INDIAN JOURNAL OF MALARIOLOGY

Volume 25 Number 2

December 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

CONTENTS

Volume 25
Number 2
December 1988

Short Notes

Note: The editor assumes no responsibility for the statements and opinions expressed by the contributors. This issue has been delayed for reasons beyond our control.

Indian Journal of Malariology
Vol. 25, December 1988, pp. 67 – 72.

Evaluation of four Azadirachtin Rich Fractions from Neem, *Azadirachta indica* A. Juss (Family: Meliaceae) as Mosquito Larvicides

D. RAGHUNATHA RAO[1], R. REUBEN[1], Y. GITANJALI[2] and G. SRIMANNARAYANA[2]

Four new azadirachtin rich fractions (ARFs), Nemidin, Vemidin, Vepeidin and Nemol, were tested for larvicidal activity against the early IV instar larvae of *Anopheles culicifacies* Giles and *Culex quinquefasciatus* Say. Among these Nemidin, which was found to have a high content of azadirachtin, showed high larvicidal activity for both *An. culicifacies* and *Cx. quinquefasciatus* larvae as shown by low LD50 values, 1.12 ppm and 3.9 ppm respectively. However, the other fractions showed moderate larvicidal activity only. Nemidin produced rectal prolapse in *Cx. quinquefasciatus* larvae but not in *An. culicifacies*.

INTRODUCTION

The neem tree, *Azadirachta indica* A. Juss, is traditionally known for its medicinal and insecticidal properties. In recent times there has been renewed interest in the chemistry of neem, because of its varied biological activities. Many studies have shown insecticidal, growth regulatory and antifeedant properties of neem products against various insect pests, and several studies have shown that neem products are effective against mosquito larvae also (Chavan, 1983; Zebitz, 1983; Singh, 1984; Matemu and Mosha, 1986). The biological effects of neem products are due to the presence of several active principles, of which the most important is azadirachtin, a tetranortriterpenoid, which is very potent. However, it is extremely expensive and difficult to isolate, and is also photolabile when pure. Hence, it is not suitable for operational use. Therefore, natural product chemists and entomologists the world over are isolating and testing partially purified azadirachtin rich fractions (ARFs) which are relatively stable and require only few steps of purification. Thus Margosan-O, an ethanol extract, was developed and is commercially available in the USA (Larson, 1986). Feuerhake (1983) studied some technical solvents for isolation of potent azadirachtin fractions. Saxena (1987) separated

Accepted for publication: 10 October 1988.

Centre for Research in Medical Entomology
Madurai - 625003, India.

[1] Natural Products Laboratory
Department of Chemistry
Osmania University
Hyderabad - 500007, India.

and tested water soluble compounds (Neem Seed Bitters) from neem seeds, and Sharma *et al.* (1983) developed fractions as pest control agents named Neemrich I, II and III. In the present study four new ARFs have been tested for their larvicidal activity against the IV instar larvae of *Culex quinquefasciatus* Say and *Anopheles culicifacies* Giles, to evaluate them as potential vector control agents.

MATERIAL AND METHODS

Isolation of ARFs

Fresh seeds of *Azadirachta indica* A. Juss were powdered and extracted with petroleum ether (60–80°C) in a soxhlet extractor. Concentration of petroleum ether yielded a light yellow oil. This was kept aside for a month for the fatty solid matter to settle down. The oil was decanted carefully. The clear light yellow oil thus obtained was designated as 'Nemol' fraction.

The residual seed powder was then extracted in a soxhlet extractor. The light brown semi-solid obtained on distillation of ethanolic extract was then taken up in benzene. The benzene solution was filtered to remove insoluble matter. The clear benzene solution was distilled in a rotary evaporator to yield a light brown semi-solid, which was designated as 'Vepcidin' fraction.

Fresh seed powder of *Azadirachta indica* was extracted with ethanol in a soxhlet extractor. Concentration of the ethanolic extract yielded a brown semi-solid residue designated as 'Vemidin' fraction.

Vemidin was partitioned between ethylacetate and water. The aqueous portion was rejected. The ethylacetate solution was washed with water and dried over anhydrous sodium sulphate. The solution was filtered and concentrated to yield a light brown semi-solid. The residue was then again partitioned between aqueous methanol (95%) and petroleum ether. The petroleum ether solution on concentration yielded an oil which was kept aside. The aqueous methanolic solution on concentration yielded a light brown semi-solid designated as 'Nemidin' fraction.

All these fractions were subjected at various concentrations to thin layer chromatography (TLC) on silica gel G to separate the groups of compounds.

Bioassay

Initially 5 ml of acetone was added to each of the fractions Nemidin, Nemol and Vepcidin for solubility, and then water was added to obtain 1,000 ppm stock solutions. For Vemidin 5 ml of DMSO (Dimethyl sulfoxide) was used as solvent. From these stock solutions serial dilutions were made in distilled water to 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9 and 0.97 ppm. Parallel controls were maintained throughout the experiment with 5 ml of suitable solvents. Control and treated beakers were kept 5 mins for evaporation of solvent from the solution. In each test, twenty early IV instar larvae of *An. culicifacies* and *Cx. quinquefasciatus* were held in separate beakers with different concentrations of the test compounds. Experiments were triplicated under identical conditions. Mortality was recorded at 24 hrs for assessing the larvicidal activity. Observations were continued till all the larvae moulted into pupae and then adults, to record various abnormal resulting stages due to the delayed effects of fractions. The results of the larvicidal activity of four ARFs were subjected to probit analysis for working out LD_{50} values.

RESULTS AND DISCUSSION

TLC examination of the neem fractions revealed that Nemidin contains azadirachtin in considerable amount, while Vepcidin was found to contain relatively less (Fig. 1a). At the same concentration in Vemidin the spot due to azadirachtin was not noticed, probably due to the low concentration of azadirachtin in Vemidin. The streak of Nemol reveals that it contains fatty oil portion in excess amount (Fig. 1b).

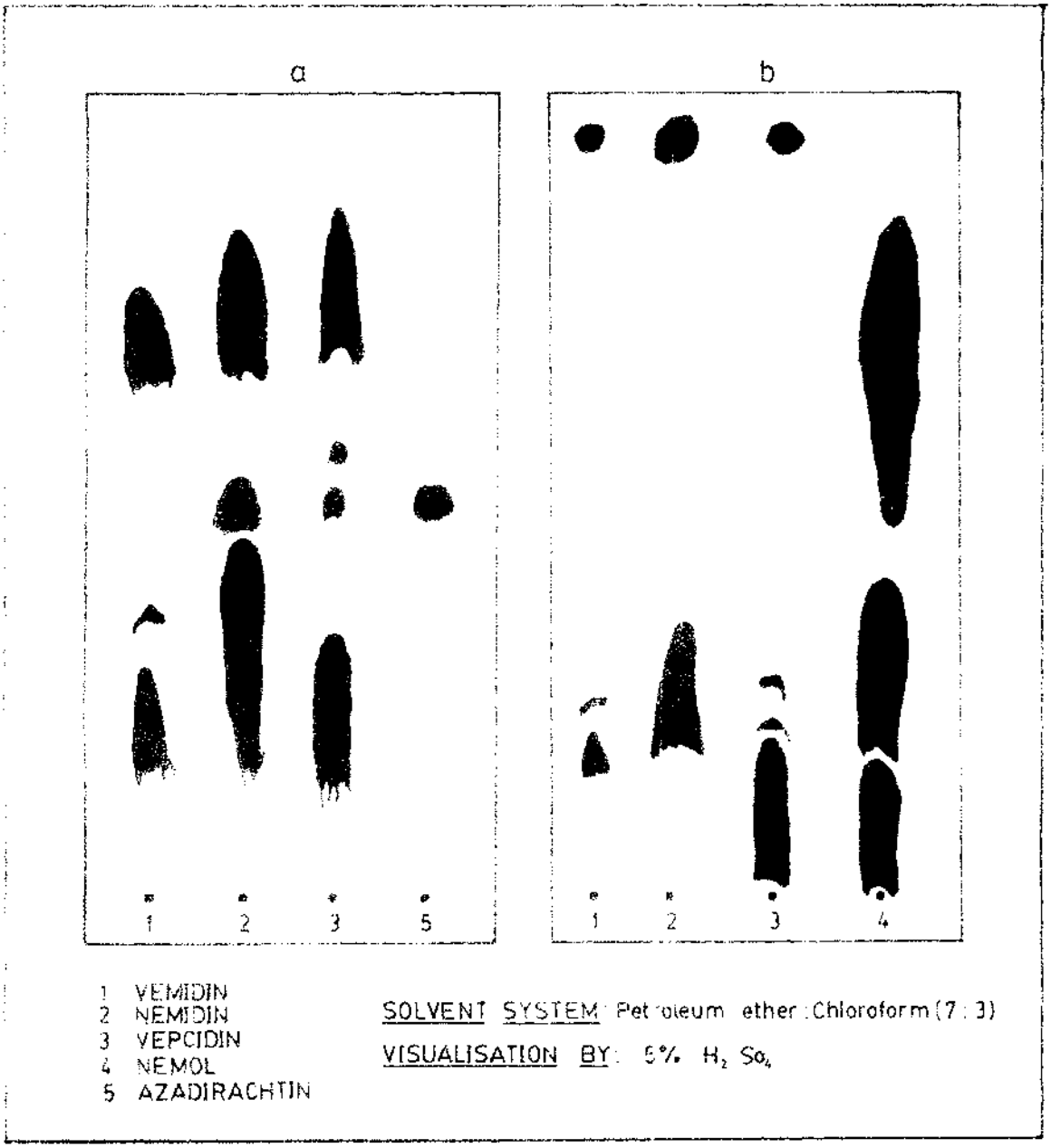


Fig 1: TLC examination of Neem seed fractions.

In bioassays all four ARFs showed larvicidal activity against the freshly moulted IV instar larvae of *Cx. quinquefasciatus*. The lowest LD_{50} value of 0.9 ppm was recorded with Nemidin, followed by Vepcidin with 223.87 ppm, Nemol with 501 ppm and Vemidin with 707 ppm for *Cx. quinquefasciatus* larvae. The LD_{50} values in descending order for the four ARFs with their fiducial limits

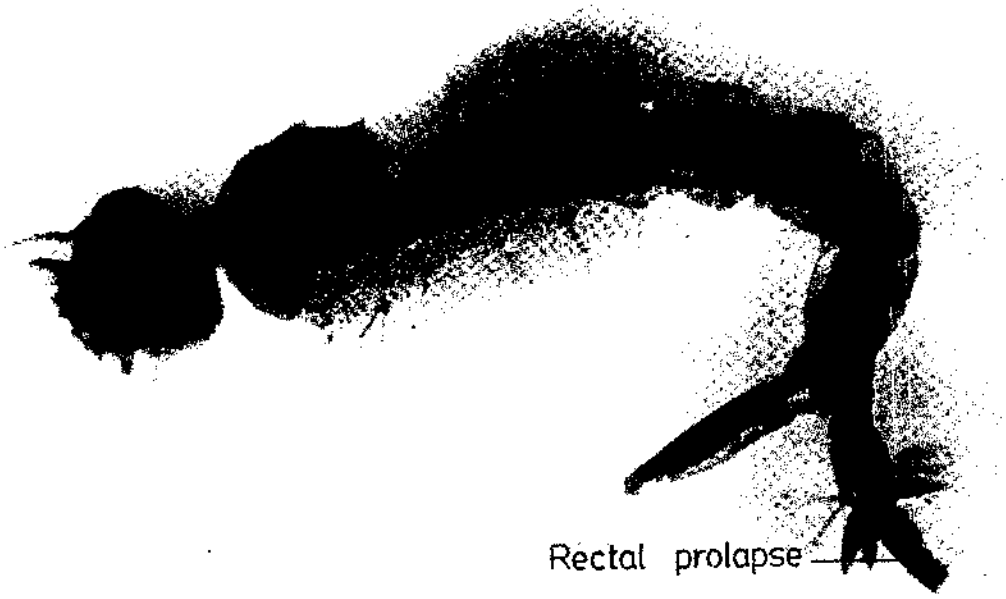


Fig. 2: **Rectal prolapse in Nemidin treated larva of *Cx. quinquefasciatus* Say.**

Table 1. Tests of susceptibility of *Culex quinquefasciatus* Say to four azadirachtin rich fractions

S. No.	Fraction	LD50 value (ppm)	Fiducial limits (ppm)	χ^2 value	Standard error
1.	Nemidin	3.9	15.1 – 1.05	0.414	0.2941
2.	Vepcidin	223.87	439.54 – 113.76	1.191	0.1498
3.	Nemol	501	650.13 – 368.13	1.226	0.0629
4.	Vemidin	707	1086 – 460.25	1.908	0.0949

Table 2. Tests of susceptibility of *Anopheles culicifacies* Giles to four azadirachtin rich fractions

S. No.	Fraction	LD50 value (ppm)	Fiducial limits (ppm)	χ^2 value	Standard error
1.	Nemidin	1.12	1.91 – 0.65	0.511	0.11885
2.	Nemol	56.23	71.78 – 44.05	1.056	0.0542
3.	Vemidin	77.62	150.66 – 40.1	0.603	0.14697
4.	Vepcidin	158	196.78 – 127.35	0.999	0.04837

and χ^2 values are given in Table 1. At higher concentrations of Nemidin from 1,000 ppm to 62.5 ppm all the test larvae which died within 24-48 hrs showed rectal prolapse (Fig. 2). The anal area was ruptured and the protruding rectum was filled with haemolymph. Similar rectal prolapse has been observed by Nagishi *et al.* (1976) in the Tobacco cutworm, *Spodoptera litura* F., when IV instar larvae were fed on artificial diet treated with juvenile hormone. With Vepeidin rectal prolapse was observed at 1,000 ppm only in 5% of the dead larvae. The two fractions, Nemol and Vemidin did not produce rectal prolapse even at 1,000 ppm.

Larvae of *An. culicifacies* were more susceptible than *Cx. quinquefasciatus* as shown by lower LD_{50} values with all the four ARFs. Among the four fractions tested, Nemidin showed the highest larvicidal activity with an LD_{50} value of 1.72 ppm followed by Nemol with 56.23 ppm, Vemidin with 77.62 ppm and Vepeidin with 98 ppm. The fiducial limits, χ^2 values and standard error of different fractions are given in Table 2. Total mortality was observed within 6 hrs in *An. culicifacies* with Nemidin at concentrations from 1,000 to 62.5 ppm, but there was no rectal prolapse in any of the dead larvae, nor was rectal prolapse produced by any of the other fractions. This suggests that the mode of action of the fractions may be different in *An. culicifacies* and *Cx. quinquefasciatus*, with possible contact poisoning in the former and stomach poisoning in the latter.

Among the larvae of *An. culicifacies* and *Cx. quinquefasciatus* which were not killed by the lower concentrations (31.2 ppm to 0.97 ppm) of ARFs within 24 hrs, some prolonged their larval life by 5 to 9 days and eventually died. The delay in moulting and the prolongation in the test larvae could be due to the effect of azadirachtin on the neuroendocrine system (Pagoonee and Lauge, 1981). Other larvae could not completely moult into pupae, resulting in larval-pupal intermediate forms. Similar abnormalities were also reported in *Aedes aegypti* when treated with neem fractions (Zebitz, 1984). In certain cases the larvae moulted into pupae but could not shed off the larval exuvium which remained attached. There were a few pupae which moulted into pupal-adult intermediates and a small precentage which gave rise to apparently normal adults.

In the present investigation Nemidin which had the highest content of azadirachtin, showed more potent larvicidal activity than the other fractions tested. Therefore, emulsifiable and granular formulations from this should be made and field tested against mosquito larvae.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. H. Rembold, Max Planck Institute of Biochemistry, München, FRG (West Germany) for supplying the sample of pure azadirachtin for our experimental purpose. Y. Giftanjali and G. Srimannarayana thank ICAR for financial assistance. The technical assistance of Shriyuts V. Kodangi Alagan and G. Baskaran is gratefully acknowledged.

REFERENCES

1. Chavan, S.R. (1983). Chemistry of alkanes separated from leaves of *Azadirachta indica* and their larvicidal/insecticidal activity against mosquitoes. In *Proceedings of the 2nd International Neem Conference*, Rauischholzhausen: 59-66.
2. Pagoonee, I. and G. Lauge (1981). Noxious effects of neem extracts on *Crocidolomia binotalis*. *Phytoparasitica*, 9:113-118.
3. Feuerhake, K.J. (1983). Effectiveness and selectivity of technical solvents for the extraction of neem seed components with insecticidal activity. In *Proceedings of the 2nd International Neem Conference*, Rauischholzhausen:103-114.
4. Larson, R.O. (1986). The development of Margosan-O TM, a shelf stable pesticide non-toxic to humans, domestic animals, birds and honey bees, made from ethanolic extract of neem seed. Abstracted in 3rd International Neem Conference, Nairobi, Kenya:31.
5. Mascma, D.P. and F.W. Mosha (1986). Toxic effects of neem (*Azadirachta indica*) berry extract on mosquitoes. *Neem Newsletter*, 3(4):44-47.

6. Negashi, I., S. Shimada and S. Asano (1976). Effect of Insect growth regulators on the Tobacco cutworm *Spodoptera litura*: Rectal prolapse. *J. Insect. Physiol.*, 22:1523-1527.

7. Saxena, R.C. (1987). Neem seed "Bitters" for management of plant hopper and leaf hopper pest of rice. In *Proceedings of the Midterm review workshop on Botanical Pest Control in Rice Based Cropping System*. TNAU, Coimbatore, 1-6 June.

8. Sharma, R.N., B.A. Nagasampagi, A.S. Bhosale, M.M. Kulkarni and V.B. Tungikar (1983). "Neemrich": The concept of enriched fractions from neem for behavioural and physiological control of insects. In *Proceedings of the 2nd International Neem Conference*, Rauischholzhausen:115-128.

9. Singh, R.P. (1984). Effect of water extract of deoiled neem kernel on second instar larvae of *Culex fatigans* Wied. *Neem Newsletter*, 2(1): 16-17.

10. Zebitz, C.P.W. (1984). Effect of some crude and azadirachtin – enriched neem (Azadirachta indica) seed kernel extracts on larvae of *Aedes aegypti*. *Entomol. Exp. Appl.*, 35:11-16.

Indian Journal of Malariology
Vol. 25, December 1988, pp. 73 - 76.

Reliability of the Fluorescent Antibody Test in the Measurement of Malaria in the Community

RAMESH KUMAR[1], Y. BHARADWAJ[1], M.A. ANSARI[2], R.K. RAZDAN[2], C.P. BATRA[2] and V.P. SHARMA[2]

A total of 1960 serum samples were collected for malarial antibody assay as well as smear examination in the month of February and March 1987 (non-transmission season). All the villages were under active surveillance by the Malaria Research Centre. The infection rates of malaria as calculated for each village from the serological data, correlated well with the number of malaria cases seen in the age group 1-4 years during the year 1986-87. The significance of the findings is discussed and it is suggested that serology should be accepted at the national level for surveillance of malaria.

