma protein)	115.3 ± 20.6	68 ± 5.9	$P < 0.05$	S
HMW-Kininogen (Bradykinin released ng/mg plasma protein)	7.3 ± 1.7	2.7 ± 0.3	$P < 0.05$	S
Kininase (μg bradykinin split/ml plasma/min.)	1.15 ± 0.4	3.57 ± 0.84	$P < 0.05$	S

S = Significant: NS = Non-significant.
In each case number of subjects investigated was six.

sma. A plastic test tube, containing 41.9 mg of TAME (Tosyl Arginine Methyl Ester) in 2 ml of 0.1 M sodium phosphate buffer (pH 7.6) and 0.15 M sodium chloride, was taken. Plasma of volume 0.3 ml was mixed with 0.3 ml of kaolin suspension (10 mg/ml) in buffer, and the kaolin plasma mixture was incubated at 25°C. Again at the end of 5 mins. 0.2 ml aliquot was withdrawn and poured in to the test tube. The test tube was placed in a water bath (37°C). After 1 min. (blank) and 31 mins. (test samples) aliquots of 1 ml were removed from each tube and added to 0.5 ml of 15% TCA. After centrifugation (1000 g × 5 mins.), 0.5 ml of the supernatant of each tube was taken and added to a separate tube which already contained 0.1 ml 2% $KMnO_4$. This was mixed thoroughly for 1 min. and 0.1 ml 10% sodium sulphide was added and again mixed. After decolorisation, 4 ml 0.2% chromotropic acid in 65% sulfuric acid was added. The sample was boiled for 15 mins. in a water bath and the test samples were read at 580 nm (EC spectrophotometer) against its own blank. The standardization of kallikrein inhibitor was assessed in six normal subjects (Table 1) which is about 95% of the normal level.

Determination of prekallikrein in plasma with the help of chromogenic substrate

Plasma prekallikrein was estimated after Friberger and co-workers (1978). The substrate is H-D-pro-phe Arg. pNA. Hageman factor of FXII is activated to FXIIa (activated Hageman factor). Prekallikrein in plasma is then activated by FXIIa and acts on HMW-kininogen. The plasma kallikrein so formed catalyzes the splitting of p-nitroaniline (pNA) from the substrate S-2302. The released pNA is then measured photometrically at 405 nm.

Estimation of total kininogen

Plasma total kininogen is determined after Diniz and Carvalho (1963) (modified by Uchida and Katori. 1978). HCl pretreated plasma was kept at 37°C for 15 mins., neutralised, added to 0.2 M — Tris/HCl buffer and incubated with 200 μg trypsin for 30 mins., the reaction was terminated by the addition of boiling absolute ethanol (70°C). After centrifugation the mixture was vacuum dried, and thereafter dissolved in saline and assayed on rat uterus.

Estimation of high molecular weight (HMW) kininogen

Plasma high molecular weight kininogen level was determined after Gallimore *et al.* (1978). A volume of 0.1 ml plasma heated at 60°C for two hours was incubated with 0.02 ml of 1:10 phenanthroline (3×10^{-2} M) for 5 mins., 0.1 ml of kallikrein (mg/ml) and 3.8 ml of de Jalon solution for 30 mins. at 37°C. Aliquot of this solution was taken and assayed for bradykinin on isolated rat uterus.

Estimation of kininase

Plasma kininase activity was estimated according to the method of Wright (1977). Eighty μl plasma in 0.92 ml Tris HCl (0.02 M, pH 7.4) buffer was incubated at 37°C with 500 ng synthetic bradykinin in 0.5 ml Tris HCl buffer. At 0 and 10 mins. aliquots were withdrawn and assayed on rat uterus. Kininase activity was expressed in terms of μg bradykinin destroyed per ml plasma per min.

Statistical test

Students' t-test was used to evaluate the significance of differences between group means.

RESULTS

Prekallikrein level in *Plasmodium vivax* infected patients was significantly lower as compared to healthy normal subjects. No significant change of kallikrein inhibitor was demonstrated in *P. vivax* infected subjects. Table I shows that total kininogen level and high molecular weight (HMW) —kininogen level were lower in the malaria infected patients than in the healthy volunteers.

Kininase activity of plasma was estimated in control and *P. vivax* infected patients. Plasma kininase level was significantly increased in malaria infected patients (Table 1).

DISCUSSION

Vasodilation and stasis in blood vessels, particularly cerebral, are likely to contribute to the pathophysiology of malaria. It is likely that endogenous pharmacologically active agents may be involved. Kinins and kallikreins with their hypotensive, vasodilator and capillary permeability increasing activity fit such a role (Onabanjo and Maegraith, 1970).

There is some information regarding the role of these two substances in experimental malaria in monkey but nothing is known about changes in *P. vivax* infected patients, nor about changes brought about by plasmodial infections in the components of kinin system.

Normally human blood contains a very small amount of kinin. This is not surprising when one considers that kinins increase capillary permeability and are potent vasodilators; bradykinin is, on molar basis, the most potent vasodilator of human vessels. Large amounts of kinin precursor, kininogen occur in blood and can be rapidly activated.

An increased blood kinin level may be the reason for vasodilation in malaria. Nevertheless, blood kinin levels are the outcome of interplay between several determinants, such as prekallikrein, factor XIIa, kallikrein, kininogenase, and kininase.

Prekallikrein is the inactive precursor of kallikrein. There does not appear to be any previous report about changes in prekallikrein level in malaria. The present experiment showed that in man plasmodial infection is followed by a lowering of prekallikrein level. The cause of such a decrease can be known only when more information is available. One may speculate whether the reduced level of prekallikrein is due to the hepatic accumulation of parasites, which may reduce the hepatic synthesis of prekallikrein. An alternate possibility is a reduction in prekallikrein level due to its heightened conversion to kallikrein. Kallikrein level measurement would have answered the question. However in spite of the high sensitivity of the chromogenic substrate method followed in the present experiments, it fails to detect plasma-kallikrein, perhaps because of its ultrashort half-life (Vinazzer, 1979). Recent findings in this laboratory have shown that plasma factor XIIa level is increased in plasmodium infected rats (Roy and Lahiri, 1984, unpublished findings). The heightened XIIa activity would suggest an increased conversion of prekallikrein, resulting in a raised kallikrein level. One may speculate whether even the reduced amount of prekallikrein formed results in

a greater amount of kallikrein due to enhanced FXIIa activity.

The prekallikrein-kallikrein mechanism is not the only pathway to kinin release. At least two other endogenous proteolytic enzymes, trypsin and plasmin, cleave kinins from kininogen (Erdös, 1966). Beraldo (1950) first observed that plasmin activation accompanied anaphylactic shock *in vivo*. One cannot, therefore, ignore the possibility of plasmodial infection affecting the plasmin system and thus influencing kinin system. The present experiments did not measure plasmin levels, but recent findings from this laboratory indicate that in plasmodial infection of rat, plasmin level is increased and antiplasmin level is decreased (Roy and Lahiri, 1984, unpublished findings). This may play a significant role in increased kinin level in malaria.

Ohtomo and Katori (1972) reported that in infected mouse blood kinin level rose only in high parasitaemia accompanied by lowered level of plasma kininogen. In *P. vivax* infected patients, present experiments yield consonant results; kininogen level has also decreased significantly in malaria infected patients.

Degradation of kininogen by plasma kallikrein is probably a major cause of its disappearance from the circulation, the result of which is bradykinin formation. However, liver damage might also have been a factor for the low level of HMW-kininogen which is also seen in experimental endotoxemia (Gallimore *et al.*, 1978).

In the present experiment, the kininase activity in plasma increased markedly in the *P. vivax* infected patients. Part of the increased activity is due to microscopical haemolysis caused by the malarial infection. In the course of malarial haemolysis, some erythrocyte kininase may escape from erythrocytes into plasma. Erythrocytes have strong kininase activity (Onabanjo *et al.*, 1970). Increased plasma kininase activity in infected monkeys and mice has been reported earlier. Increased plasma kininase activity indicates rapid destruction of kinins, therefore, balancing their increased production, may serve as a regulatory mechanism and thus control the inconvenient effects of peptides on the capillary endothelium. Kininase also converts angiotensin I to angiotensin II. Increased kininase activity may assist in maintaining blood pressure also.

ACKNOWLEDGEMENT

We are grateful to the Indian Council of Medical Research for financial assistance and providing research fellowships to S.R. and U.B.