INTRODUCTION

The role of serological procedures like Indirect Fluorescent Antibody test is very promising in the epidemiologic interpretation of data in malaria (Draper *et al.*, 1972a; 1972b; Voller, 1971). The period prevalence of malaria in the community as seen by the age related antibody profile has been shown to be a more sensitive tool for surveillance as compared to parasite index which provides only point prevalence data (Draper *et al.*, 1972a; Bruce-Chwatt *et al.*, 1975; Lobel *et al.*, 1976; Kumar *et al.*, 1986; 1987). Serologic data seem to be more consistent with parasite indices in stable transmission areas than in unstable or low transmission areas (Draper *et al.*, 1972 b).

We have recently reported the use of serological procedures in the measurement of malaria in a community and shown it to be more reliable than conventional methods like the parasite index (Kumar *et al.*, 1987). The infection rates of malaria in the community as calculated from the serological data should correlate with the number of malaria cases occurring in these communities. We report here our findings which show such a correlation and confirm the reliability of serology in the measurement of malaria.

Accepted for publication: 24 October 1988.

[1] Department of Microbiology
All India Institute of Medical Sciences
New Delhi - 110029, India.

[2] Malaria Research Centre
22-Sham Nath Marg,
Delhi - 110054, India.

MATERIAL AND METHODS

A double blind controlled study was carried out in 28 villages of District Ghaziabad. These villages are under active surveillance by the Malaria Research Centre and all cases of malaria are properly recorded. The overall incidence of malaria in the study areas has been high for the last several years with both *P. vivax* and *P. falciparum* infections. Blood samples from these areas were collected during the months of February and March 1987 (non-transmission season). From each village at least 30 samples were collected from each of the age groups 0-1 year, 1-2 years, 2-3 years, 3-4 years, 4-5 years, 5-10 years and 10-15 years. The collection of samples, their transport, storage, smear examination, recording and analysis of data was undertaken as reported previously (Kumar *et al.*, 1987).

Serology

IFA test was performed (Voller, 1971) using an antigen prepared from *in vitro* cultivated *Plasmodium falciparum* containing >5% mature schizonts (Trager and Jensen, 1976). Twelve smears were prepared on each microscopic slide, which after drying was wrapped and preserved at -70°C till tested. The filter paper eluates of each sample were taken for antibody estimation (Kumar *et al.*, 1986) and diluted serially from 1/20 to 1/640. The prepared slides were removed from -70°C and allowed to remain at room temperature for 30 mins and then covered with diluted serum samples. Positive and negative control serum samples were included with each batch of tests. After incubation at 37°C for 30 mins, in a moist chamber the slides were washed thoroughly thrice with PBS pH 7.2 and covered with optimum dilution of anti-human IgG-FITC conjugate (Dakopatts). These were incubated at 37°C for 30 mins, and washed as before. The slides were mounted in 50% glycerol and examined under incident light fluorescent microscope (Carl-Zeiss). The results were expressed as reciprocal of the highest dilution of the test sample giving a clear positive reaction. The serum titres of 20 and above were taken as positive. End titres were not determined in samples giving a positive reaction on 1:640 dilution.

Statistical methods

The mathematical model used for calculation of infection rate in the study population is described in detail by Draper *et al.* (1972a) Briefly, the probability of a child never having been infected in n days = $(1-\lambda)^n = e^{-n\lambda}$ where λ is the probability of a child getting infected in one day.

According to the above method, the probability of being infected in one year i.e., infection rate will be = $1-e^{-\mu A}$ where μ is $35 \times \lambda$ and A is age measured in years.

RESULTS

A total of 1960 peripheral smears were examined of which 20 were positive for *P. falciparum* and 17 for *P. vivax*. It may be noted that the samples were examined during the non-transmission season of malaria and all the individuals were healthy and afebrile at the time of sample collection. The percentage positive for IFA antibodies in each age group is shown in Fig. 1. (The percentage has been calculated for 280 cases in each age group). The age-related increase in the number of persons positive for antibodies is quite evident. In the same figure the percentage positive for malarial parasite in the peripheral blood is also shown (the individuals positive for *P. vivax* and *P falciparum* have been pooled). It is evident that the number of individuals with positive peripheral blood smears increase upto the age of 4 years and then there is a sharp decline.

The infection rate for all the 28 villages was calculated from the serological data restricted to the age groups of 1-5 years. It was found to be 26.9. The number of cases taken from each village was sufficient to enable us to calculate the infection rates for each village individually, which ranged

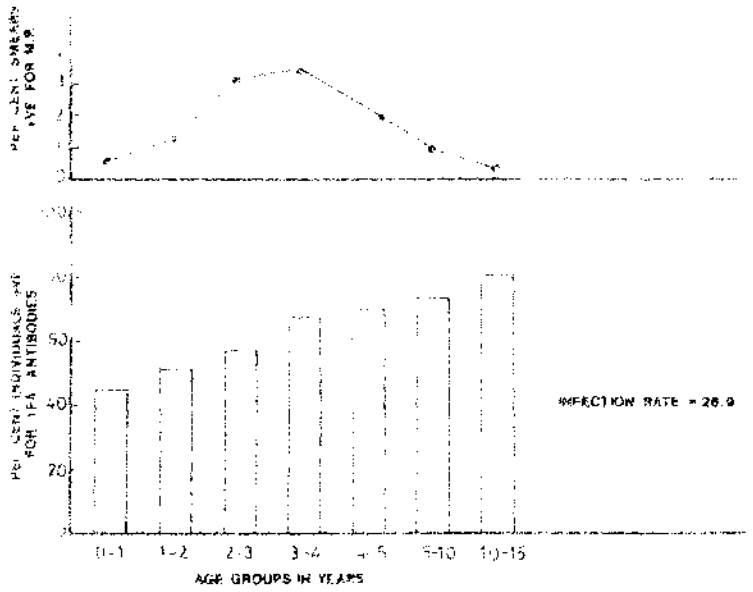


Fig. 1. Percentage of positive smears for malarial parasite showing a rise upto 4 years and later decline. The lower panel shows age related increase in IFA antibodies.

from 0.7 to 63.2. In Fig. 2, a correlation is shown between the infection rates for each village and the total number of malaria cases as percentage of the entire population of each village in the lower panel and with percentage of malaria cases restricted to 1-4 years age groups in the upper panel. There was no correlation between the former while there was a very good correlation in the latter which was highly significant (p value < 0.01).

DISCUSSION

From 1946 onwards, in the Malaria Control Programme the malariometric indices used were child spleen rate, child parasite rate and infant parasite rate. These were replaced by the ABER, API, SPR, SfR under the NMEP which came into effect since 1961 (Sharma, 1984). All these indices depend upon peripheral smear examination by competent workers. The main difficulties in assessment of the extent of malaria in its true figures have been constraints of administrative nature e.g., manpower deployment, expense, antimalarial drug usage and organisational problems.

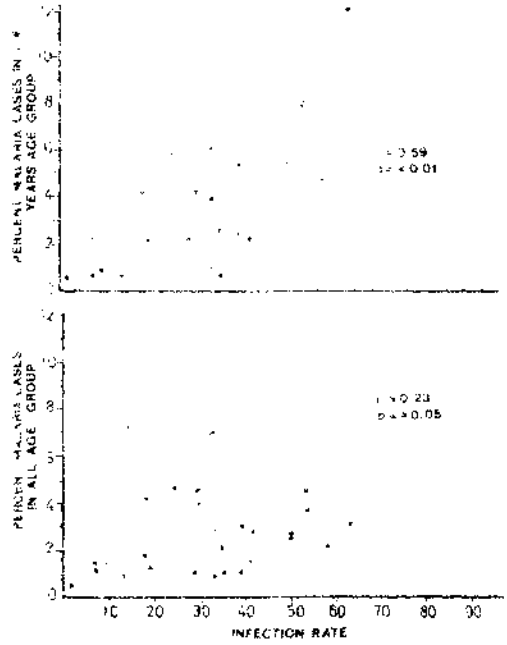


Fig. 2. Correlation between infection rates for each village and the percentage of malaria cases in the age group of 1-4 years is shown in the upper panel and in the total population in the lower panel in 28 villages studied.

In high incidence areas, malaria infection may not be followed by clinical disease. Therefore, particularly in higher age groups there may not be parasites in the blood due to immunity. Thus, peripheral smear examination has certain limitations. In view of this, serology seems promising. Our results indicate that in areas of high malaria incidence, the age groups above 4 years acquire immunity induced by repeated infections, resulting in considerably more number of unrecognised cases and, therefore, no correlation with overall incidence; but within the age groups of 1-4 years of age as expected, there is a good correlation of antibody status with incidence of malaria. However, the incidence may vary greatly even at short distances e.g., in one PHC (Razapur) in different villages infection rate varied from 1.7 to 72.8.

We, therefore, advocate serological indices for malaria surveillance at the national level. However, since serology cannot distinguish between *P. vivax* and *P. falciparum* infections active surveillance by smear examination should continue.

REFERENCES

1. Bruce-Chwatt, L. J., J. C. Draper, D. Avramidis, and D. Kazandzoglou (1975). Seroepidemiological surveillance of disappearing malaria in Greece. *J. Trop. Med. Hyg.*, 78:194-200.
2. Draper, C.C., A. Voller and R.G. Carpenter (1972a). The epidemiologic interpretation of serologic data in malaria. *American J. Trop. Med. Hyg.*, 21:696-703.
3. Draper, C.C., J.L.M. Lutyweid, V.G. Matola and G.B. White (1972b). Malaria in the Para area of N.E. Tanzania IV. Malaria in the human population 11 years after the suspension of residual insecticide spraying with special reference to the serologic findings. *Trans. R. Soc. Trop. Med. Hyg.*, 66:905-912.
4. Kumar, R., S.N. Rao, M.A. Ansari, R.K. Razdan, A. Srivastava and V.P. Sharma (1986). Feasibility of IHA and ELISA in seroepidemiology of malaria. *Indian J. Malariol.*, 23:75-80.
5. Kumar, R., Y. Bharadwaj, M.A. Ansari, R.K. Razdan and V.P. Sharma (1987). Immunofluorescence test in the seroepidemiology of malaria around Delhi. *Indian J. Malariol.*, 24:119-124.
6. Lobel, H.O., A.J. Najera, W.L. Chien, P. Munroe and H.M. Mathews (1976). Seroepidemiological investigations of malaria in Guyana. *J. Trop. Med. Hyg.*, 79:275-284.
7. Sharma, G.K. (1984). Review of malaria and its control in India. In *Proceedings of the Indo-UK Workshop on Malaria*, edited by V.P. Sharma. (Malaria Research Centre, ICMR, Delhi): 13-40.
8. Trager, W. and J.B. Jensen (1976). Human malaria parasites in continuous culture. *Science*, 193:673-675.
9. Voller, A. (1971). Immunofluorescence method suitable for large-scale application to malaria. *Bull. WHO*, 45:524-529

Indian Journal of Malariology
Vol. 25, December 1988. pp. 77 – 82.

Sporogonic Stages of *P. berghei* (NK 65): An Ultrastructural Study

M. RASTOGI[1], S. C. MAITRA[1], ABHA ARYA[1] and A. B. SEN[2]

Ultramicroscopic characteristics of the sporogonic stages of *P. berghei* (NK 65) were studied in *A. stephensi*. Banana shaped ookinetes were observed from 12 hr post-feed mosquitoes. Each ookinete had a small protuberance 'conoid' at the anterior end. The surface of ookinete was smooth. The development of oocysts was asynchronous. Immature oocysts had smooth wall and mature ones had wrinkled surface. The oocysts had no specific pore or opening for liberation of sporozoites. No morphological difference was observed between the sporozoites of oocysts and those of salivary glands except size variation.

INTRODUCTION

The surface ultrastructure of sporogonic cycle of some species of malaria parasites has been studied by Strome and Beaudoin (1974), Sinden (1975), and Sinden and Strong (1978). The present study shows the sequential development of sporogony cycle from ookinete to sporozoites. This would be helpful in understanding the sporontocidal effect of standard antimalarials and new synthesized compounds on *P. berghei* (NK 65) in *A. stephensi*.

Accepted for publication 22 November 1988.

[1] B. M. Unit, Division of Parasitology
Central Drug Research Institute
Chattar Manzil,
Lucknow-226001, India.

[2] Rajendra Memorial Research Institute of
Medical Sciences
Agam Kuan,
Patna-800007, India.

MATERIAL AND METHODS

Parasite – *Plasmodium berghei* (NK 65)
Vector – *Anopheles stephensi*
(NICD, Delhi Strain)
Host – *Mastomys natalensis*

Mosquitoes (*A. stephensi*) were fed on infected *Mastomys* containing mature gametocytes. After 10-24 hrs the midguts of mosquitoes were removed and blood content from midgut was mixed with 3.8% sodium citrate. A drop of this suspension was put over coverslips coated with 0.1% poly-l-lysine for adherence of parasites. For oocysts, the midguts were removed on day 10 post-feed. To observe the sporozoites from oocysts and salivary glands, heavily infected midguts and glands were taken out on day 16 post-feed in RPMI 1640 medium from the same batch of mosquitoes. The midguts and salivary glands

were teased separately and centrifuged at 1000 rpm for 5 mins. Supernatant containing sporozoites was put over pre-coated coverslips.

All the samples, thus prepared were fixed with 3% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) for 3 hrs in cold. The specimens were washed with same buffer, dehydrated in graded alcohol and isoamylacetate. These were finally dried in a Balzer's critical point drier taking CO_2 as critical point drying agent, coated with gold-palladium alloy in a Polaron sputter coater and examined in Philips 515 scanning electron microscope.

RESULTS

The ookinetes were typically banana shaped structures with broad anterior and narrow posterior ends. At the anterior end a small protuberance (conoid) was observed in each ookinete. In some ookinetes a small depression was observed (Figs. 1-2).

Oocysts at the 10th day post-feed showed asynchronous development. These were measured 10-32 μm in diameter. The oocysts lay beneath the basal lamina. Numerous tracheoles were found around the oocysts. The surface structure of the oocysts was of two types – smooth and wrinkled. Some broken or ruptured oocysts were still attached to the gut wall (Figs. 3-5). Oocysts were mainly confined to the middle region of the midgut. Sporozoites of the salivary glands were long, narrow, curved bodies 10-12 μm long (Fig. 6). The widest region of the sporozoites approximately at the middle of the body, corresponded to the site of the nucleus (Fig. 7). Sporozoites liberated from the oocysts were pleomorphic varying from 6-18 μm in length.

DISCUSSION

All the ookinetes were typically banana shaped as reported by Garnham (1965), Yoeli and Most (1960), and Sinden and Strong (1978). In some ookinetes, a small depression was found but biological significance of this depression was not known. A small conoid at the anterior end was supposed to contain the organelles of apical complex (Canning and Sinden, 1973). During the examination of numerous preparations, we observed some ookinetes in movement postures. The postures seemed to suggest a gliding movement. Freyvogel (1967), Ferguson *et al.* (1954) also reported the same findings.

Although infected mosquitoes were maintained under the same conditions of food, temperature and humidity, the development of oocysts was considerably asynchronous in the individual mosquito. Such differential development may give rise to separate broods of sporozoites (Bafort, 1971). Many oocysts had undulating surfaces, suggesting that these were mature ones. Sinden (1975) suggested that highly convoluted oocysts were degenerated and had not been seen to release sporozoites. Strome and Beaudoin (1974) suggested the cyst wall becomes deformed by the contained sporozoites. Duncan *et al.* (1960) and Aikawa and Sterling (1974) all supported a nutritive role for the oocyst wall, although Vanderberg *et al.* (1967) suggested that these were pitfalls.

The excystment of sporozoites from oocysts is still a subject of controversy. Garnham (1966) assumed that excystment of sporozoites occurred by rupturing of the oocyst wall. Our studies are also in agreement with the observation of Garnham and no pore was seen for release of sporozoites in case of *P. berghei* (NK 65) strain. Few ruptured oocysts were seen which clearly indicated that sporozoites are released by rupturing of the cyst wall. Sinden (1974) reported that sporozoites escaped through the oocyst wall by small perforations. It is interesting to note that in *P. cynomolgi bastianellii*, most of the oocysts were ruptured by internal pressure developed by sporozoites in them. It appears that the cyst wall becomes very brittle and can rupture at any place, ultimately the whole wall peeled off leaving sporozoites attached to residual body. Simultaneously, few sporozoites

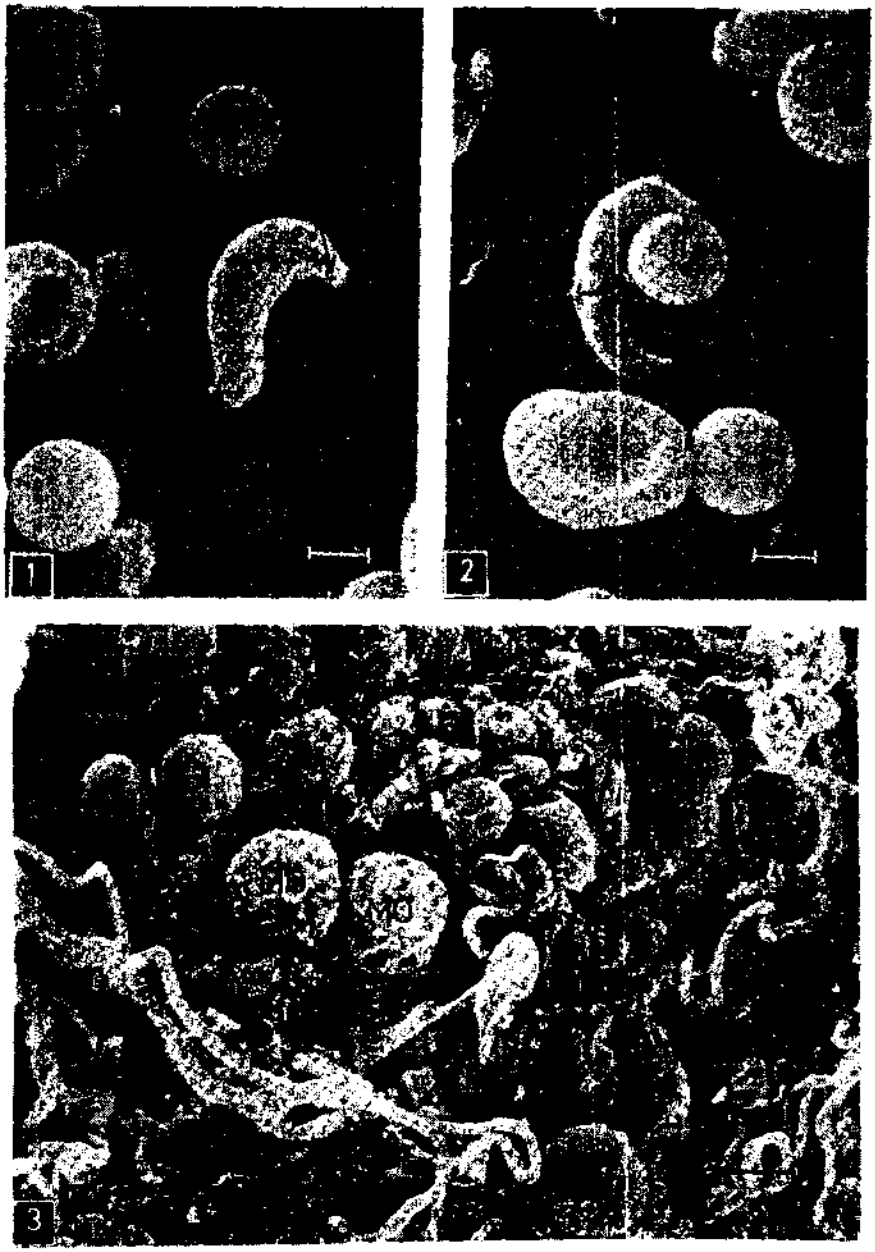


Fig. 1: SEM micrograph of ookinete 20 hrs post-feed showing 'conoid' (arrow). Bar = 1μm.