REFERENCES

1. Beraldo, W.T. (1950). Formation of bradykinin in anaphylactic and peptone shock. *American J. Physiol.*, **163:** 283–289.
2. Colman, R.W., W.T. Mason and S. Sherry (1969). The kallikreinogen-kallikrein enzyme system of human plasma. *Ann. Intnatl. Med.*, **71:** 763–773.
3. Diniz, C.R. and I.F. Carvalho (1963). A micromethod for determination of bradykininogen under several conditions. *Ann. N.Y. Acad. Sci.*, **104:** 77–89.
4. Erdös, E.G. (1966). Hypotensive peptides : bradykinin, kallidin and eledoisin. *Adv. Pharmacol.*, **4:** 1–90.
5. Friberger *et al.* (1978). *KABI DIAGONISTICA*: Determination of prekallikrein in plasma with S-2302. Laboratory Instruction, March '78.
6. Gallimore, M.J., A.O. Aasen, K.H.N. Lyngaas, M. Larsbraaten and E. Amundsen (1978). Falls in plasma levels of prekallikrein, high molecular weight kininogen and kallikrein inhibitors during lethal endotoxin shock in dogs. *Thromb. Res.*, **12:** 307–318.
7. Ohtomo, H. and M. Katori (1972). Reliable evidence of involvement of the kinin system in mouse malaria. *Japan J. Pharmacol.*, **22:** 493–509.
8. Onabanjo, A.O. and B.G. Maegraith (1970). Kallikrein as a pathogenic agent in *Plasmodium knowlesi* infection in *Macaca mulatta*. *British J. Exp. Pathol.*, **51:** 523–533.
9. Onabanjo, A.O. (1974). Pathophysiological role of plasma kinins in malaria. *W. African J. Pharmacol. Drug Res.*, **1:** 2.
10. Onabanjo, A.O., A.R. Bhabani and B.G. Maegraith (1970). The significance of kinin destroying enzyme activity in *Plasmodium knowlesi* malarial infection. *British J. Exp. Pathol.*, **51:** 534–540.
11. Tella, A. and B.G. Maegraith (1966). Studies on bradykinin and bradykininogen in malaria. *Ann.*

Trop. Med. Parasitol., **60:** 304–317.

12. Uchida, Y. and M. Katori (1978). An improved method for determination of the total kininogen in rabbit and human plasma. *Biochem. Pharmacol.*, **27:** 1463–1469.
13. Vinazzer, H. (1979). Assay of total factor XII and of activated factor XII in plasma with a chromogenic substrate. *Thromb. Res.*, **14:** 155–166.
14. Wright, I.G. (1977). Kinin, kininogen and kininase levels during acute *Babesia bovis (= B. argentina)* infection of cattle. *British J. Pharmacol.*, **61:** 567–572.

SHORT NOTE

Indian Journal of Malariology
Vol. 25, June 1988, pp. 59-62.

Natural Parasitic Infections in Anopheline Larvae of Mandya district, Karnataka state

C. ACHUTHAN[1]*

The Malaria Investigation Centre, Mandya (Karnataka state) undertook studies on the breeding, resting and feeding behaviour of anophelines during 1964–1967. The studies were carried out in 16 study villages located in the neighbourhood of Mandya town (Shamasastry *et al.*, 1961). During the course of the studies, efforts were made to obtain knowledge on the natural parasitic infections in anopheline larvae, host specificity and per cent parasitism in different months. The observations recorded are summarised in this note.

Larval collections were made in all the 16 study villages on weekly basis. Anopheline larvae were collected from different habitats such as ricefields, field channels, pools and swamps using a dipper of 250 ml capacity. The larvae collected were transferred to containers habitat-wise on the spot itself and transported to the laboratory. They were identified under a microscope and screened for the presence or absence of parasitic infections.

Accepted for publication: 27 January 1988.
[1]Malaria Investigation Centre
Mandya, Karnataka, India.

During the course of the study spread over a period of 31 months between June 1964 and December 1967, a total of 23987 larvae belonging to 13 species of anohelines were collected. The anophelines recorded in the collections were: *A. annularis*, *A. aconitus*, *A. barbirostris*, *A. culicifacies*, *A. fluviatilis*, *A. hyrcanus*, *A. jamesii*, *A. jeyporiensis*, *A. pallidus*, *A. subpictus*, *A. splendidus*, *A. vagus* and *A. varuna*. Of these, *A. subpictus* predominated the collection with 16220 specimens (67.6%), followed by *A. vagus* (4251 specimens or 17.7%). *A. culicifacies* constituted 7.04% (1690 specimens) of the collection.

The entomopathogenic aquatic fungi *Coelomomyces indicus* and the mermithid nematode *Romanomermis iyengari* were the two major parasites found infecting anopheline larvae. Out of the 13 species of anophelines encountered, only three species i.e., *A. subpictus*, *A. vagus* and *A. culicifacies* were found infected with *Coelomomyces indicus*. The per cent parasitism by *Coelomomyces indicus* in these three species according to breeding sites monthwise have been summarised in Tables 1 and 2 respectively.

It was evident that the incidence of fungal infection was highest in *A. subpictus* larvae. The monthly incidence of parasitism ranged from 0

Table 1. Incidence of *Coelomomyces* infection in wild caught larvae by species

Year/ Month	Per cent infection in			
	A. subpictus	*A. vagus*	*A. culicifacies*	Total
1964				
Jun	0.2	0	0	0.2
Jul	0	0	0	0
Aug	25.9	0	0	24.3
Sep	0	0	0	0
Oct	5.1	0	0	5.0
Nov	0	0	0	0
Dec	7.5	17.7	0	9.0
1965				
Jan	2.4	9.5	0	3.9
Feb	2.1	1.1	0	0.9
Mar	38.4	3.8	0	29.9
Apr	10.6	15.0	0	10.8
May	16.4	20.8	25.0	17.1
Jun	24.7	1.1	0	20.8
Jul	5.5	4.8	0	4.6
Aug	26.6	1.2	4.6	18.3
Sep	22.1	15.4	5.0	19.8
Oct	9.3	23.3	2.6	9.0
Nov	0.3	0	0	0.1
Dec	11.1	2.8	0	7.4
1966				
Jan	3.1	0	0	2.1
Feb	1.5	0	0	1.2
Mar	20.0	4.6	2.1	13.6
Apr	19.7	17.2	6.4	16.6
May	8.3	0	0	7.2
Jun	30.0	4.8	1.5	26.1
Jul	0	0	0	0
Aug	10.4	32.3	24.1	20.2
Sep	0	0	0	0
Oct	6.1	4.5	0	5.9
Nov	1.4	0	0	1.1
Dec	11.1	2.4	0	6.8

to 38.4% in *A. subpictus*, 0 to 23.3% in *A. vagus* and 0 to 25.0% in *A. culicifacies* in different months. Natural parasitism was encountered in all the months during 1965 in pools, for four months in channels and for three months in swamps. However, the ricefield habitat exhibited highest per cent parasitism. There was a double peak in the incidence of infection, the first during March after a few pre-monsoon showers and the second one during May—September coinciding with the local irrigation practices.

The infection of larvae of *A. culicifacies* with *Coelomomyces indicus* is reported for the first time in India. There are reports of *Coe. indicus* infection in larvae of *A. splendidus* and *A. stephensi* in Bangalore City (Iyengar, 1963, Personal communication). Chandrahas and Rajagopalan (1979) reported *Coe. indicus* infection in *A. subpictus* larvae in Pondicherry ricefields. Gugnani *et al.* (1965) recorded *Coe. indicus* from *A. subpictus* in Ghaziabad area of Uttar Pradesh.

Table 2. Analysis of larval infestations according to the nature of breeding sites (1965)

Type of breeding place	Particulars of larval collection	Month												Total
		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	
Pools*	No. collected	607	683	1713	252	378	1010	562	978	555	511	1190	261	8705
	No. infected	31	8	513	27	29	233	28	11	22	37	2	20	961
	% infected	5.1	1.1	29.9	10.7	7.6	23.1	4.9	1.1	3.9	7.2	0.1	7.6	11.0
Paddy fields	No. collected	86	83	—	446	1013	108	—	1117	679	37	282	162	4013
	No. infected	0	0	—	53	235	0	—	375	223	13	0	12	911
	% infected	0	0	—	11.8	23.2	0	—	33.5	32.8	35.1	0	7.4	22.7
Channels	No. collected	40	80	—	50	—	—	—	—	—	6	—	—	176
	No. infected	0	0	—	1	—	—	—	—	—	0	—	—	1
	% infected	0	0	—	2.0	—	—	—	—	—	0	—	—	0.5
Swamps	No. collected	46	—	—	—	178	—	36	—	—	—	—	—	260
	No. infected	0	—	—	—	6	—	0	—	—	—	—	—	6
	% infected	0	—	—	—	3.3	—	0	—	—	—	—	—	2.3
Total	No. collected	779	846	1713	748	1569	1118	598	2095	1234	554	1472	423	13149
	No. infected	31	8	513	81	270	233	28	386	245	50	2	32	1879
	% infected	3.9	0.9	29.9	10.8	17.9	20.3	4.6	18.4	19.8	9.0	0.1	7.6	14.28

*Pools: Water collections with marginal vegetation inclusive of tank bed pools and turf pools.