Fig. 2: Ookinete showing circular depression (D). Bar = 1μm

Fig. 3: SEM micrograph of infected midgut at day 10 showing all the mature oocysts (MO). Bar = 10μm.

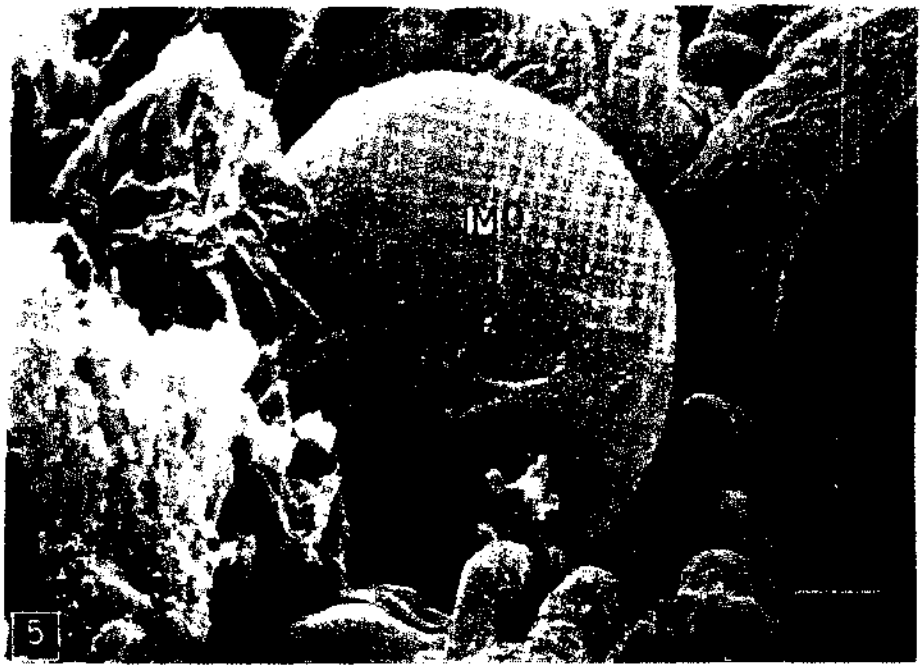


Fig. 4: Infected midgut at day 10 showing immature (IMO), mature (MO) and ruptured (RO) oocysts. Bar = 10 μm.

Fig. 5: Immature oocyst (IMO) surrounded by tracheoles (arrow). Bar = 2 μm.

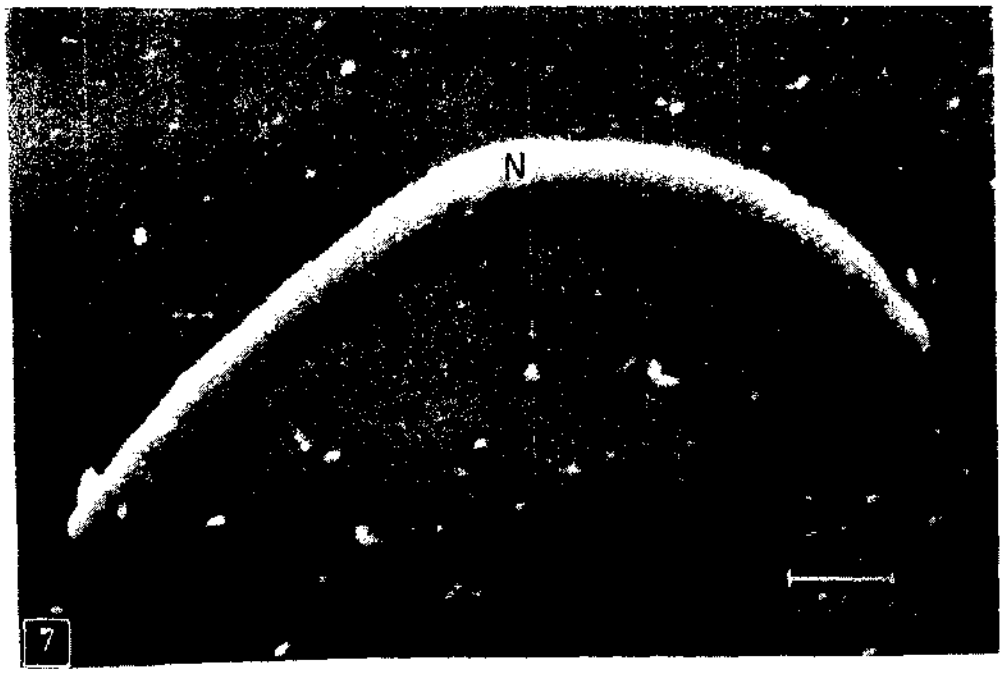


Fig. 6: SEM micrograph of salivary gland's sporozoites. Bar = 10 μm.

Fig. 7: Sporozoite from salivary gland showing site of nucleus (N). Bar = 1 μm.

were also observed coming out from the hole (unpublished results of Mohan *et al.*, CDRI). These results are in confirmation of the findings of Chen *et al.* (1985).

Morphologically sporozoites of salivary glands and oocysts could not be differentiated. Sporozoites from oocysts showed marked variation in their size. This is perhaps responsible for different broods of pre-erythrocytic schizonts in vertebrate hosts.

ACKNOWLEDGEMENT

Authors are grateful to Indian Council of Medical Research, New Delhi for providing financial support.

REFERENCES

1. Aikawa, M. and C. R. Sterling (1974). Intracellular parasitic protozoa. (Academic Press, New York)
2. Bafort, J. M. (1971). The biology of rodent malaria with particular reference to *P. vinckei vinckei* Rodhain 1952. *Ann. Soc. Belg. Med. Trop.*, **51**:1-204.
3. Canning, E. U. and R. E. Sinden (1973). The organization of the ookinete and observations on nuclear division in oocysts of *P. berghei*. *Parasitol.*, 67:29-40
4. Chen, Peihui, Wu Hong, Zhang Sui, Shi Yunlin and Guo Baozhong (1985). Observations on the oocysts, sporoblasts and sporozoites of *P. cynomolgi* by scanning electron microscopy. *Act. Zool. Sin.*, **31**(3):232-235.
5. Duncan D., J. Eades, S. R. Julian and D. Micks (1960). Electron microscope observations on malaria oocysts (*P. cathemerium*). *J. Protozool.*, 7:18-26.
6. Ferguson, M. S., G. B. Wolcott and M. D. Young (1954). Mosquito stages of *P. falciparum*. Film No. CDC, 138b. (Centre for Disease Control, Atlanta, Georgia, USA).
7. Freyvogel T. A. (1967). Shape, movement *in situ* and locomotion of plasmodial ookinetes. *Acta Tropica*, **23**:201-222.
8. Garnham, P. C. C. (1965). The structure of the early sporogonic stages of *P. berghei*. *Ann. Soc. Belg. Med. Trop.*, **45**:259-266.
9. Garnham, P. C. C. (1966). *Malaria Parasites and other Haemosporidia*. (Blackwell, Oxford).
10. Sinden, R. E. (1974). Excystment of sporozoites of malaria parasite. *Nature*, **252**:314.
11. Sinden, R. E. (1975). The sporogonic cycle of *P. yoelii nigeriensis*: A scanning electron microscope study. *Protistologica*, **11**:31-39.
12. Sinden, R. E. and K. Strong (1978). An ultrastructural study of the sporogonic development of *Plasmodium falciparum* in *A. gambiae*. *Trans. R. Soc. Trop. Med. Hyg.*, **72**:477-491.
13. Strome, C. D. A. and R. L. Beaudoin (1974). The surface of the malaria parasite. I. Scanning electron microscopy of the oocyst. *Exp. Parasitol.*, **36**:131-142.
14. Vanderberg, J. P. J. A. G. Rhodain and M. Yoeli (1967). Electron microscopic and histochemical studies of sporozoite formation in *P. berghei*. *J. Protozool.*, **14**:82-103.
15. Yoeli, M. and H. Most (1960). The biology of a newly isolated strain of *P. berghei* in a rodent host and in experimental mosquito vectors. *Trans. R. Soc. Trop. Med. Hyg.*, **54**:549-555.

Indian Journal of Malariology
Vol. 25, December 1988, pp. 83 – 87.

Ultrastructural Studies on Morphogenesis of Rhoptries in Sporozoites of *Plasmodium berghei* (NK 65) in *Anopheles stephensi*

M. RASTOGI[1], S. C. MAITRA[1] and A. B. SEN[2]

The development of rhoptries in the sporozoites of *P. berghei* (NK 65) was studied in *A. stephensi*. Sporozoite formation occurred around the peripheral plasmalemma of sporoblastoids. The sporozoite formation is initated by electron dense pellicle at certain points, known as primordia of sporozoites. Adjacent to each primordium, a nucleus was observed suggesting that sporozoite formation is a nucleus controlled process. Later a vesicle, precursor of rhoptry, was also seen adjacent to the sporozoite bud. Golgi complex seemed to take part in the formation of these vesicles. With the development of sporozoites, these vesicles also became elongated and pear-shaped. In the fully formed sporozoites, paired rhoptries were elongated and uniformly electron dense.

INTRODUCTION

The malaria parasites undergo cyclical development in anopheline mosquitoes and vertebrate hosts. Sporozoites are the stage responsible for establishing the infection in vertebrate host. In spite of their importance for onset of infection, it is still unclear how sporozoites invade the salivary glands of mosquitoes and hepatocytes of the vertebrate host. These invading stages have many organelles in the apical portion of their body and one of the most important organelles are rhoptries. It is commonly believed that rhoptries are associated with the penetration of the host cell membrane. Some workers have suggested that this organelle may be secretory in function (Garnham *et al.*, 1960; Ladda *et al.*, 1969) which helps in cellular invasion possibly by releasing proteolytic enzyme/s. Meis *et al.* (1985) reported that sporozoites first entered the Kupffer cells by phagocytosis to infect the hepatocytes. Keeping the importance of rhoptries in mind, we have undertaken a study on their origin and morphogenesis in the sporozoites of *P. berghei* (NK 65) infection.

Accepted for publication: 9 December 1988.

[1] E.M. Unit, Division of Parasitology
Central Drug Research Institute
Chattar Manzil
Lucknow-226001, India.

[2] Rajendra Memorial Research Institute of Medical Sciences
Agam Kuan
Patna-800007, India.

MATERIAL AND METHODS

Mosquitoes were fed on *Mastomys natalensis* infected with *P. berghei* (NK 65) having 0.5-1.0% parasitaemia. The fed mosquitoes were maintained in a climatically controlled insectary (27 ± 1°C and 80-85% RH). Midguts from infected mosquitoes containing sporogonic stages were removed at different time intervals after the blood meal. These were fixed in glutaraldehyde and paraformaldehyde (according to Karnovsky, 1965). After adequate fixation specimens were washed with 0.1 M cacodylate buffer thrice at 10 mins intervals and were osmicated in 1% O_5O_4 in 0.1 M cacodylate buffer (pH 7.3) for 1 hr at 4°C. Following osmication, the specimens were washed in distilled water several times and dehydrated in graded acetone before being embedded in Epon-araldite medium (Mollenhauer, 1964). Thin sections were cut on LKB ultratome and stained in 1% aqueous uranyl acetate and in lead citrate (Reynolds, 1963). These were viewed under Philips 410 LS transmission electron microscope.

RESULTS

Sporoblastoids were formed by condensation of cytoplasm of the immature oocyst and a clear space appeared all along the capsule wall leaving the cytoplasm at the centre. Simultaneously, the cytoplasm divided by the formation of clefts to form several sporoblastoids. Budding of sporozoites was initiated through the formation of electron dense pellicle at certain points. The nucleus was generally found near the electron dense site. It was a pear-shaped body with fine granular material somewhat denser than cytoplasm. The nuclear land near the site of sporozoite budding was invariably very dense and it appeared that some material was oozing out from the condensed nuclear portion, to the newly formed pellicle (Fig.1).

An electron dense vesicle (250-300 nm) was found at the site of sporozoite budding. This vesicle is the precursor of rhoptry. Concomitant with this some Golgi bodies with one or two cisternae ter-

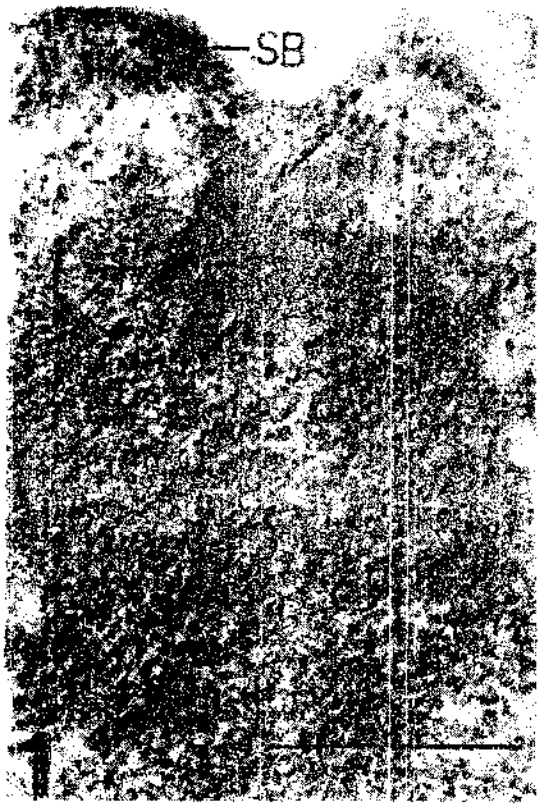


Fig. 1: Part of sporoblastoid showing budding site (SB) and nucleus (N) nearby oozing out some substance (arrow). Bar = 0.5 µm.

minating in vesicles (240 – 300 nm) were found (Fig. 2). The elongation of sprozoite bud continued along with the elongation of vesicles. These vesicles became electron dense and flask-shaped and were connected to the anterior pole of the sporozoites (Fig. 3). The fully formed sporozoites showed elongated paired organelles known as rhoptries (Figs. 4 and 5).

DISCUSSION

In case of *P. berghei* (NK 65), the nucleus can be seen near the electron dense site of sporozoite budding. The close association of these two structures suggests that the site of sporozoite budding is predetermined and nucleus controlled. The hypothesis about the morphogenesis of rhoptries

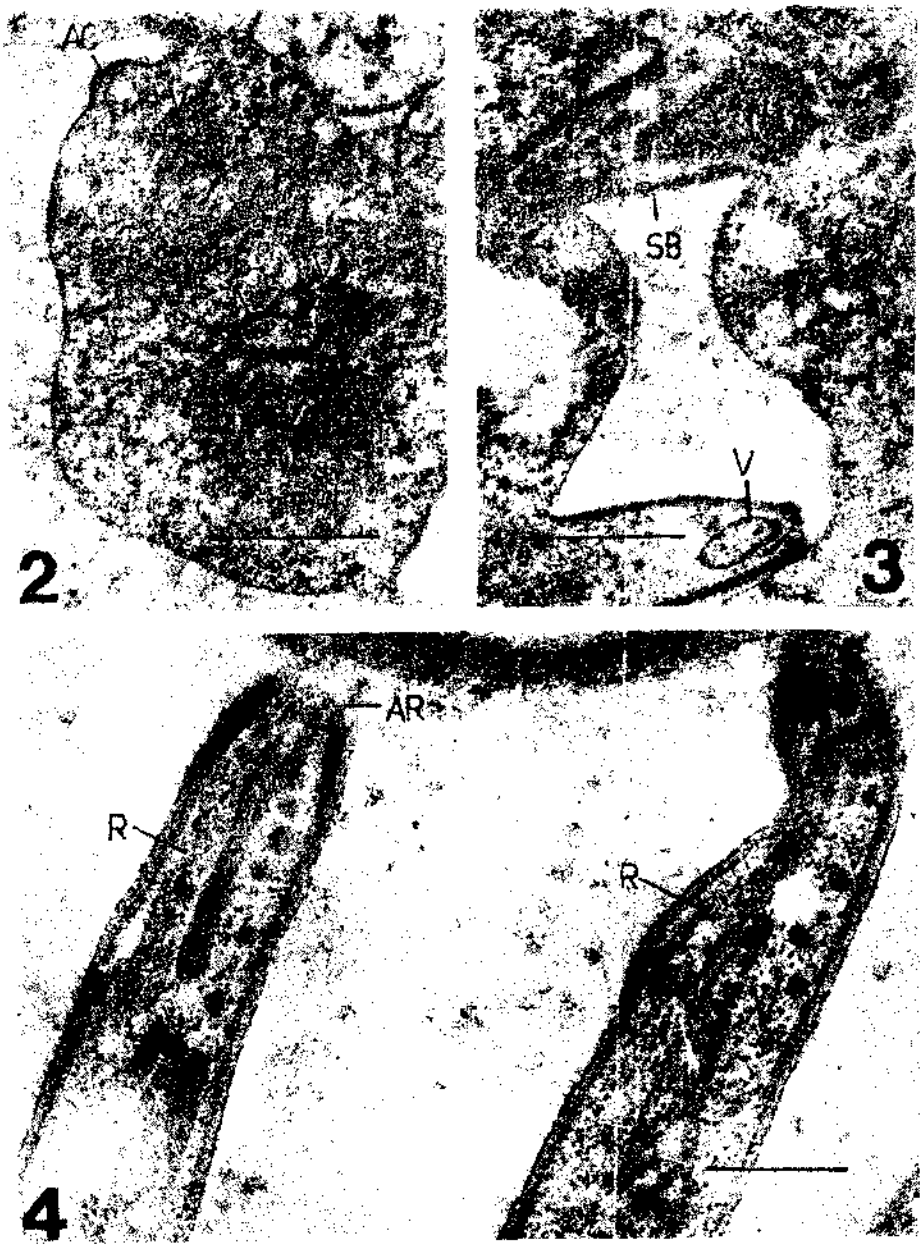


Fig. 2: Part of sporoblastoid showing electron dense area, initiation of apical complex (AC) in close association of vesicle (V). Note the presence of Golgi (GC) with vesicles (V) in the cytoplasm. Bar = 1 μm.