Romanomermis iyengari infection was not as widespread as that of *Coe. indicus* and produced non-significant levels of infection. Only five *A. vagus* larvae showed infection.

ACKNOWLEDGEMENTS

Grateful thanks are due to the late Dr. M.O.T. Iyengar for confirming identification of *Coe. indicus* and *Romanomermis iyengari* and to all the field staff of the Malaria Investigation Centre, Mandya for their faithful work. Special thanks are due to the entomologists, Sh. L. Raghuveera Rao, Sh. R.K. Chandrahas and Sh. L.R. Loganathan. Facilities extended by the Director of Public Health in Bangalore, Karnataka, for field work are gratefully acknowledged.

REFERENCES

1. Chandrahas, R.K. and P.K. Rajagopalan (1979). Mosquito breeding and the natural parasitism of larvae by a fungus *Coelomomyces* and a mermithid nematode *Romanomermis*, in paddy fields in Pondicherry. *Indian J. Med. Res.*, **69:** 63–70.
2. Gugnani, H.C., B.L. Wattal and N.L. Kalra (1965). A note on *Coelomomyces* infection in mosquito larvae. *J. Ind. Soc. Mal. Com. Dis.*, **2:** 333.
3. Shamasastry, H., A.R. Sunder Rao, T.S. Rama Rao, N.L. Sitaraman and C. Achuthan (1961). A note on the interruption of spraying of residual insecticides in some villages of Visvesvaraya Canal area, Mandya district, Mysore state. *Indian J. Malariol.*, **15:** 233–238.

OBITUARY

C.P. Pant

(1932–1987)

The very sad and untimely death of Dr. Chandra P. Pant, on 15 December 1987, in Geneva, is indeed a great loss to the profession of medical entomologists.

Dr. Pant was born on 12 January 1932 in the beautiful hill resort of Nainital, Uttar Pradesh state, India. After high school education in Almora, he entered the Agra University from which he graduated and where he also took his postgraduate M.Sc. degree in entomology standing first in the order of merit in the University. He received his Ph.D. degree in entomology with top honours from the Iowa State University and an M.P.H. from Harvard University, both in the USA. Later he also attended a number of WHO refresher training courses on various aspects of vector biology and control in several parts of the world.

After brief assignments with the Department of Agriculture and the Ministry of Defence in India, Dr. Pant joined the World Health Organization in 1958 as an entomologist with the Malaria Project in Nepal and subsequently in Burma. After a short period as a member of intercountry programmes in Venezuela and Colombia, he joined the Interregional Malaria Team in Kankiya, Nigeria, and subsequently was assigned as Project Leader of the Interregional Research Team for Testing of Insecticides in Lagos and Kaduna, also in Nigeria. His next assignments took him to South-East Asia as Project Leader of the Interregional Team on *Aedes* Research in Bangkok and on Vector Control Research in Jakarta. After over 17 years of field work, Dr. Pant was assigned, in 1975, as a scientist to the headquarters Division of Vector Biology and Control in Geneva and, since 1981, was Chief of the unit of Ecology and Control of Vectors.

Dr. Pant was universally acclaimed for his technical competence and dedication to work by all his associates both within and outside WHO. An excellent scientist, as authenticated by over one hundred publications, he was also a first-class administrator, a rare combination. He was a man of high intellectual and moral integrity, constantly striving for quality, precision and perfection. He had many characteristics which greatly endeared him to his colleagues and earned him their respect: natural dignity, uprightness, pro-

found humanity, discretion, humility, politeness, geniality and ever-readiness to share his knowledge and busy time.

His dedication to his profession and job did not prevent him from taking a lively interest in all aspects of life. Very friendly and sociable by nature, his hospitality knew no bounds. He was a sports enthusiast, represented his Indian university in cricket and badminton, and was an excellent tennis player and golfer in later life. He was a man of robust health until cruel fate struck him down with an illness which, however, brought to light yet another trait—that of immense courage. He did not let his ailment affect his performance of duties or social obligations or his sang-froid, and the infectious cheerfulness never left him, even when bedridden. One colleague recalls with admiration how he was even providing moral encouragement to somebody he had never met and who was similarly affected.

A thorough gentleman, a great friend, a respected colleague, a well-wisher to all without distinction of nationality, colour or creed, a true international civil servant, his death will be a personal loss to all those who met him even casually and who will remember him for a long time. He is survived by his wife, Mrs. Shanti Pant.

Division of Vector Biology and Control
World Health Organization
1211 Geneva 27, Switzerland.

INDIAN JOURNAL OF MALARIOLOGY

Instructions to Authors

Editorial Policy

The Indian Journal of Malariology is devoted to the publication of original research which contributes significantly to the knowledge of any field of malariology. Papers of routine and repetitive nature dealing with gross observations may not be included. Articles will be published at the editor's discretion in the order accepted. Date of acceptance will be that date on which copy is accepted in final form for publication. Manuscripts should be submitted in triplicate to:

The Editor
Indian Journal of Malariology
Malaria Research Centre (ICMR)
22-Sham Nath Marg
Delhi—110054, India.

Classes of Items Published

In addition to regular papers the journal publishes short notes and invites review articles. Book reviews may be published at the end of the journal.

Format

The matter should be arranged in the following order: Title, Name(s) of the author(s) with address of the Institute/University (as footnotes and numbered serially in superscript), Abstract, Introduction, Material and Methods, Results, Discussion, Acknowledgement and References. Authors should provide a short title to be used as running title, of not more than 5 words in case the title of the paper is long.

Preparation of Copy

Manuscripts should be typewritten in English on one side of the paper leaving 1½ inch left-hand margins. The entire matter should be typed double space including references, tables and captions. Abstract, tables, references and legends for illustrations should be typed on separate sheets of paper. Pages are to be numbered consecutively.

Tables should be placed singly on sheets of paper, along with relevant headings and footonotes. Tables should be self-explanatory and referred to in the text. Designation of tables must be with arabic numerals (e.g., 1, 2), avoid roman numerals (e.g., I, II). Do not use any horizontal or vertical lines in the body of the table.

Footnotes to the text should be avoided as far as possible, parenthetical insertions are preferable.

Illustrations should be sent in duplicate. All illustrations including figures, photographs, graphs and maps should be numbered consecutively in order of appearance in the text. Captions and legends should be typed separately and must not appear on the face of illustrations. Authors should identify each illustration on the reverse side with author's name, fig. no. and abbreviated captions. Line drawings should be clear and letters and numerals should be planned for legibility following reduction. Labelling should be neat and accurate. Photographs should be sharp, glossy, black and white prints, preferably mounted and covered with a transparent overlay for protection. Photographs should have allowance for reduction to 1/3 size. Size of art work for quarter page should be 24 × 21 cms; half page 45 × 24 cms and full page 57 × 45 cms approximately.

Data for tables, graphs etc., should be carefully verified. All statistics, percentages and other

calculations should be checked thoroughly before submission of a communication. Once a paper is accepted for publication, data in it should be treated as final.

Nomenclature. Authors of scientific names should be omitted in abstract and title. Authors of scientific names of insects should be included at first instance in the body of the text.

Numbers less than one should have a zero set before the decimal point e.g., 0.1.

Measurements should follow the international system of units (SI Units). Kindly see WHO publication *The SI for the Health Professional*, WHO, Geneva, 1977. Use of the 24-hour time system (e.g., 0830 hours, not 8:30 A.M.) is preferable.

References should include only published references and papers in press. References in the text may be cited as Sharma *et al.* (1976) or (Mount *et al.*, 1972; Coombes *et al.* 1973; Mount *et al.*, 1974). For references to a paper accepted for publication, the word 'in Press' should appear after the title of the periodical. Citations of unpublished work should be incorporated in the text itself (e.g., R.G. Roy, unpublished data or S. Pattanayak, personal communication). If the reference is to an article published without any authorship in a periodical, in place of author's name the term "Anonymous" should be used. Titles of Periodicals cited in the references are to be abbreviated as in the *World List of Scientific Periodicals*.