Fig. 3: Elongation of sporozoite buds (SB) with elongation of vesicles (V) and migration of nucleus (N) into bud (SB). Bar = 1 μm.

Fig. 4: L.S. of sporozoites showing apical ring (AR), rhoptries (R). Bar = 0.5 μm.

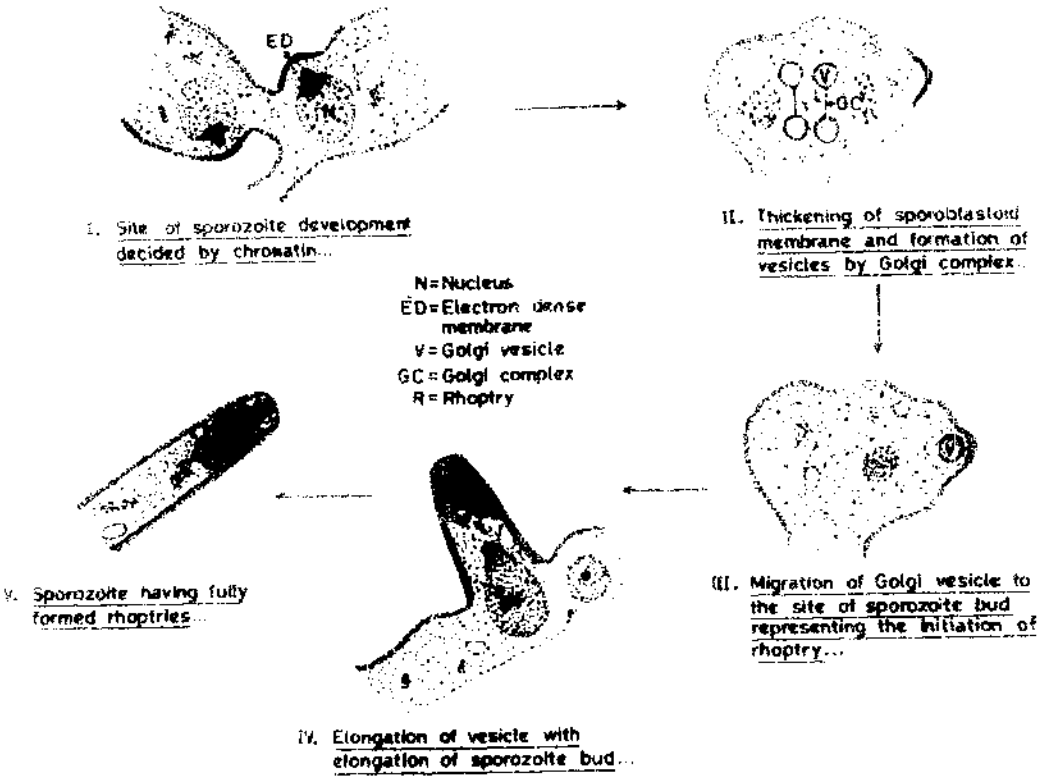


Fig. 5. Diagrammatic representation of the formation of rhoptries.

and its secretory function has been made by numerous authors (in *Eimeria magna*), Jensen and Edgar 1976, Scholtyseck and Mehlhorn 1970 in sporozoa and Vivier and Petitprez, 1972 in *Toxoplasma gondii*). The rhoptries are formed by a large (0.4 μm diameter) 'empty' sphere limited by a dense membrane (Sinden and Garnham, 1973). Wong and Desser (1976) in *Leucocytozoon dubreuili* observed oval sac-like bodies near the apex of each sporozoite bud, believed to be precursors of the rhoptries. These findings are corroborated by our observations. Terzakis *et al.* (1966) revealed that the development of rhoptry was the outcome of the condensation of numerous small particles. In the present study, such particle accumulation has not been seen. In *Eimeria*, small vesicles are formed initially on Golgi complex and later coalesce to form the large vesicle (Dubremetz, 1975). In *P. berghei* (NK 65) infection, it is clearly seen that vesicles produced by Golgi complex have a similar look and are of the same size as that found adjacent to the sporozoite primordium.

Thus, it can be concluded that sporozoite budding is, to a certain extent, a nucleus controlled phenomenon and that the rhoptries which are supposed to take part in the penetration process are formed from the Golgi complex.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the financial support from ICMR to Meena Rastogi. We are also thankful to Mrs. Abha Arya for her technical help during the work.

REFERENCES

1. Dubremetz, J. P. (1975). La genese des merozoites chez la coccidie *Eimeria necatrix*. Etude Ultrastructurale. *J. Protozool.*, 22:71-84.
2. Garnham, P. C. C., R. G. Bird and J. R. Baker (1960). Electron microscope studies of motile stages of malaria parasites. I. The fine structure of the sporozoites of *Haemamoeba* (*Plasmodium*) *gallinacea*. *Trans. R. Soc. Trop. Med. Hyg.*, 54:274-278.
3. Jensen, J. B. and S. A. Edgar (1976). Possible secretory function of the rhoptries of *Eimeria magna* during penetration of cultured cells. *J. Parasitol.*, 62:988-992.
4. Karnovsky, M. J. (1965). A formaldehyde glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell. Biol.*, 27:137A.
5. Ladda, R., M. Aikawa and H. Sprinz (1969). Penetration of erythrocytes by merozoites of mammalian and avian malarial parasites. *J. Parasitol.*, 55:633-644.
6. Meis, J. F. G. M., J. P. Verhave, A. Brouwer and J. H. E. Th. Meuwissen (1985). Electron microscopic studies on the interaction of rat kupffer cells and *Plasmodium berghei* sprozoites. *Z. Parasitenkd.*, 71:473-483.
7. Mollenhauer, H. H. (1964). Plastic embedding mixture for use in electron microscopy. *Stain. Tech.*, 39:111-114.
8. Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell. Biol.*, 17: 208-212.
9. Scholtyseck, E. and H. Mehlhorn (1970). Ultrastructural study of characteristic organelles (Paired organelles, micronemes, micropores) of sporozoa and related organisms. *Z. Parasitenkd.*, 34:97-127.
10. Sinden, R. E. and P. C. C. Garnham (1973). A comparative study on the ultrastructure of *Plasmodium* sporozoites within the oocyst and salivary glands, with particular reference to the incidence of the micropore. *Trans. R. Soc. Trop. Med. Hyg.*, 67:631-637.
11. Terzakis, J. A., H. Sprinz and R. A. Ward (1966). Sporoblast and sporozoite formation in *Plasmodium gallinaceum* infection of *Aedes aegypti*. *Milit. Med.* 131:984-992.
12. Vivier, E. and A. Petitprez (1972). Donnees ultrastructurales complementaires, morphologiques et cytochimiques, sur *Toxoplasma gondii*. *Protistologica*, 8:193-221.
13. Wong, T. C. and S. S. Desser (1976). Fine structure of oocyst transformation and the sporozoites of *Leucocytozoon dubreuili*. *J. Protozool.*, 23:115-126.

Indian Journal of Malariology
Vol. 25, December 1988, pp. 89 – 96.

Clinical Trial of Fansimef in Indian Patients of *P. falciparum* Malaria

OMKAR P. ASTHANA[1], A.N. TANGRI[1] and SWARN NITYANAND[1]

Clinical trial was carried out to evaluate safety, tolerance and antimalarial efficacy in patients with *P. falciparum* infection. Thirty one male patients from Haldwani district, a malaria endemic area, were studied for a period of 35 days after drug administration. Fansimef was well tolerated and safe as evident from the absence of any significant drug related changes in the various haematological and biochemical parameters determined. This antimalarial drug combination of mefloquine, sulphadoxine and pyrimethamine, in a single oral dose of three tablets controlled pyrexia, a predominant feature of malaria within 24 hours after drug administration in 100% cases, while parasitaemia got cleared on day 2 in 35.4% on day 7 in 70.9% and 100% on day 35 post-drug. Re-infection was not noticed in any case during the period ending 35 day post-drug. Undesirable effects due to Fansimef included, nausea, vomiting, vertigo, pain in abdomen, anorexia, loss of sleep, headache, weakness, constipation and restlessness during first 24 hrs of medication. These side effects were mild to moderate, self limiting and did not require any special management. Thus Fansimef was found to be a safe and effective drug for the management of *P. falciparum* malaria in Indian patients.

INTRODUCTION

There is an urgent need to develop better, safe and effective antimalarial chemotherapeutic agents for the effective implementation of National Malaria Eradication Programme in India. Treatment given over several days to two weeks on outpatient basis often leads to poor patient compliance, once symptoms like fever and rigor subside and this may be a cause of the increasing incidence of resistance to conventionally used antimalarials like chloroquine.

Mefloquine, a quinoline methanol derivative, is active against all forms of plasmodium infection. Its value against resistant cases of *P. falciparum* infections has been well documented (Rozman and Canfield, 1979; Rieckmann *et al.*, 1974; Hall *et al.*, 1977; Doberstyn *et al.*, 1979).

It has been observed that the administration of mefloquine alone may lead to the development of

Accepted for publication, 20 December 1988.

[1] Division of Clinical and Experimental Medicine
Central Drug Research Institute
Chattar Manzil,
Lucknow - 226001, India.

Table 1. Clinical trial protocol

Parameters	Observation/Investigation: Days (Post-drug)									
	0	1	2	3	5	7	14	21	28	35
A. Clinical examination										
Medical history	X	X	X	X	X	X	X	X	X	X
General examination	X	X	X	X	X	X	X	X	X	X
Systemic examination	X	X	X	X	X	X	X	X	X	X
Vitals (pulse rate respiration and B.P. record)	X	X	X	X	X	X	X	X	X	X
Pyrexia (temperature chart)	X	X	X	X	X	X	X	X	X	X
B. Laboratory investigation										
1. *Routine*										
(a) Urine examination	X								X	
(b) Faeces examination	X								X	
2. *Haematological* (Haemoglobin, red blood cell count, total and different WBC count)	X								X	
3. *Biochemical* (Serum)										
Glucose	X								X	
Urea	X								X	
Bilirubin	X								X	
Total proteins	X								X	
Albumin	X								X	
Globulin	X								X	
SGOT	X								X	
SGPT	X								X	
C. Parasitological										
Smear examination for parasitaemia	X	X	X	X	X	X	X	X	X	X

resistance to *P. falciparum* (Peters *et al.*, 1977; Kazim *et al.*, 1979). The use of mefloquine in combination with other antimalarials delays the development of resistance to individual components (Peters, 1974). Fansimef® (Hoffman-La-Roche, Basel) is a new antimalarial drug combination of 250 mg mefloquine (base), 500 mg sulfadoxine, and 25 mg pyrimethamine per tablet. Safety and efficacy of Fansimef after a single oral dose of 3 tablets in Brazilian male subjects has been established recently (Desouza *et al.*, 1985).

Phase I/II studies with mefloquine were undertaken at Central Drug Research Institute, Lucknow, where after establishing its safety in human volunteers, kinetics in normal subjects and patients of malnutrition have also been studied (Asthana *et al.*, 1986).

The present study with Fansimef was undertaken to assess tolerance and efficacy in patients with *P. falciparum* infection. This clinical trial was conducted at Haldwani, an area in the foothills of the Himalayas where malaria endemicity is very high.

MATERIAL AND METHODS

The clinical trial was an open prospective study. The total duration of the study was 37 days which included two days observation before actual administration of the drug.

Patient selection

Thirty one male patients aged 17 to 50 years (mean 26.8 ± 8.2 years) executed their informed consent to undergo the present clinical trial. The patients were Indians from the endemic area of Haldwani district, Nainital. Their body weight ranged from 48 to 69 kg (mean 49.4 ± 7.4 kg) and height was in the range of 135 to 175 cms (mean 162.6 ± 7.5 cms). These patients acquired *P. falciparum* malaria naturally and had not received any antimalaria drugs for four weeks prior to inclusion in the clinical trial.

Drug administration

Each patient was given a single oral dose of 3 tablets with a glass of water after a light breakfast on the morning of day 0 in the presence of the Medical Officer. Patients were allowed supportive treatment in the form of analgesics, antipyretics, multivitamins and tranquilo-sedatives as and when required. Worm infestations, detected on stool examination, were treated at the end of the trial.

Clinical examination

Prior to drug administration, each patient was subjected to a detailed history taking and thorough clinical examination. Clinical follow-up and laboratory investigations were performed as per protocol (Table 1).

RESULTS AND DISCUSSION

Vital parameters and pyrexia

In patients treated with single oral dose of Fansimef, no drug induced changes were observed on vital parameters (pulse rate, respiration rate and blood pressure).

Pyrexia, a predominant feature of malaria was recorded in the range of 100 to 102.4°F (mean 101.6° ± 0.6°F). The body temperatures were normal in all patients within 24 hrs after drug administration, and thereafter all patients remained afebrile during the entire study period.

Haematology

Pre-drug (0 day) and post-drug (28 day) determinations did not reveal any significant change in the various haematological parameters (Table 2).

Biochemical investigations

The results are given in Table 3, values for serum glucose, urea, bilirubin, total proteins, albumin,

Table 2. Showing pre-drug and post-drug values Mean ± SD (range) of haematological parameters in patients treated with Fansimef

Haematological parameters	Units (SI)	0 Day pre-drug	28 Day post-drug
Haemoglobin	m mol/l	5.75 ± 1.66(2.79–9.3)	7.05 ± 2.31(4.81–9.6)
Red blood cell count	10^{12}/l	3.10 ± 0.87(1.55–5.2)	3.79 ± 1.31(2.7–5.2)
White blood cell count	10^{9}/l	7.238 ± 2.240(4.6–13.1)	7.657 ± 2.390(5.6–9.9)
Differential white cell count	%		
Neutrophil		61.39 ± 7.70(50–77)	61.3 ± 8.55(53–72)
Eosinophil		3.64 ± 2.50(0–11)	3.09 ± 2.67(0–7)
Lymphocyte		27.12 ± 8.75 (10–38)	31.37 ± 8.92(24–42)
Monocyte		6.90 ± 2.31(2–11)	4.14 ± 2.62(2–8)

Table 3. Showing pre-drug and post-drug values Mean ± SD (range) of biochemical parameters in patients treated with Fansimef

Biochemical parameters	Units (SI)	0 Day pre-drug	28 Day post-drug
Serum			
Glucose	m mol/l	4.51 ± 0.384(3.75–5)	4.53 ± 0.53(3.83–5.5)
Urea	m mol/l	9.01 ± 2.24(5.76–13.68)	8.6 ± 2.01(6.12–12.24)
Bilirubin (Total)	μ mol/l	34.85 ± 17.68(11.9–85)	9.52 ± 3.06(5.1–17.0)
Proteins (Total)	gm/l	66.1 ± 5.8 (50–80)	70 ± 8.2 (60–82)
Albumin	gm/l	32.2 ± 5.2 (22–44)	39.1 ± 5.8 (28–48)
Globulin	gm/l	33.9 ± 7.7 (14–48)	30.6 ± 6.8 (20–36)
SGOT	iu/l	21.60 ± 4.96 (15–30)	9.89 ± 3.06 (7–15)
SGPT	iu/l	30.20 ± 7.11 (15–45)	11.50 ± 4.25 (8–17)

Table 4. Patients showing parasitaemia clearance on different days after Fansimef administration

	Parasitaemia clearance								
Day (post-drug)	1	2	3	5	7	14	20	21	35
Patient code No.	01,02	06,18	20,22	29,30	04,08	46	44	14,17	09,10
	16,42	34,35	32	45	13,23			21	11,12
	43	38,41			48				
Total number of cases	5	6	3	3	5	1	1	3	4
Percentage	16.1	19.3	9.7	9.7	16.1	3.2	3.2	9.7	12.9

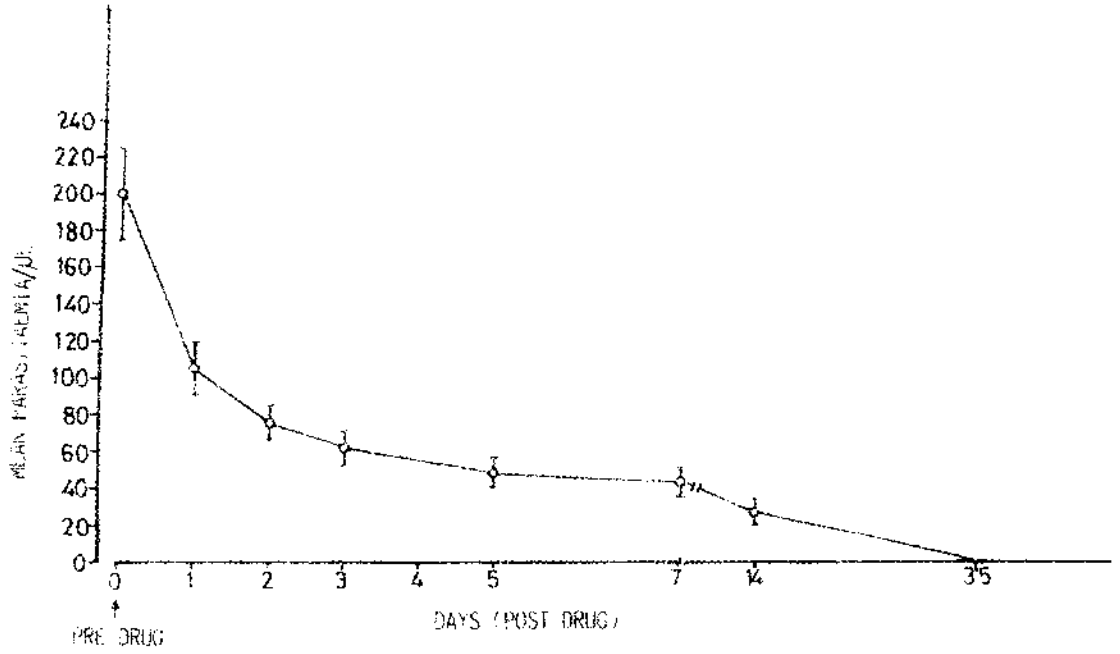


Fig. 1. Showing mean parasitaemia (count/μl) before and after single dose treatment with Fansimef. Vertical bars indicate the standard error of the mean.

globulin, SGOT, and SGPT were determined before the drug administration and on 28 day post-drug. No significant drug related changes were observed except initial high values of serum bilirubin (mean 34.85 ± 17.68, range 11.9–85 μmol/litre and SGPT (mean 30.2 ± 7.11, range 15-45) in 80% cases which returned to normal at 28 day post-drug without any special management (it seems to be due to haemolysis caused by *P. falciparum* infection).