References to the literature, arranged alphabetically and numbered consecutively, must be placed at the end of the paper. The following style is acceptable for this journal:

Research Paper

Sharma, V.P. (1976). Elimination of aziridine residues from chemosterilised mosquitoes. *Nature*, **261** (5556): 135.

Book/Monographs

Rao, T. Ramachandra (1981). *The Anophelines of India*. (W.Q. Judge Press, Bangalore).

Landau, I. and Y. Boulard (1978). In *Rodent Malaria*, edited by R. Killick-Kendrick and W. Peters. (Academic Press Inc., London): 53–84.

Paper presented at Symposium/Conference

Subbarao, S.K. (1981). *Cytoplasmic incompatibility in mosquitoes*. Paper presented at the International symposium on recent developments in the genetics of insect disease vectors, Bellagio, Italy, 20–24 April.

Authors are requested to verify spelling, punctuation, titles and dates of all references. The address of the publisher should be given for books. References are attributable to authors, not to editors in case of compilations or contributory texts e.g.:

Killick-Kendrick, R. and W. Peters (1978). Ed. *Rodent Malaria*. (Academic Press Inc., London): 406. **(Incorrect).**

Landau, I. and Y. Boulard (1978). In *Rodent Malaria*, edited by R. Killick-Kendrick and W. Peters. (Academic Press Inc., London): 53 –84. **(Correct)**.

Correct references are the responsibility of the author.

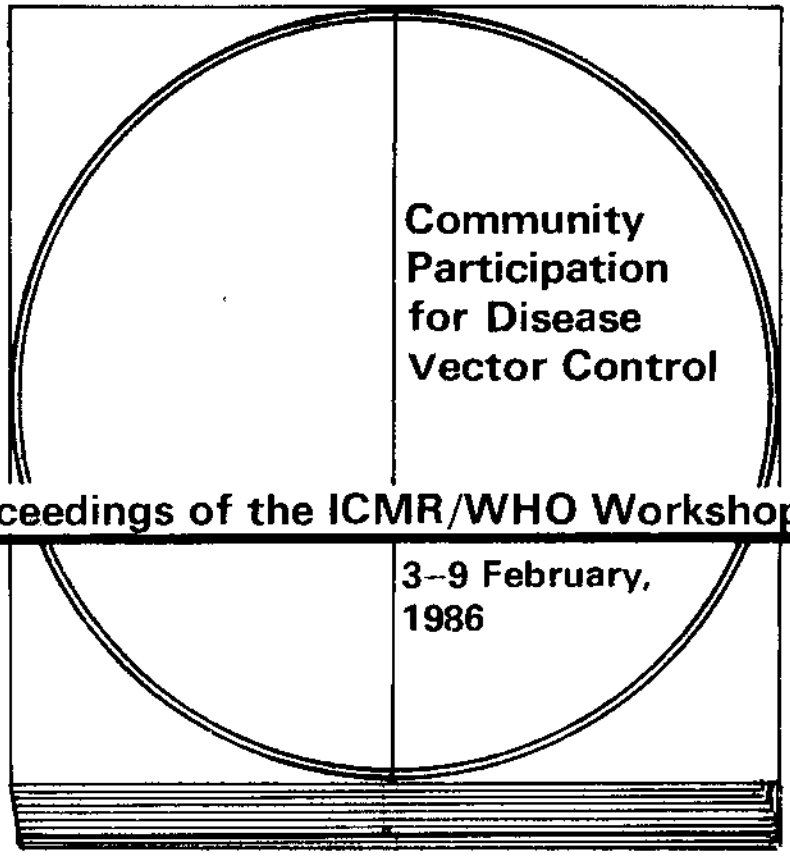
Short notes should be prepared in a manner similar to the research papers and will have title, name(s) of author(s) with address of Institute/University as footnotes, acknowledgement and references.

Proofs

Page proofs of the articles will be sent to the authors for correction. Corrected proofs must be returned promptly to the editor or else the article may not be printed in the current issue or may be printed as it stands. Only minimal changes i.e., those that do not substantially alter the page make-up are allowable at the proof stage in exceptional cases. Alterations which are disallowed by the editor shall be deleted or charged to the author.

Reprints

Reprints will be mailed to the senior author. Twenty-five reprints will be supplied free of charge to single authors and thirty for joint authors to be distributed on an equal basis. Additional reprints may be ordered on payment when the proofs are returned. A reprint order form will be supplied with the proofs.