Urine and faeces examination

In all cases urine analysis and faeces examination was carried out before drug administration and at 28 day post-drug. Urine examination showed no significant changes except presence of urobilinogen in 35% patients with high initial values of serum bilirubin, which might be possibly due to haemolysis caused by malarial infection. It cleared off rapidly after Fansimef administration and was absent on day 28.

Blood smear examination

Effect on parasitaemia

The response of Fansimef on mean parasitaemia is depicted in Fig. 1. A consistent decline in mean parasitaemia has been observed after a single oral dose of Fansimef reaching 0 at 35 day post-drug. The number and percentage of patients showing parasitaemia clearance on different days after Fansimef administration, have been shown in Table 4

Table 5 shows the effect of Fansimef treatment on *P. falciparum* infection precisely on asexual and sexual forms appearing in peripheral blood. During the post-treatment period both rings and gametocytes were seen in peripheral blood smears but a large number of gametocytes did not show normal morphology showing distorted and altered shapes which could be due to the effect of Fansimef and it may be quite possible that

Table 5. Effect of [illegible] on different stages of *P. falciparum* after single dose treatment†

Patient code no.	0 Day		1 Day		2 Day		3 Day		5 Day		7 Day		14 Day		20 Day		21 Day	
	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S
01	208	-		-	-													
02	528	-	-	-	-	-												
16	199	-	-	-														
42	99	-	-															
43	88	-																
06	263	-	75*	-														
18	-	196		36*														
34	84	-		42		-	-	-										
35	-	60		30*	-	-	-											
38	98		42		-	-	-	-										
41	102	-	-	20*			-	-										
20	166		114	-	83	-	-	-										
22	167		91		30	-		-										
32	44	44	-	44*	-	22*	-	-										
29	109		68	19*	28	28*	-	28*		-								
36	112		56		28	-	14	14*		-								
45	74			53	-	42		21		-								
04	192	182	101	95	-	81	-	81		51								
08	215	-	123	-	50		26	-	13		-							
13	306	90	169	31	77	62*	77	16*		32*								
23	137	49	56	98		66*		49		33*								
46	92	42	37	47		28		18	-	18	-	-						
46	135	83	62	21		21	-	21	-	10	-	10	-	-				
44	88	-		39*	-	39*		30		20*	-	10*	-	10*				
14	288	26	262	52*	79	131*	79	131*	26	157*	-	131*	-	52*	-	26*	-	-
17	171	390		293*		147*		98*	-	49*	-	26*	-	52*	-	13*	-	
21	108	-	19	59*		59*	-	49*	-	39*	-	49*	-	37*		25*		-
09	-	290		116		81		46		35	-	23	-	35	-	23	-	23
10	182	36	30	36*	-	36*	-	36*	-	54*	-	90*	-	30*	-	30*	-	36*
11	204	61	61	61	41	61		82		67	-	41	-	41	-	30	-	30
12	134	166	99	116*	17	116*	-	66*		33*		17*	-	17*		17*	-	17*

† Values expressed show parasite density: A = Asexual forms (Rings); S = Sexual forms (Gametocytes)
* Gametocytes distorted and altered in shape.

As per WHO (1984) Parasite density = $\frac{\text{Parasite counted} \times \text{Total WBC count/mm}^3}{\text{Leucocytes counted (500)}}$

= Parasite counted per mm³ of blood

Note: There was complete clearance of parasitaemia on Day 35.

Table 6. Side effects observed in patients treated with single dose of Fansimef

Side effect	Patient code No.	Total number of patients showing side effects
Nausea, vomiting	02, 04, 06, 08, 09, 10, 12, 15, 16, 17, 18, 20, 21, 22, 30, 45	16
Nausea, vomiting and vertigo	23, 29, 32, 34, 38, 41, 42, 48	8
Pain in abdomen	02, 04, 10, 12, 20, 21, 35, 38, 43, 45	10
Loss of appetite	06	1
Constipation	01, 30, 38, 48	4
Weakness	01, 04, 06, 08, 13, 18, 20, 22	8
Restlessness	13	1
Loss of sleep	01, 06, 10, 12, 17, 20, 22, 23 29, 30, 32, 34, 38, 42, 45	15
Headache	02, 04, 10, 18, 20, 21, 22, 23 30, 42	10

Side effects were observed in first 24 hrs of medication.

gametocytes not showing normal morphological appearances are devoid of infectivity potential.

Side effects

Side effects were frequent after single dose administration as evident from the incidence shown in Table 6, but in majority of the cases the nature and severity of side effects was never so severe as to require any special management. The incidence of side effects is more than that observed with mefloquine alone (Asthana *et al.*, 1986). However, in Brazilian male subjects Fansimef produced lesser side effects (Desouza *et al.*, 1985) this may due to regional or ethnic variation in drug tolerance.

The present clinical study carried out in male patients with naturally acquired *P. falciparum* malaria from an endemic area in the foothills of the Himalayas in the town of Haldwani has established Fansimef to be a safe and effective antimalarial drug. Though side effects were frequent they could be managed without any specific treatment. These results are in conformity with the findings reported in a study conducted in Brazil (Desouza *et al.*, 1985).

ACKNOWLEDGEMENTS

Authors wish to thank Dr. M. M. Dhar, Ex-Director, Central Drug Research Institute, Lucknow, Prof. R.N. Misra, Ex-Head of the Department of Medicine, King George's Medical College, Lucknow, Dr. S.C. Bhalla, Additional Director, State Medical and Health Services, U.P., Lucknow, Dr. M.S. Malhotra, Scientist-in-charge, Malaria Research Centre (ICMR), Haldwani, Dr. B. D. Nariyal, Medical Officer, Civil Hospital, Haldwani for extending their help in completing this project and to Dr. Nitya Anand, Ex-Director, CDRI, Lucknow for arranging the supply of Fansimef tablets through the World Health Organization, Geneva. Technical assistance by Mr. A.K.

Nigam and manuscript typing by Mr. Shyam Lal, Central Drug Research Institute, Lucknow is gratefully acknowledged.

REFERENCES

Asthana, O.P., S. Satyanand, S. Singh and S.K. Das (1986). Abstract published in the Proceedings of II Asian Congress of Parasitology held at Lucknow, India, Feb. 13-16.

2. Desouza, J.M., U.K. Sheth, R.M.G. De Oliverira, A.T. Gomes and E.Q. Cavalcante (1985). A phase I Clinical trial of Fansimef (Mefloquine plus sulfadoxine-pyrimeth) in Brazilian male subjects. *Bull. WHO*, 63 (3): 611-615.

3. Doberstyn, E.B. *et al. (1979)*. Single dose therapy of falciparum malaria with mefloquine or pyrimethamine-sulphadoxine. *Bull. WHO*, 57(2):275-279.

4. Hall, A.P., B. Edward, Doberstyn, C. Karnchanachetanee, S. Samaransamryuajkit, B. Laixuthai, E.J. Pearlman, R.M. Lampe, C.F. Miller and P. Phinyothin (1977). Sequential treatment with quinine and mefloquine or quinine and pyrimethamine-sulfadoxine for falciparum malaria. *Trans. Roy. Soc. Trop. Med. Hyg.*, 71: 625-626.

5. 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.

6. Peters, W. (1974). Prevention of drug resistance in rodent malaria by the use of drug mixtures. *Bull. WHO*, 51: 379-383.

7. Peters, W., J. Portus and B.L. Robinson (1977). The chemotherapy of rodent malaria, XXVIII: The development of resistance to mefloquine (WR 142490). *Ann. Trop. Med. Parasitol.*, 71: 419-427

8. Rieckmann, K.H., G.M. Trenholme, R.L. Williams, P.E. Carson, H. Frischer and R.E. Desjardins (1974). Prophylactic activity of mefloquine hydrochloride (WR 142490) in drug-resistant malaria. *Bull. WHO*, 51: 375-377.

9. Rozman, R.S. and C.J. Canfield (1979). New supermental antimalarial drugs. *Adv. Pharmacol. Chem.*, 16: 1-43.

10. WHO (1984). Advances in malaria Chemotheraphy. *Tech. Rep. Ser.*, 711:185-187.

SHORT NOTES

Indian Journal of Malariology
Vol. 25, December 1988, pp. 99 – 101.

Chloroquine Resistant *Plasmodium falciparum* Malaria in Calcutta A case report

M.S. DAS[1], S. DAS[1] and A. MUKHERJEE[1]

A case of chloroquine resistant *P. falciparum* malaria in an adult male of south Calcutta is reported. Two weeks extended *in vivo* test for sensitivity of *P. falciparum* to chloroquine (1500 mg) revealed RI pattern of resistance. Treatment with metakelfin terminated the infection.

Calcutta almost exclusively a *P. vivax* area since resurgence, has recently been encroached upon by *P. falciparum* in 1980 (NMEP). Dwivedi *et al.* (1981) remarked that monitoring of chloroquine resistance should be done where *P. falciparum* cases reappeared. Resistance of *P. falciparum* to chloroquine has been reported from different parts of India (WHS, 1986) since its first appearance from Karbi Anglong, Assam (Sehgal *et al.*, 1973). Earlier studies on chloroquine resistant in *P. falciparum* in Cooch Bihar district of West Bengal in 1975 showed 100% susceptibility (Pattanayak *et al.*, 1979). But recent studies have shown emergence of chloroquine resistant falciparum strains in Jalpaiguri and Purulia districts of West Bengal (WHS, 1986). A focus of chloroquine resistant falciparum strains has also been identified in Calcutta (Sinha *et al.*, 1987). As chloroquine is the drug of choice for the treatment of falciparum malaria, they stressed the need of further studies in Calcutta using standard WHO *in vivo* test. The present study was, therefore, undertaken to ascertain the susceptibility status of *P. falciparum* strain to chloroquine in Calcutta by WHO *in vivo* test.

An elderly (45 years) male postgraduate medical student of south Calcutta attended the Department of Microbiology of All India Institute of Hygiene and Public Health, Calcutta on 28.12.87 for investigation of fever with chill and rigor. His blood examination revealed the presence of *P. falciparum* rings, the density of which was found to be 270 per 300 leucocytes viz., 6750/l. He was given 600 mg chloroquine and 45 mg primaquine and kept under surveillance. His temperature came down to normal on day 2 but as he complained of uneasiness and malaise, his blood was examined again on day 7. The blood smear showed *P. falciparum* rings, the density of which was 135 per 300 leucocytes. After giving 600 mg chloroquine the day to day density of parasite over 7 days was not estimated as the case was not initially suspected to be chloroquine resistant. He was given 1500 mg chloroquine base for three consecutive days as per recommendation of WHO (1973). Before administering chloroquine his urine was examined by the technique of Dill and

Accepted for publication: 8 July 1988.

[1] All India Institute of Hygiene and Public Health
110, Chittaranjan Avenue
Calcutta - 700073, India.

Gilazko (Leijveld and Kortmann, 1970) for the presence of chloroquine but no chloroquine was detected. The patient was kept under conditions where reinfection with *P. falciparum* was not possible. The parasite count was not done as per WHO guidelines due to refusal of patient in giving blood daily for first 7 days. Therefore, his blood was collected on some selected days viz., day 3, 7 and 14 and urine on day 3. Urine showed presence of chloroquine on day 3. Blood smear examination showed complete clearance of rings on day 3 and day 7 and reappearance on day 14, the density of which was 12 per 300 leucocytes. Instead of extending the study for another 2 weeks he was given 3 tablets of metakelfin (each tablet contains 500 mg sulfamethopyrazine and 25 mg pyrimethamine) which showed good clinical response without further recrudescence till today. During the clinical attack of malaria, the patient had no history of diarrhoea and vomiting. He gave a history of two such episodes due to *P. vivax* malaria on 27.7.87 and 12.11.87 which were confirmed on blood smear examination. He was treated with 600 mg chloroquine and 75 mg primaquine for the first time and 600 mg chloroquine and 210 mg primaquine during the second attack.

The present case study has shown emergence of RI resistant *P. falciparum* in Calcutta after standard dose of chloroquine. The minimum possibility of reinfection with *P. falciparum* during the gap between disappearance of asexual parasites on day 3 and their reappearance on day 14 which falls within the incubation period was ruled out by keeping the patient under adequate preventive measures. Chloroquine resistant falciparum malaria has recently been reported in 6 (21%) out of 28 patients in Calcutta metropolitan area using standard WHO *in vitro* test (Sinha *et al.*, 1987). But there is no such report of chloroquine resistance of *P. falciparum* by WHO *in vivo* test. This is the first report of RI resistance of *P. falciparum* to chloroquine in Calcutta by *in vivo* test. The study has revealed the degree of chloroquine resistance of *P. falciparum* case.

The patient showed considerable reduction of parasites after single dose of 600 mg chloroquine but no complete clearance on day 7. Earlier studies in Koraput, Sambalpur and Bolangir districts of Orissa state in 1977 by Guha *et al.* (1979) showed that in *P. falciparum* infection parasitaemia failed to clear in 3.3% cases even after presumptive treatment with single dose of 600 mg chloroquine. Whether the refractory phenomenon of *P. falciparum* to chloroquine (600 mg) in the present study was due to inadequacy of drug or due to resistance was confirmed by the extended *in vivo* test after 1500 mg chloroquine administration. The recrudescence on day 14 corresponded with RI resistance. Circumstantial evidence suggests that it is a local strain of *P. falciparum* which has developed RI pattern of resistance to chloroquine and not an imported one as the patient did not move outside Calcutta during last one year before being attacked with *P. falciparum* malaria.

Further studies are indicated to assess the efficacy of chloroquine in *P. falciparum* malaria in Calcutta.

REFERENCES

1. Dwivedi, S.R., R.C. Rastogi, [illegible] and S. Bhatia (1981). A focus of chloroquine resistance in *Plasmodium falciparum* in Shaktinagar area of Mirzapur district, Uttar Pradesh. *Indian J. Malariol.*, 18:35-40.
2. Guha, A.K., R.G. Roy and S. Pattanayak (1979). Results of presumptive treatment of malaria in Orissa state. *Indian J. Med. Res.*, 70:52.
3. Leijveld, J. and H. Kortmann (1970). The cosin colour test of Dill and Gilazko: A simple field test to detect chloroquine in urine. *Bull. WHO*, 42:496.
4. Pattanayak, S., R.G. Roy and S. Sen (1979). Response to chloroquine with or without pyrimethamine in *Plasmodium falciparum* in West Bengal, Tripura, Mizoram, Manipur and Arunachal Pradesh. *Indian J. Med. Res.*, 70:48.
5. Sehgal, P.N., M.L.D. Sharma, S.L. Sharma and S. Gogai (1973). Resistance to chloroquine in falciparum malaria in Assam state, India. *J. Com. Dis.*, 5:175-180.

6. Sinha, S., D.S. Choudhury, S.K. Ghosh, C. Usha Devi and V.P. Sharma (1987). *In vitro* chloroquine resistant *Plasmodium falciparum* in Calcutta and its sensitivity to Qinghaosu (Artemisitene). *Indian J. Malariol.*, 24:107-109.

7. WHO (1973). Chemotherapy of malaria and resistance to antimalarials. *Tech. Rep. Ser.* 529.

8. WHS (1986). World Health Statistics, 39 (2):199-200.

Indian Journal of Malariology
Vol. 25, December 1988, 103 – 105.

A Note on *Anopheles dirus* Peyton and Harrison, 1979 [*An. balabacensis (sensu lato)* Baisas, 1936] in India

H.R. BHAT[1]

Christophers (1933) designated the Indian representatives of *Anopheles leucosphyrus* group as *An. leucosphyrus* of Donitz (1901). Under this he also placed *An. elegans* James, 1903 as a synonym. This status continued to be accepted until Kalra and Wattal (1962) split the taxon of Christophers into two distinct species, namely *An. balabacensis balabacensis* Baisas, 1936 and *An. elegans* James, 1903 on the basis of Reid (1949) and Colless (1956; 1957) after re-examination of specimens of the Malaria Institute of India. Their locality records of *An. balabacensis balabacensis* included western ghats from Sawantwadi southwards, eastern India east of Darjeeling district, Andaman Islands and Kasauli in Himachal Pradesh; and the locality records of *An. elegans* included western ghats from Sawantwadi southwards.

Recently Peyton and Harrison (1979) re-examined different variants of *An. balabacensis* designated by Colless (1956; 1957). On a critical comparison of Thailand population with those of typical population of *An. balabacensis* they raised the Thailand form to the status of a distinct species and named it *Anopheles dirus*. They delineated the geographical range of *An. dirus* within Thailand but indicated its possible occurrence elsewhere in mainland southeast Asia North of 8° latitude.

According to the previously accepted concept *An. balabacensis balabacensis* had a very wide geographic range extending almost all over the Oriental region, but this species was subsequently divided into different taxa by Colless (1956; 1957) and the typical form *An. balabacensis balabacensis* was restricted to Thailand, Burma, Assam and Bangladesh (Peyton and Harrison, 1979). In India, *An. balabacensis balabacensis* was reported to occur in the forested areas receiving heavy rainfall in eastern India and the western ghats in southwestern India. In the western ghat region *An. balabacensis balabacensis* was reported to be sympatric with *An. elegans*. There is a great gap in the geographic range across central India between the eastern and the western ghat region of southwestern India (Peyton and Harrison, 1979). However, there is a single record of a single specimen from Kasauli in the western Himalayas (Kalra and Wattal, 1962). Except this record, there has been no record of this species anywhere between Kasauli and Darjeeling district. Perhaps the Kasauli record is an error and the species does not occur West of Darjeeling district in northern India.

Accepted for publication: 12 October 1988.

[1] National Institute of Virology
Pune - 411001, India.

Subsequent to the description of *An. dirus*, on the suggestion of Dr. Peyton, the present author has examined 398 adult specimens of *An. balabacensis balabacensis (sensu lato)* collected from the field in Kyasanur forest disease area around Sagar, Shimoga district, Karnataka, between August 1978 and 1981. The field collected material included 173 males and 174 females reared from immatures collected from breeding places in the forests and betel nut gardens, 48 females collected on human baits, one male and one female collected from vegetation and one engorged female collected from a cattleshed. Sixty associated skins each of larvae and pupae were also examined along with the adults. In addition, several F_1 generation larvae, pupae and adults reared from the field collected females and three females collected at Sirsi on 19 January 1981 and 5 adult specimens designated as *An. balabacensis* from Defence Research Laboratory, Tejpur, collected in Mizoram (Malhotra *et al.*, 1984; Das and Baruah, 1985) were examined. They all conform with *An. dirus* (Peyton and Harrison, 1979) morphologically, and it is suggested that the Indian species which has been so far designated as *An. balabacensis balabacensis* be called *An. dirus* (Rao, 1984).

Moreover, recent cytogenetic and taxonomic studies have enabled the differentiation of *An. balabacensis* complex into at least seven genetic species in the southeast Asian region: *balabacensis*, *takasagoensis*, *dirus* A, B, C, D and E. These cryptic (sibling) species are only partially distinguishable morphologically, but each can be separated on the basis of karyotype analysis and cross-mating experiments. *An. balabacensis sensu stricto* is confined to the original locality in Balabac Island and neighbouring areas such as Palawan Island, Sabah and North Kalimantan (Peyton and Harrison, 1979), while another sibling species *An. takasagoensis* is restricted to Thailand (Peyton and Harrison, 1980). *An. dirus* is a species complex of at least five distinct genetic species provisionally designated: *dirus* A, B, C, D and E. *An. dirus* A is widespread in central and northern Thailand, Burma, eastern India, part of Bangladesh, Laos, Kampuchea, Vietnam and the adjoining part of P. R. China, while *dirus* B is confined to southern Thailand and the Thai-Malaysian border. Apparently *dirus* D occurs in central and southern Thailand towards central peninsular Malaysia and exists sympatrically with A, B and C forms. At present *dirus* C is known only from Kanchanaburi province, West Thailand.

The western ghat form in peninsular India is designated as *dirus* E (Baimai *et al.*, 1984; Peyton, personal communication). However, the status of Andaman population (Nagpal and Sharma, 1983) of *An. balabacensis* designate has not yet been determined.

An. balabacensis sensu lato is known to be an important vector of malaria. The discovery of *dirus* complex within *An. balabacensis* requires a critical re-assessment of the vector potential of each species for malaria.

REFERENCES

1. Baimai, V., C.A. Green, R.G. Andre, J.A. Harrison and E.L. Peyton (1984). Cytogenetic studies of some species complexes of *Anopheles* in Thailand and southeast Asia. *Southeast Asian J. Trop. Med. Pub. Hlth.*, **15**:536-546.
2. Christophers, S.R. (1933). *The fauna of British India, including Ceylon and Burma*, Diptera v 4.—Family *Culicidae*, Tribe *Anophelini*. (Taylor and Francis, London):271.
3. Colless, D. H. (1956). The *Anopheles leucosphyrus* group. *Trans. R. Entomol. Soc. Lond.*, **108**:37-116.
4. Colless, D.H. (1957). Further notes on the systematics of the *Anopheles leucosphyrus* group (Diptera: Culicidae). *Proc. R. Entomol. Soc. Lond., Ser. B.*, **26**:131-139.
5. Das, S.C. and J. Baruah (1985). Incrimination of *Anopheles minimus* Theobald and *Anopheles balabacensis balabacensis* Baisas (*A. dirus*) as Malaria vectors in Mizoram. *Indian J. Malariol.*, **22**:53-55.
6. Kalra, N.L. and B.L. Wattal (1962). A note on *Anopheles leucosphyrus* Donitz adults in the collection of the Malaria Institute of India with distribution

records of *An. balabacensis baisasi* Baisas and *Ano. elegans* James in India. *Bull. Nat. Soc. Ind. Mal. Mosq. Dis.*, **10**:159-167.

7. Malhotra, P.R., M. Bhuyan and I. Baruah (1984). Mosquitoes of Mizoram. *Indian J. Malariol.*, **21**:125-126.

8. Nagpal, B.N. and V. P. Sharma (1983). Mosquitoes of Andaman Islands. *Indian J. Malariol.*, **20**:7-13.

9. Peyton, E.L. and B.A. Harrison (1979). *Anopheles (Cellia) dirus* a new species of the *Leucosphyrus* group from Thailand. (Diptera: Culicidae). *Mosq. Syst.*, **11**:40-49.

10. Peyton, E.L. and B.A. Harrison (1980). *Anopheles (Cellia) takasagoensis* Morishita 1946, and additional species in the *balabacensis* complex of southeast Asia. (Diptera:Culicidae). *Mosq. Syst.*, **12**:335-347.

11. Rao, T. Ramachandra (1984). *The Anophelines of India*. (Rev. ed.). (Malaria Research Centre, ICMR, Delhi):518.

12. Reid, J.A. (1949). A preliminary account of the forms of *Ano. leucosphyrus* Donitz. *Proc. R. Entomol. Soc. Lond., Ser. B.*, **18**:42-53.

Indian Journal of Malariology
Vol. 25, December 1988, pp. 107 – 108.

Vertical Distribution of *Anopheles stephensi* Larvae in Calcutta

K.K. CHATTERJEE[1], D. BISWAS[1], G. CHANDRA[1], AMRITA BHATTACHARYYA[1] and A.K. HATI[1]

Historically, *Anopheles stephensi* is associated with transmission of malaria in Bombay, when Liston (1908) and Bentley (1910) for the first time associated malaria with this species. Subsequently Covell (1928), carried out a comprehensive survey of Bombay and studied in-depth the bioecology of this species. According to Covell (1928), *An. stephensi* breeds with equal facility in dark places and those exposed to direct sunlight and the larvae flourish in any depth. The breeding places may be situated below the level of the ground or on the roof of a building 80-100 ft (24.4-30.5 m) high. He identified among others, roof cisterns as one of the permanent breeding sites in Bombay along with improperly graded roof gutters and terraces. Generally large roof cisterns connected with mills and railways (situated at a great height) were invariably found breeding for this species. In Calcutta, again, the work of Covell (1932) was the first attempt to gather qualitative information on *An. stephensi*.

In Calcutta, the existence of *Anopheles stephensi* is known from the beginning of this century and the species has been incriminated (Siddons, 1946; Hati *et al.*, 1987) as a vector of malaria as in other cities of India. Precise information regarding the larval breeding of this vector mosquito in relation to height above the ground level is seriously lacking. Hence a systematic two-year search involving the ovitrap collection of *An. stephensi* larvae was made at different vertical sites of a multi-storied building with a view to elucidate its breeding habits at different heights above the ground.

Twelve earthen vats, each measuring about 18 cm in depth and 21 to 49 cm in cross section, to be used as ovitraps, were placed two at each of the different vertical situations of a five storied building, selected in an area in central Calcutta. Three-fourths of each ovitrap was always kept filled with tap water. Mosquito larvae from these ovitraps were collected at a regular interval of ten days for two consecutive years (September 1985 to August 1987). The collected larvae were identified and monthly records were kept on the number and species of mosquito larvae collected from each floor. Five days after each collection, additional visits were made to investigate whether the ovitraps were in proper condition or not. Altogether 50782 mosquito larvae were collected, of which, 14011, 35435 and 1336 were identified as the larvae of *An. stephensi*, *Aedes aegypti* and *Culex*

Accepted for publication: 27 October 1988.

[1] Department of Medical Entomology
Calcutta School of Tropical Medicine
Calcutta - 700073, India.

Table 1. Ovitrap collection of mosquito larvae at different heights of a multi-storied building during September 1985 to August 1987

Species	Height above the ground level						Total
	0 m	3.6 m	7.2 m	10.8 m	14.4 m	18.0 m	
Anopheles stephensi	126 (0.9)	971 (6.9)	1682 (12.0)	2537 (18.1)	5360 (38.2)	3335 (23.8)	14011
Aedes aegypti	19130 (53.9)	13363 (37.7)	2516 (7.1)	342 (0.9)	43 (0.1)	41 (0.1)	35435
Culex quinquefasciatus	890 (66.6)	357 (26.7)	89 (6.6)	0	0	0	1336

Figures in parentheses indicate the percentages of total individual larval species collected.

quinquefasciatus respectively. Out of 14011 *An. stephensi* larvae, 0.9%, 6.9%, 12.0%, 18.1%, 38.2% and 23.8% were collected at the ground, first, second, third, fourth floors and the roof respectively (Table 1), thereby indicating that *An. stephensi*, once a well-breeder (Afridi *et al.*, 1938; Russell and Rao, 1941), might be to some extent geophobic so far as its breeding habits are concerned as compared to *Ae. aegypti* and *Cx. quinquefasciatus*.

These observations should be helpful in formulating an effective integrated control strategy against the larvae of *An. stephensi* in sky-scrapers of big cities.

ACKNOWLEDGEMENTS

The authors are thankful to Prof. A.K. Bhattacharyya, Director, Calcutta School of Tropical Medicine for allowing them to publish this paper. Technical assistance given by Mr. A. Ghosh and Mr. G. Das is also acknowledged. The study received financial support from the Science and Technology Committee, Govt. of West Bengal.

REFERENCES

1. Afridi, M.K., S. Abdul Majid and J. Singh (1938). Malaria in Kutch state. *J. Mal. Inst. Ind.*, 1:187-213.
2. Bentley, C.A. (1910). The natural history of Bombay Malaria. *J. Bom. Nat. Hist. Soc.*, 20:392-422.
3. Covell, G. (1928). *Malaria in Bombay, 1928.* (Government Central Press, Bombay):113.
4. Covell, G. (1932). Malaria in Calcutta. *Rec. Mal. Surv. Ind.*, 3:1-82.
5. Hati, A.K., K.K. Chatterjee and D. Biswas (1987). Daytime resting habits of *Anopheles stephensi* in an area of Calcutta. *Indian J. Malariol.*, 24:85-87.
6. Liston, W.G. (1908). The present epidemic of malaria in the part of Bombay. *J. Bom. Nat. Hist. Soc.*, 18:872-881.
7. Russell, P.F. and T. Ramachandra Rao (1941). On seasonal prevalence of *Anopheles* species in South Eastern India. *J. Mal. Inst. Ind.*, 4:263-296.
8. Siddons, L.B. (1946). *Anopheles stephensi* as a vector of malaria in Calcutta. *J. Mal. Inst. Ind.*, 6:367-376.

Indian Journal of Malariology
Vol. 25, December 1988, pp. 109 - 111.

Mosquitoes of Daman

J. S. KHAMRE[1] and M. B. KALIWAL[2]

Information on the mosquito fauna of the Union Territory of Daman is very scanty. Only 3 species of *Anopheles viz., A. vagus, A. culicifacies, A. subpictus* and *Culex* spp. were recorded earlier (J.S. Khamre, unpublished report, 1982). There has been no other information on mosquito fauna of Daman. Daman is endemic for malaria and API is more than 2. DDT was sprayed from 1965 to 1971 under NMEP. BHC is being sprayed since 1972. Susceptibility tests were not done. Therefore, the studies were carried out to know complete mosquito fauna, relative abundance, role of suspected malaria vectors and susceptibility status to insecticides during September 1983 and the results are briefly summarised here.

Union Territory of Daman is bounded by Valsad district on the East, Arabian sea on the West, Bhagvan river on the North and Kalem river on the South. It has an area of 88 sq kms with 21 villages and one town. Population is 55,000. Most part of Daman is low land and little portion is plateau. Temperature varies from 22°C to 31°C. Annual rainfall is 1600 mm. Paddy is the main crop.

Accepted for publication: 7 December 1988.

[1] Regional Office
Health and Family Welfare
Pune - 411009, India.

[2] National Malaria Eradication Programme
Directorate of Health Services
Panaji - 403001, India

Mosquito collections were done by suction tube method from cattlesheds, human dwellings, mixed dwellings, other man-made structures, surrounding shrubs and nearby cattlesheds and human dwellings. The collections were done both by day (0600-1000 hrs) and night (2000-2400 hrs). Mosquitoes biting man and cattle were collected during night hrs. Larval collections were also taken from wells, cement tanks, water pools, pits, drums, etc. and were held in cages until adult emergence. Identification of the mosquitoes was done by using the keys of Barraud (1934), Christophers (1933), Puri (1955) and Rao (1984).

Susceptibility tests with healthy, uninjured and fully fed *A. culicifacies* adults were done against DDT 4%, dieldrin 0.4%, malathion 5% and fenitrothion 1% using WHO susceptibility test kit. Mosquitoes were exposed for 1 hr, keeping control sets and mortalities were recorded after a post-exposure period of 24 hrs. When, the mortality in the control groups was over 5% but less than 20%, a correction of mortality is made by applying Abbott's formula.

Table 1 gives the details of mosquito collection. A total of 494 mosquitoes were collected. Identification of the mosquitoes revealed that the mosquito fauna of Daman comprised of 9 species belonging to 3 genera viz., (i) 475 specimens of genus *Anopheles* consisting of 5 species, (ii) 12

Table 1. Results of mosquito collection in Daman

S.No.	Species collected	No. collected	PMHD
1.	*Anopheles annularis* Van der Wulp, 1884	3	0.15
2.	*A. barbirostris* Van der Wulp, 1884	27	1.32
3.	*A. culicifacies* Giles, 1901	228	11.12
4.	*A. stephensi* Liston, 1901	4	0.20
5.	*A. subpictus* Grassi, 1899	213	10.39
6.	*Culex gelidus* Theobald, 1901	4	0.20
7.	*C. tritaeniorhynchus* Giles, 1901	8	0.39
8.	*Mansonia annulifera* (Theobald), 1901	2	0.10
9.	*M. indiana* Edwards, 1930	5	0.24
	Total	494	-

Table 2. Results of adult susceptibility tests with *A. culicifacies*

Insecticide Conc.	No. of replicates	No. exposed	% Mortality
DDT 4 %	3	50	32.0
Dieldrin 0.4 %	3	40	42.5
Malathion 5 %	3	45	11.1
Fenitrothion 1 %	1	15	100.0

specimens of genus *Culex* consisting of 2 species and (iii) 7 specimens of genus *Mansonia* consisting of 2 species. It may be noted that *A. culicifacies* (46.2%) and *A. subpictus* (43.1%) were the most prevalent species in Daman. *A. barbirostris* (5.46%), *A. stephensi* (0.8%) and *A. annularis* (0.6%) were collected in small numbers. Two *Culex* species viz., *C. tritaeniorhynchus* and *C. gelidus* formed only 2.43%. *Mansonia annulifera* and *M. indiana* formed 1.41%.

Results of the susceptibility tests with *A. culicifacies* are summarised in Table 2. It is evident from the data that there was only 32% mortality with DDT 4%, 42.5% mortality with dieldrin 0.4%. The vector was highly resistant to malathion and susceptible to fenitrothion. Resistance to DDT, dieldrin and malathion has been reported from the neighbouring states of Gujarat (Rajagopal, 1977) and from Maharashtra (Vittal *et al.*, 1982). The resistance to DDT and dieldrin is due to the use of DDT and HCH under NMEP for a long period. Resistance to malathion may be attributed to its use in agriculture.

The mosquito fauna of Daman comprises of known malaria vectors viz., *A. annularis*, *A. culicifacies* and *A. stephensi*. Mosquito collections carried out in Daman had reported only 4 *A. culicifacies* and mass survey in 2 villages, one with high incidence and the other with low incidence revealed that all 225 blood smears covering all age groups, collected and examined were found negative for malaria parasite during April (J. S.

Khamre, unpublished report, 1982) indicating the absence of active transmission. In the present survey, a large number of *A. culicifacies* were collected and the analysis of monthwise cases revealed that maximum cases occurred during August–October months when the density of *A. culicifacies* is high and the incidence was practically low during the rest of the year. *A. stephensi* is an urban vector and *A. annularis* a known vector of local importance elsewhere in India (Rao, 1984). But, these two species were collected in small numbers. Though dissections were not done to incriminate the vector, the role of *A. culicifacies* in malaria transmission in Daman, cannot be ruled out.

ACKNOWLEDGEMENTS

We are grateful to Dr. Estibeiro, Director of Health Services for Goa, Daman and Diu, Dr. J.M. Pereira, CMO (Malaria) and Dr. Bhattacharya, Regional Director (H & FW), Pune for their encouragement and kind help for carrying out the present studies.

REFERENCES

1. Barraud, P.J. (1934). *The fauna of British India including Ceylon and Burma*, v. 5. (Taylor and Francis, London): 1-463.
2. Christophers, S.R. (1933). *The fauna of British India including Ceylon and Burma*, v. 4. (Taylor and Francis, London): 1-360.
3. Puri, I.M. (1955). Synoptic table for the identification of the anopheline mosquitoes of India. *Health Bulletin No. 10, IV ed.* (Manager of Publications, Delhi).
4. Rajagopal R. (1977). Malathion resistance in *A. culicifacies* in Gujarat. *Indian J. Med. Res.*, **66**: 27-28.
5. Rao, T. Ramachandra (1984). *The Anophelines of India*. (Rev. ed.) (Malaria Research Centre, ICMR, Delhi): 518.
6. Vattal, M., S.M. Mustafa, R.B. Deobhankar, L.B. Deshpande and R.R. Deo (1982). Insecticide susceptibility status of malaria vectors in Maharashtra. *Indian J. Malariol.*, **19**(1): 59-61.

Indian Journal of Malariology
Vol. 25, December 1988, pp. 113 – 116.

Epidemiological Implications of Population Migration: Part I. Imported Malaria in Kheda district, Gujarat

R.C. SHARMA[1] and V.P. SHARMA[2]

In Kheda district, Gujarat, agriculture is well developed due to fertile soil, good annual rainfall and an excellent network of canals and tributaries. Almost all villages of the district have an approach road and small industrial units are commonly found in the area. The district attracts labour mainly from backward areas of Gujarat and some other neighbouring states. This labour often brings malaria infection from endemic areas, a factor of human behaviour and ecology that has not received sufficient attention in the past by malariologists (Prothero, 1983). The project on bioenvironmental control of malaria was started in 1983 in 7 villages (population 26,000) and subsequently extended in 1986 to cover the entire Nadiad taluka comprising of 100 villages (population 3,50,000). During epidemiological investigations it was revealed that immigrant labour had high incidence of malaria. Results of a 4-year study on the prevalence of malaria in experimental villages is reported in this paper.

Accepted for publication: 13 December 1988

[1] Malaria Research Centre (Field Station)
Civil Hospital
Nadiad-387001, India.

[2] Malaria Research Centre
22-Sham Nath Marg
Delhi-110054, India.