Community Participation for Disease Vector Control

Proceedings of the ICMR/WHO Workshop on

3–9 February, 1986

A review of the process, role and outcome of people's involvement in vector control. Invaluable for workers and decision makers in Primary Health Care and health-related projects.

Statement about ownership and other particulars of Indian Journal of Malariology

FORM IV
(*See Rule 8*)

1.	Place of Publication	Malaria Research Centre (ICMR) 22-Sham Nath Marg Delhi—110054
2.	Periodicity of its publication	Half yearly
3.	Printer's Name Nationality Address	V.P. Sharma Indian Malaria Research Centre (ICMR) 22-Sham Nath Marg Delhi—110054
4.	Publisher's Name Nationality Address	Dr. V.P. Sharma Indian Malaria Research Centre (ICMR) 22-Sham Nath Marg Delhi—110054
5.	Editor's Name Nationality Address	Dr. V.P. Sharma Indian Malaria Research Centre (ICMR) 22-Sham Nath Marg Delhi—110054
6.	Names and addresses of individual who own the newspaper and partners or shareholders holding more than one per cent of the total capital	Malaria Research Centre (ICMR)

I, V.P. Sharma, hereby declare that the particulars given above are true to the best of my knowledge and belief.

Sd/-
(V.P. SHARMA)
Signature of publisher

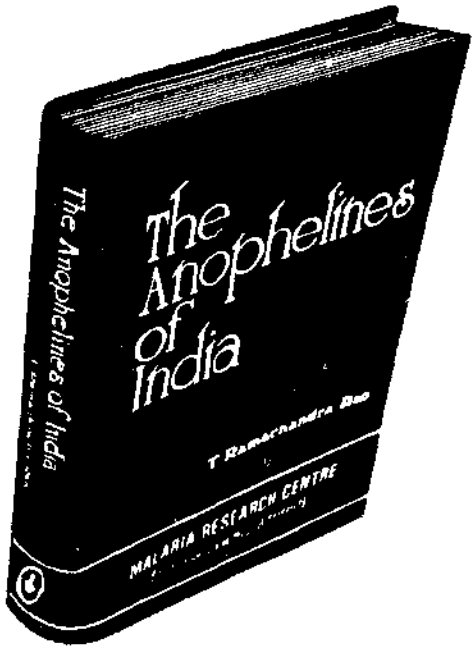
30 June 1988

THE ANOPHELINES OF INDIA

Revised Edition 1984

by

T. Ramachandra Rao



An authoritative and comprehensive publication which brings together all that is known about distribution, bionomics, ecology, relation to disease and basic principles of control of anopheline mosquitoes.

Size CROWN 4 TO pp 538 (Hardbound)

Rs. 150.00 (India) US $ 45.00 (Other countries)

25% Discount per copy

The monograph can be obtained by sending M.O./bank draft/postal order/cheque crossed and marked payable to Malaria Research Centre (ICMR), 22-Sham Nath Marg, Delhi-110054, INDIA.

MALARIA RESEARCH CENTRE

PRICED PUBLICATIONS

The Anophelines of India (Rev. ed.) 1984 by T. Ramachandra Rao

Price: India Rs. 150/- / Foreign US $ 45/-] 25% Discount

Indian Journal of Malariology

Volume 18 Nos. 1&2 (1981) *
Volume 19 Nos. 1&2 (1982) *
Volume 20 Nos. 1&2 (1983) *
Volume 21 Nos. 1&2 (1984) *
Volume 22 Nos. 1&2 (1985) *
Volume 23 Nos. 1&2 (1986) *
Volume 24 Nos. 1&2 (1987) **
Volume 25 Nos. 1&2† (1988)**

(† To be released in December)

** Annual Subscription — India Rs. 75.00 ● / Foreign US $ 20.00

● 25% discount for individuals.

* Back issues are available at old rates i.e. Rs. 30/- and $ 10/-

---

The Editor
Indian Journal of Malariology
Malaria Research Centre
22-Sham Nath Marg
Delhi-110054 (India)

Sir,

I enclose herewith a bank draft/postal order No/s.....for $/Rs.....(in the name of Malaria Research Centre, Delhi–110054) towards subscription/payment for Indian Journal of Malariology for the year.....(2 Nos.)/The Anophelines of India (Revised edition) 1984. The journals/publication should be mailed to me/my client at the following address.

.....
.....
.....
.....