The study was taken up in Nadiad taluka as part of the evaluation of the impact of intervention measures under the bioenvironmental control strategy (Sharma *et al.*, 1986). House-to-house surveillance of fever cases was done on weekly basis by the resident worker(s) of the village. In case of fever, blood smear was prepared and presumptive treatment of 600 mg chloroquine adult dose was given. All blood smears were brought to the laboratory for examination. Persons positive for *P. falciparum* malaria were given additional 900 mg chloroquine adult dose (600 mg on day 1 along with 45 mg primaquine and 300 mg on day 2). *P. vivax* adult positive cases were given 75 mg primaquine (15 mg daily for 5 days). Children received proportionately low dosages. Surveillance workers also recorded all population movement regarding the incoming guests and labour etc. In addition to this, all positive cases were investigated about their movement. In case a person was found positive for malaria and had spent 7 nights outside the experimental area and diagnosed positive within 10 days of his return he was considered to have brought in malaria from the place of his visit. Mass blood surveys of incoming labour were carried out with the people's cooperation and slides examined for malaria. The place from where the labour had come was

Table 1. Analysis of imported cases in Kheda

Year	Population covered	Indigenous cases			Imported cases			Origin of imported cases		
		Total	Pf	API	Total	Pf	API	Kheda district	Other districts of Gujarat	Other states of India
1984	26,000	141	64 (45.4)	2.5	72	45 (62.5)	6.9	50 (69.4)	21 (29.2)	1 (1.4)
1985	60,000	154	52 (33.7)	0.9	90	24 (40.0)	1.6	31 (51.7)	28 (46.7)	1 (1.6)
1986	3,50,000	626	211 (33.7)	0.6	312	188 (60.3)	2.1	124 (39.7)	153 (49.1)	35 (11.2)
1987	3,50,000	1287	371 (28.8)	1.1	954	573 (60.1)	6.5	187 (19.6)	653 (68.5)	114 (11.9)
	Total	2208	698 (31.6)		1398	830 (59.4)		392 (20.0)	855 (61.2)	151 (10.8)

Table 2. Monthwise parasite distribution in migrant population in Kheda

Month 1987	B.S. examined	Total cases	Pf +ve	SPR	SFR
Jan	481	45	43	9.35	8.93
Feb	547	33	27	6.03	4.94
Mar	238	3	2	1.26	0.84
Apr	330	17	12	5.15	3.64
May	961	29	22	3.01	2.29
Jun	135	6	3	4.44	2.22
Jul	414	25	17	6.03	4.11
Aug	391	26	19	6.65	4.86
Sep	464	17	10	3.66	0.22
Oct	630	49	22	7.78	3.49
Nov	1433	112	72	7.81	5.02
Dec	1210	141	106	11.65	8.76
Total	7234	503	355	6.95	4.91

recorded but no separate record of labour and other travellers was maintained for the years 1984-86.

Table 1 provides information on the high rate of *P. falciparum* in cases coming from outside the study areas. Increase in the total number of malaria cases from 1984-87 was due to the expansion of the project area from original 7 villages in 1984 to 100 villages in 1986. This was also reflected in the increase of imported cases. Four years of study showed that in a total of 2208 indigenous cases 31.6% were *P. falciparum*, whereas *P. falciparum* percentage among imported cases was higher i.e, 59.4%. Origin of imported cases revealed that in the years from 1984-87, 69.4%, 51.7%, 39.7% and 19.6% cases respectively originated from Kheda district. The proportion of cases that originated from other districts of Gujarat were 29.2%, 46.7%, 49.1% and 68.5% respectively, during the same period. The proportion of cases originating from other states was 1.4% in 1984 and 1.6% in 1985. It increased to 11.2% and 11.9% in 1986 and 1987 respectively. One of the interesting observations from Table 1 is that the API in migrant labour is 2 to 5 times higher during the period of observation.

Table 2 shows blood smear collection and positives for 1987. It was revealed that in migrant population SfR and SPR was low in February, March, June and September showing that epidemiologically first group of migrants do not show high incidence of malaria but subsequent batches bring higher infection. *P. falciparum* rate is low in September commensurate with general trend of species distribution in the area.

For comparison observations of 1986 and 1987 were important because the same area and population were available for analysis. Results presented in Table 1 indicate that the proportion of *P. falciparum* cases among indigenous cases reported declined from 33.7% in 1986 to 28.8% in 1987. However, *P. falciparum* percentage among the imported cases remained constant at 60% level during the same period. Increase in the number of imported cases in 1987 could perhaps be due to the successive years of drought in Gujarat, which was severe in 1987 resulting in large-scale migration of labour to those districts where conditions were better. For example, conditions in Kheda district were far better in spite of scanty rainfall due to the well developed canal and tubewell irrigation facilities. This becomes evident from the fact that from other districts of Gujarat the percentage of imported malaria cases in the project area rose considerably from 49.1% in 1986 to 68.5% in 1987. Contributions from other states remained between 11 and 12% in both the years.

Observations on imported malaria through labour and other travellers (Table 3) revealed that in 1987

Table 3. Classification of migrants, Kheda (1987)

Category	Total cases	Pf cases	% Pf
Labourers	503 (52.72%)	355	70.6
Other travellers	451 (47.27%)	218	48.3
Total	954	573	60.1

Table 4. Activities influencing labour movement in the experimental villages

	Nature of Work	Labour		Duration of stay in months
		Arrival	Departure	
1.	Road construction	September	June	10
2.	Paddy cultivation	February	May	4
		July	October	4
3.	Tobacco cultivation	July	October	4
		November	April – May	6 – 7
4.	Chicory cultivation	November	April – May	6 – 7
5.	Wheat harvesting	February	March	2
6.	Brick making	February	May – June	4 – 5

both labour and travellers contributed almost equally i.e., 52.72% and 47.27% respectively, and out of a total of 954 cases 573 (60.1%) were *P. falciparum*. Proportion of *P. falciparum* was found to be 70.6% in labourers against 48.3% among other travellers.

To identify the type and period of attraction of labour in the project area investigations were made so that the labour camps could be followed for screening and treatment and also to ascertain and control the epidemiological risk. Results of investigations have been presented in Table 4 which indicated that labourers are mainly attracted towards road construction work, paddy, tobacco and chicory cultivation, wheat harvesting and brick making. Labour starts coming in the project area in the month of February for brick making and leaves in May/June before rains. Agricultural labourers come to Kheda for paddy cultivation in the months of February and July and leave in the months of May and October respectively, as two crops of paddy are taken in a year. Road construction activities start in the month of September and labourers remain engaged in the work for about 10 months till June. November is the month for tobacco and chicory cultivation when labour comes to work in the fields and leaves in April/May after about 7 months of stay. Wheat harvesting in February/March attracts labour for two months. Thus, the influx of labour continues almost throughout the year for different jobs and moves from village to village. There are no records of population movement but it may be anywhere between 20-30% of the resident population. Thus, population migration is an important source of infection in the event of non-detection.

ACKNOWLEDGEMENT

Authors are thankful to Mr. S.M. Banerjee for technical assistance during the course of studies.

REFERENCES

1. Prothero, R.M. (1983). Malaria: socio-demographic and geographical problems. In *Proceedings of the Indo-UK Workshop on Malaria*, edited by Dr. V.P. Sharma. (Malaria Research Centre, ICMR, Delhi): 41-52.

2. Sharma, V. P., R. C. Sharma and A. S. Gautam (1986). Bioenvironmental control of malaria in Nadiad, Kheda district, Gujarat, *Indian J. Malariol.*, 23(2): 95-137.

Indian Journal of Malariology
Vol. 25, December 1988, pp. 117 – 118.

Epidemiological Implications of Population Migration: Part II. Evidence of Chloroquine Resistant *Plasmodium falciparum* Malaria in Kheda district, Gujarat

R.C. SHARMA[1] and V.P. SHARMA[2]

A demonstration project on the bioenvironmental control of malaria was launched in 1983 in Nadiad taluka, Kheda district, Gujarat. This area was prone to malaria epidemics and in 1981 an outbreak of falciparum malaria resulted in high morbidity and deaths in some villages, although insecticides (DDT and malathion) were being sprayed by the NMEP to interrupt transmission. The study was initially launched in 7 villages (population 26,000) in 1983 and gradually extended to cover Nadiad and Kapadwanj talukas by 1986 (Sharma *et al.*, 1986). An important component of the study was weekly active surveillance, rapid slide examination and administration of radical treatment within 24-36 hrs. Surveillance was carried out by project staff who belonged to the same village. As a result performance of passive surveillance was very good and radical treatment and follow-up of cases was easy and more reliable. At present chloroquine and primaquine are being used, the former clears asexual and sexual forms of *P. vivax* and the latter provides radical cure by the elimination of the hypnozoites of *P. vivax*. In *P. falciparum* chloroquine is used as a schizontocidal and primaquine as a gametocytocidal.

In vivo P. falciparum resistance was monitored following the WHO (1973) extended field test for 28 days. In the project area fever cases were given 600 mg chloroquine as presumptive treatment. Soon after the results of slide examination, *P. falciparum* cases were given a total dose of 1500 mg chloroquine (600 mg on day 0 as presumptive followed by 600 mg on day 1 and 300 mg on day 2) and 45 mg primaquine. Children were given proportionately low dosages. All *P. falciparum* cases were monitored on day 4, 7 and then weekly upto day 28 to record any recrudescence. All chloroquine resistant cases were treated with 2 tablets of metakelfin and 45 mg primaquine and followed for four weeks. This was done keeping in view the fact that *P. falciparum* resistance to chloroquine has been reported from various parts of India (Sharma, 1984). In the project area all

Accepted for publication 13 December 1988.

[1] Malaria Research Centre (Field Station)
Civil Hospital
Nadiad-387001, India.

[2] Malaria Research Centre
22-Sham Nath Marg
Delhi-110054, India.

Table 1. *P. falciparum* **resistance to chloroquine in Kheda district**

Year	Population	Positive cases		Results (level of resistance)			Total Pf resistant cases*
		Total	Pf	RI	RII	RIII	
1984	26,000 **	141	64	10	0	0	10
1985	60,000 **	154	52	2	0	0	2
1986	3,50,000 **	626	211	16	0	0	16
1987	7,00,000 ***	1938	511	75	0	0	75
Total		2859	838	103	0	0	103

* Children upto 5 years of age have been excluded ** Nadiad taluka. *** Nadiad and Kapadwanj talukas.

cases of drug resistance were to be detected and given radical cure to prevent further dissemination of the parasite.

A record of *P. falciparum* resistant cases from among the total cases is given in Table 1. In 1984, in a population of 26,000, a total of 141 malaria cases were recorded out of which 64 were *P. falciparum*. Ten *P. falciparum* cases did not respond to 1500 mg chloroquine and parasitaemia reappeared after 7 days and within 28 days of chloroquine therapy. Similarly, 2 cases did not respond to chloroquine in 1985. In 1985 the area was further extended to cover the entire Nadiad taluka comprising of 3,50,000 population. A total of 211 *P. falciparum* cases were detected in 1986 and out of these 16 showed chloroquine resistance. Similarly, in 1987 when the area was extended to cover Kapadwanj taluka a total of 511 *P. falciparum* cases were detected in 0.7 million population. Seventy five of these showed resistance. In terms of percentage there were 15.62% Pf cases resistant to chloroquine in 1984 and 3.85%, 7.58% and 14.68% in 1985, 1986 and 1987 respectively. Thus, in the study area out of 838 *P. falciparum* cases detected, there were 103 chloroquine resistant cases producing an average of 12.3 from 1984-87.

In all cases i.e., 103 *P. falciparum* resistant cases the parasitaemia cleared within 7 days of the administration of chloroquine and reappeared before day 28. Thus, all the cases showed RI level of resistance.

From the foregoing, it is evident that the problem of chloroquine resistance in *P. falciparum* in Kheda district has started surfacing and there is need of careful monitoring and proper treatment. There was no resistance to metakelfin but the widespread use of sulpha drugs (metakelfin/fansidar) without slide examination may shorten the life of these useful drugs. There is, therefore, need of more widespread monitoring of drug resistance in the country and eradication of foci to prevent further build-up of cases resistant to one or more antimalarials.

ACKNOWLEDGEMENT

Authors are thankful to Mr. S.M. Banerjee, Technical Assistant for his help during the course of the studies.

REFERENCES

1. Sharma, V.P. (1984). Drug resistant *Plasmodium falciparum* malaria in India. In *Proceedings of the Indo-UK Workshop on Malaria*, edited by Dr. V.P. Sharma. (Malaria Research Centre, ICMR, Delhi): 169-184.
2. Sharma, V.P., R.C. Sharma and A.S. Gautam (1986). Bioenvironmental control of malaria in Nadiad, Kheda district, Gujarat. *Indian J. Malariol.*, 23(2): 95-117.
3. WHO (1973). Chemotherapy of malaria and resistance to antimalarials. *Tech. Rep. Ser.*, 529.

Indian Journal of Malariology
Vol. 25, December 1988, pp. 119—124.

Synthesis and Evaluation of 4, 6-Diamino-1, 2-Dihydro-2, 2'-Dialkyl or Aryl-1-(substituted)-s-Triazines as Antimalarial Agents

ZARINE KHETY[1], VIJAYLAXMI KAMATH[2], D.M. RENAPURKAR[2] and B.G. KHADSE[1]

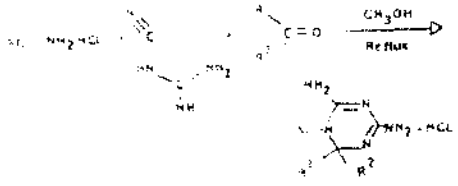
Many new compounds, structurally modified at the 1 and 2 position of the s-triazine moiety, were synthesized from basic raw materials. These were tested for their antimalarial activity against *P. berghei* in mice. Two compounds showed 100% elimination of *P. berghei* infection in mice for more than 25 days at a dose of 5 mg/kg × 4. The results are presented and discussed.

Dihydro-s-triazines are reported as antimalarial agents (Osdene *et al.*, 1967). In our synthesis programme, compounds of the type 4, 6-diamino-1, 2-dihydro-2, 2'-dimethyl-1-(p-substituted) phenyl/heterocyclic/allycyclic-s-triazines have been synthesized and screened against *P. berghei* by Rane's method and Peter's 4 days test (1965) for blood schizontocidal action. The general scheme for synthesis of compounds is described below.

Chemistry

The s-triazine compounds were prepared in 45 to 55% yield by the three component synthesis of Modest (1956). The required arylamines were prepared by known methods in the literature. The heterocyclic amines and adamantyl were available from the laboratory. Representative s-triazine hydrochlorides were studied for IR, UV, NMR spectra which are in agreement with the assigned structures. All the compounds were checked by the elemental analysis of C, H and N.

Mode of synthesis

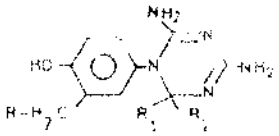


Accepted for publication: 22 December 1988.

[1] Department of Chemotherapy
Haffkine Institute
Parel
Bombay-400012, India.

[2] Department of Zoonoses
Haffkine Institute
Parel
Bombay-400012, India.

Table 1. Synthesis of 6-cresol-3-(4,6-diamino-2,2'-dialkyl or aryl-1, 2-dihydro-s-triazine)- α- substituted amino methyl



No	R	R_1	R_2	M.p. °C	Yield %	Mol. formula	Elemental analysis					
							Calculated %			Found %		
							C	H	N	C	H	N
1.	$N(C_2H_5)_2$	CH_3	CH_3	255	56.0	$C_{16}H_{26}N_6O.2HCl$	49.35	7.19	21.59	49.29	7.35	20.80
2.	$N(C_2H_5)_2$	CH_3	C_2H_5	245	43.2	$C_{17}H_{28}N_6O.2HCl$	50.37	7.46	20.74	50.51	7.44	20.39
3.	$N(C_2H_5)_2$	CH_3	$CH_3(CH_2)_4$ - CH_3	Oily	-	$C_{21}H_{36}$ $N_6O.2HCl$	54.66	7.83	18.22	54.29	7.52	18.42
4.	$N(C_2H_5)_2$	H	C_6H_5	165	28.0	$C_{20}H_{28}N_6O.2HCl$	52.52	5.92	18.38	52.24	5.36	18.69
5.	$N(C_2H_5)_2$	H	$p\text{-}N(CH_3)_2$ C_6H_4	-	26.0	$C_{22}H_{31}N_7O.2HCl.$ H_2O	52.80	6.60	19.60	52.51	6.74	19.66
6.	$N(C_2H_5)_2$	H	(Cl) (OH) (OCH_3) C_6H_5		19.9	$C_{21}H_{27}N_6ClO_3$ $2HCl.H_2O$	46.88	5.39	15.63	46.61	5.30	15.56
7.		CH_3	C_2H_5	240	20.5	$C_{17}H_{26}N_6O.2HCl$	50.63	6.45	20.84	50.45	6.32	21.20
8.		CH_3	C_6H_5	264	48.6	$C_{20}H_{24}N_6O.2HCl$	55.05	5.50	19.26	55.32	5.72	19.39

Where Ar = (i) Substituted aromatic;
(ii) Heterocyclic;
(iii) Allycyclic.

4, 6-Diamino-1, 2-dihydro-2, 2'-dimethyl-1-p-(p-chlorobenzyloxy) phenyl-s-triazine hydrochloride

A mixture of 4-chloro 4'-aminodiphenyl benzyl ether hydrochloride 0.5 gm (0.001 m), dicyandiamide 0.21 gm (0.0025 M), dry methanol (1.5 ml) and acetone (10-15 ml) was stirred and refluxed for 6 hrs. under anhydrous conditions. The reaction mixture became a clear brownish solution within 30 mins. The reaction was completed after 17 hrs, cooled, the product collected by filtration and washed with acetone and ether. It was recrystallised from aq. CH_3OH to obtain pale pink prismatic crystals of the title compound.

Yield 0.270 gm (85.6%), m.p. 234°C.
Elemental analysis for $C_{18}H_{20}ClN_5O.HCl$:
Calculated C, 54.82; H, 5.07; N, 17.17%
Found C, 54.65; H, 5.28; N, 17.03%

o-Cresol-4-(4, 6-diamino-2-phenyl-1, 2-dihydro-s-triazine)-α-diethyl amino methyl hydrochloride

3-Diethylamino methyl-4-hydroxy aniline hydrochloride 0.89 gm (0.003 M) was condensed with 0.280 gm (0.0033 M) of dicyandiamide, 0.53 gm (0.005 M) of benzaldehyde and 1-2 ml of sodium-dry methanol. The reaction mixture was refluxed at 60-70°C for 5-6 hrs under anhydrous conditions. The dark solution was then treated with charcoal, filtered and concentrated. The oily residue obtained was triturated with ether and refrigerated for over a week to afford a greyish black solid. The UV absorbance of the compound showed a max. at 236μ indicating the presence of an s-triazine as per the assigned structure (Table 1).

Yield 0.42 gm (28.0%), m.p. 165°C.
Elemental analysis for $C_{20}H_{26}N_6O.2HCl.H_2O$:
Calculated C, 52.52; H, 6.13; N, 18.38%
Found C, 52.24; H, 6.36; N, 18.69%

4, 6-Diamino-1, 2-dihydro-2, 2'-dimethyl-1-(1-adamantyl)-s-triazine hydrochloride

1.87 gm (0.01 M) of 1-amino adamantane hydrochloride was dissolved in sodium dry ethanol (5ml) and placed in a 3-necked flask fitted with a mechanical stirrer, a condenser with a calcium chloride guard tube and a stopper. 1.092 gm (0.013 M) of cyanoguanidine was added to the flask together with excess of acetone and refluxed on steam-bath with stirring for 12 hrs. A solid white crop precipitated from the homogenous reaction mixture, which was found to be quite pure (Table 2).

Crystallization from aq. methanol provided an analytically pure sample.

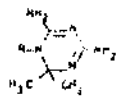
Yield 1.15 gm (36.8%), m.p. 174°C (uncorr.).
Elemental analysis for $C_{15}H_{26}N_5.HCl$:
Calculated C, 57.60; H, 8.64; N, 22.40%
Found C, 57.42; H, 8.46; N, 22.15%

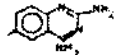
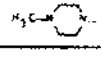
4, 6-Diamino-1, 2-dihydro-1-[1-(phenoxy) adamantyl] -2, 2'-dimethyl-s-triazine hydrochloride

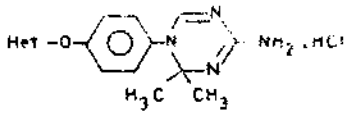
1.0 gm (0.0036 M) of 4-(adamantyloxy) aniline hydrochloride, 0.37 gm (0.0044 M) of cyanoguanidine, concentrated hydrochloric acid 0.146 gm (0.004 M) (0.178 Ml), and dry acetone 20-25 ml was refluxed on a water bath for 20 hrs, until a light purple coloured solution was obtained. The reaction solution was treated with charcoal, filtered and concentrated. On standing for two days pink flakes of the title compound were obtained. The flakes were collected, washed with ether and a very small quantity of alcohol to obtain a white product of analytically pure grade.

Yield 0.82 gm (57.2%), m.p. 236°C.
Elemental analysis for $C_{15}H_{22}N_6O.HCl$:
Calculated C, 62.45; H, 7.19; N, 17.34%
Found C, 62.39; H, 7.10; N, 17.52%

Table 2. Synthesis of 6, 6-diamino-1, 2-dihydro-4, 2-dimethyl-s-triazines with heterocyclic substituents at 1 position of the triazine ring



No	R	M.p. °C (uncorr.)	Yield %	Mol. formula	Elemental analysis					
					Calculated %			Found %		
					C	H	N	C	H	N
1.		174	36.8	$C_{15}H_{26}N_5.HCl$	57.60	8.64	22.40	57.42	8.46	22.15
2.		285	50.2	$C_{13}H_{17}N_9.3HCl$	58.19	4.13	30.84	58.29	4.26	30.81
3.		239	48.0	$C_{14}H_{16}N_6.HCl$	55.26	5.26	27.63	55.32	5.09	27.57
4.		216	62.1	$C_{14}H_{16}N_6.HCl$	55.26	5.26	27.63	55.07	5.49	27.47
5.		210	72.0	$C_{10}H_{21}N_6.3HCl.2H_2O$	28.77	6.23	23.50	28.52	6.45	23.21



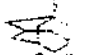
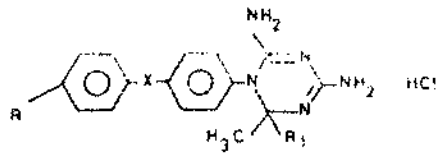
No.	Het	M.P. °C	Yield	Mol. formula	Elemental analysis					
					Calculated %			Found %		
					C	H	N	C	H	N
6.		182	52.0	$C_{16}H_{18}N_6O.2HCl$	55.41	5.19	22.04	55.71	5.28	22.19
7.		245	41	$C_{15}H_{17}N_7O.2HCl.H_2O$	49.18	5.46	24.24	49.51	5.72	24.63
8.		210	43.6	$C_{15}H_{15}N_7OCl_2.2HCl$	43.12	3.32	23.60	43.56	3.12	23.38
9.		260	57.2	$C_{21}H_{29}N_5O.HCl$	62.45	7.19	17.34	62.39	7.10	17.52
10.		284		$C_{20}H_{20}N_6O.2HCl.2H_2O$	57.90	5.30	20.20	57.81	5.55	20.00

Table 3. Synthesis of 4,6-diamino-1,2-dihydro-2,2'-dimethyl-1-(substituted)-phenyl-s-triazine hydrochloride



No	R	X	R_1	M.p. °C (uncorr.)	Yield %	Mol. formula	Elemental analysis					
							Calculated %			Found %		
							C	H	N	C	H	N
1.	Cl	CH_2O	CH_3	234	85.6	$C_{18}H_{20}N_5ClO.HCl$	54.82	5.08	17.17	54.65	5.28	17.03
2.	Br	CH_2O	CH_3	225	15.2	$C_{18}H_{20}N_5BrO.HCl$	49.25	4.56	15.96	49.15	4.73	15.69
3.	Cl	-O-	CH_3	215	57.0	$C_{17}H_{18}N_5ClO.HCl$	52.44	4.88	17.99	52.82	5.01	17.62
4.	3,4-Dichloro	-O-	CH_3	221	57.5	$C_{17}H_{17}N_5Cl_2O.HCl$	47.16	4.16	16.18	47.31	4.28	16.48
5	NO_2	-O-	CH_3	212	22.3	$C_{17}H_{18}N_6O_3.HCl$	52.24	4.16	21.52	52.48	4.39	21.82
6	$NHCOCH_3$	O	CH_3	242	63.6	$C_{19}H_{22}N_6O_2.HCl$	56.71	5.47	20.89	56.69	5.69	20.72
7.	$NHCOOCH_3$	-S	C_3H_7	Oily	29.3	$C_{20}H_{24}N_6O_2S.HCl$	53.51	5.35	18.72	53.62	5.42	18.62
8.	$NHCOOCH_3$	-S-	CH_3	215	75.6	$C_{19}H_{22}N_6O_2S.HCl$	52.46	5.29	19.32	52.09	5.08	19.50

Primary screening (Rane's method)

Normal albino male mice weighing 22-25 gms were inoculated intraperitoneally with 1×10^6 parasitised red blood cells of *P. berghei* infected mice. Test drugs were suspended in distilled water and Tween-80, and injected subcutaneously 72 hrs after inoculation.

A group of 5 mice was taken for each dose level and one group kept as control which died normally between 6 and 7 days.

Those test compounds where the animals survived almost twice the infected untreated controls were considered active.

Secondary screening

Evaluation by the Peter's 4-day suppressive test was carried out. Those compounds which showed activity by the Rane's single dose test were subjected to a divided dose regimen to observe the suppressive blood schizontocidal activity. The test compounds were given by both oral and subcutaneous routes starting from the day of infection. Blood smears were taken on the fourth, sixth, seventh, ninth day, etc. upto fourteenth day (of the surviving animals) and per cent parasitaemia noted.

Structure activity relationship

Among all the synthetic triazines, it was found that the gem-dimethyl grouping is the most essential feature in symmetrical triazine required for antimalarial activity, and if it is changed to methyl or ethyl the activity is *nil*.

Among the N-substituted grouping it was found that simple phenyl grouping is better than substituted or N-heteroaryloxy or N-hetero group like quinoline, quinazoline or N-methyl piperazine.

The most promising compounds among these s-triazine series were found to be 4, 6-diamino-1, 2-dihydro-2, 2'-dimethyl-1-(p-chlorobenzyloxy phenyl)-s-triazine and 4, 6-dimethyl-1, 2-dihydro-2, 2'-dimethyl-1-(p-bromobenzyloxy phenyl)-s-triazine.

These two compounds possess promising antimalarial activity at 5 mg/kg and 2.5 mg/kg dose by subcutaneous or post-operative route. Among these two promising compounds (Table 3, compd. no.2) is better and has low toxicity i.e., LD_{50} value/kg (1650 mg/kg).

Thus, the p-bromobenzyloxy phenyl grouping along with the symmetrical triazine with gem-dimethyl group at one position is the most favourable combination.

ACKNOWLEDGEMENTS

The authors are indebted to Director, Haffkine Institute, Bombay, for providing facilities. They are also thankful to Mr. M.T. Jaokar and Mr. D.S. Achrekar for the elemental analysis. One of the authors (ZK) thanks the Lady Tata Memorial Trust, Bombay, for the award of a Junior Research Scholarship.

REFERENCES

1. Modest, E.J. (1956). Chemical and biochemical studies on 1, 2-dihydro-s-triazines. II. Three component. *Synthesis*, 27 (1):1-13.
2. Osdene, T.S., P.P. Russell and L. Rane (1967). 2,4-7-Triamino-6-ortho-substituted aryl pteridines--A new series of potent antimalarial agents. *J. Med. Chem.*, 10:431-434.
3. Peters, W. (1965). Drug resistance in *Plasmodium berghei*, Vincke and Lips, 1948. I. Chloroquine resistance. *Exp. Parasitol.*, 17: 80-89.

INDIAN JOURNAL OF MALARIOLOGY

Editorial Acknowledgement

The Editor gratefully acknowledges the help of the following scientists who have kindly reviewed the papers for the 1988 issues of the Indian Journal of Malariology.

Dr. S.S. Agarwal, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow; Dr. A.P. Bhaduri, Central Drug Research Institute, Lucknow; Dr. Alok Bhattacharya, Jawaharlal Nehru University, New Delhi; Dr. D.S. Choudhury, Malaria Research Centre, Delhi; Dr. S. Chatterjee, Jawaharlal Nehru University, New Delhi; Dr. C.M.S. Dass, Delhi University, Delhi; Dr. G.P. Dutta, Central Drug Research Institute, Lucknow; Dr. N.K. Ganguly, Postgraduate Institute of Medical Education and Research, Chandigarh; Dr. T.N. Ghosh, Calcutta School of Tropical Medicine, Calcutta; Dr. N.L. Kalra, National Malaria Eradication Programme, Delhi; Dr. K.V. Kondrashin, World Health Organisation, New Delhi; Dr. A.N. Malaviya, All India Institute of Medical Sciences, New Delhi; Dr. K.N. Mehrotra, Indian Agricultural Research Institute, New Delhi; Dr. Indira Nath, All India Institute of Medical Sciences, New Delhi; Dr. M.V.V.L. Narasimham, National Malaria Eradication Programme, Delhi; Dr. S. Pattanayak, World Health Organisation, New Delhi; Dr. M.K.K. Pillai, Delhi University, Delhi; Dr. Ramesh Kumar, All India Institute of Medical Sciences, New Delhi; Dr. K.M. Rashid, World Health Organisation, New Delhi; Dr. R. Reuben, Centre for Research in Medical Entomology, Madurai; Dr. R.G. Roy, Calcutta; Dr. A.B. Sen, Rajendra Memorial Research Institute of Medical Sciences, Patna; Dr. S.D. Seth, All India Institute of Medical Sciences, New Delhi; Dr. G.K. Sharma, National Malaria Eradication Programme, Delhi; Dr. Sarala K. Subbarao, Malaria Research Centre, Delhi; Dr. Shobha Sehgal, Postgraduate Institute of Medical Education and Research, Chandigarh.

INDIAN JOURNAL OF MALARIOLOGY

Author Index

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, if 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 footnotes. 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 x 21 cms; half page 45 x 24 cms and full page 57 x 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

We accept MSS. on floppy, please specify software used.

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/Monograph

Rao, T. Ramachandra (1984). *The Anophelines of India.* (WQ), 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.

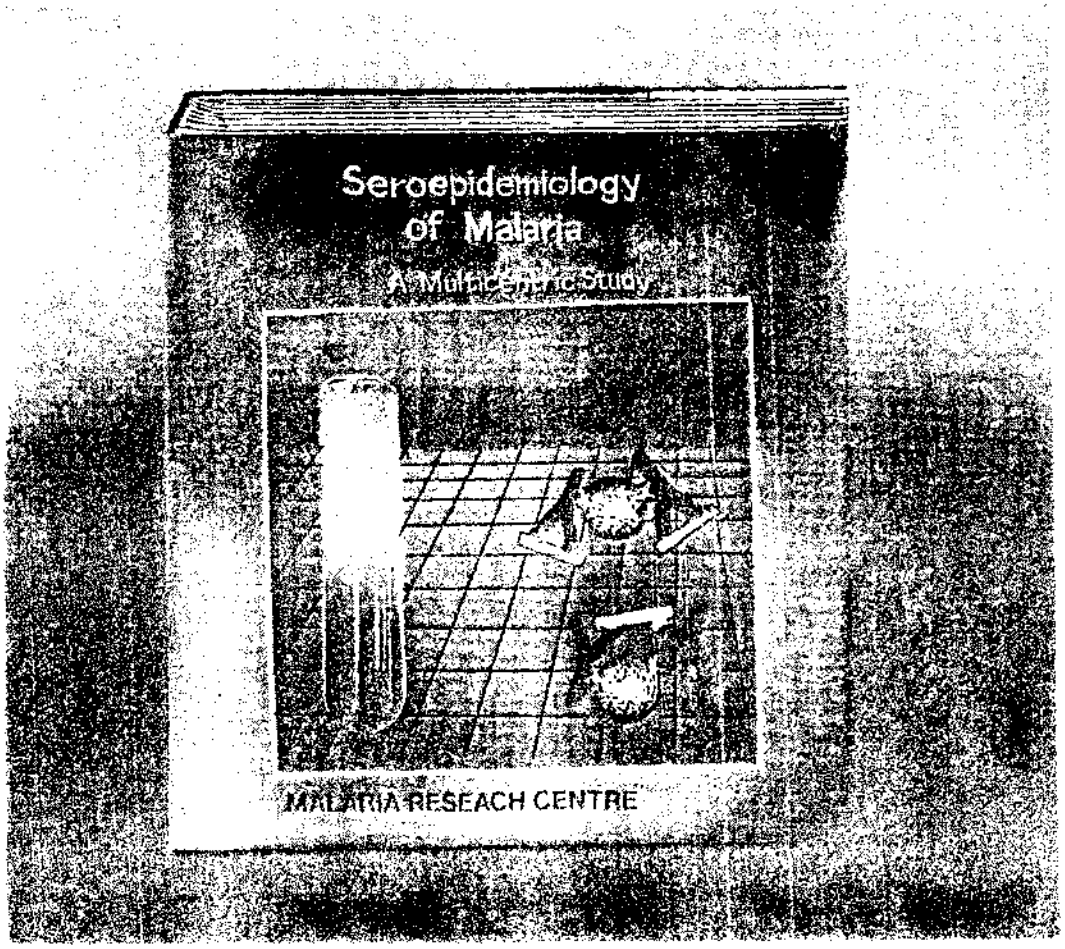
Proofs

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

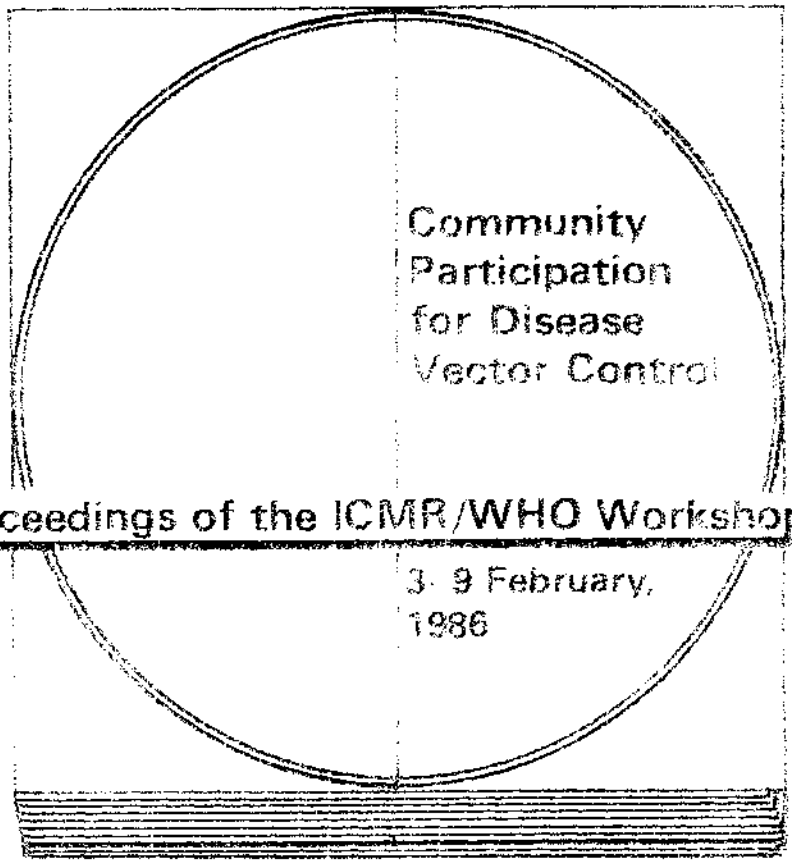
Reprints will be mailed to the senior author. Twenty-five reprints will be supplied free of charge to single authors and thirty for joint authors to be distributed on an equal basis. Additional reprints may be ordered on payment when the proofs are returned. A reprint order form will be supplied with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

SEROEPIDEMIOLOGY OF HUMAN MALARIA



Results of Multicentric Trials conducted by the ICMR
Demi-quarto, hardbound volume to be released in 1989

MALARIA RESEARCH CENTRE



Proceedings of the ICMR/WHO Workshop on

3-9 February, 1986

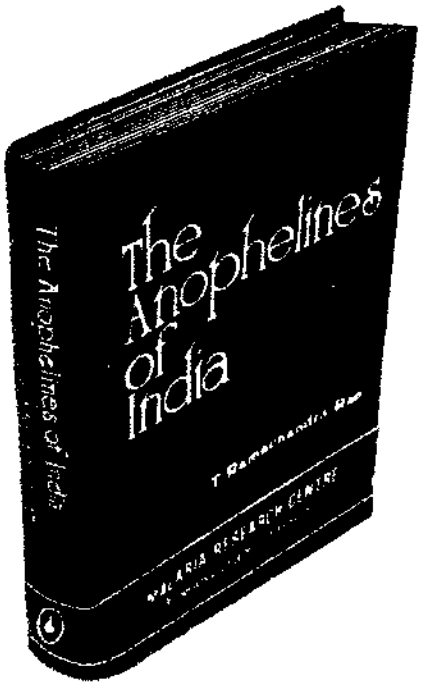
A review of the process, role and outcome of people's involvement in vector control. Invaluable for workers and decision makers in Primary Health Care and health-related projects.

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) **
Volume 26 Nos. 1-4 † (1989) **

† To be released in March, June, September and December 1989).

**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.

.....

.....

.....

.....