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Feasibility of IHA and ELISA in Seroepidemiology of Malaria

RAMESH KUMAR[1], S.N. RAO[2], M.A. ANSARI[2], R.K. RAZDAN[2], A. SRIVASTAVA[2] and V.P. SHARMA[2]

The IHA and ELISA tests were assessed for their suitability for seroepidemiological studies in malaria. The IHA test was done using antigen prepared from blood obtained from *P. knowlesi* infected monkeys. For ELISA, *in vitro* cultivated *P. falciparum* served as the source of antigen. The blood samples were collected on filter paper from three different areas with known high, mediunt and low prevalence of malarial infection. From each area samples were collected in the non-transmission season as well during peak transmission of *P. vivax* and *P. falciparum*. The results indicate that both the IHA and ELISA mean titres correlate well with SPR in the non-transmission period. Such a correlation is lacking in other periods. The expected age-related increase in antibody titres was evident only with ELISA and not IHA. In the peak transmission period of *P. vivax* and *P. falciparum*, the antibody titres were lower than in the non-transmission period, in areas with high prevalence of malarial infection. The significance of findings in relation to the seroepidemiology of malaria is discussed.

INTRODUCTION

In order to evaluate the role of seroepidemiology in malaria detection, we have carried out Indirect Haemagglutination test (IHA) and Enzyme Linked Immunosorbent Assay (ELISA) in samples collected from three different localities around Delhi. This presentation describes our results.

MATERIAL AND METHODS

The number of samples tested by IHA and ELISA from three localities around Delhi is shown in Table I. The first survey was carried out during the non-transmission season (February–March, 1984, second during the peak transmission of *P. vivax* (August–September, 1984), and the third during the peak transmission of *P. falciparum* (November–December, 1984). The three areas were chosen due to the known differences in the incidence of malarial infections. Sonepat having the lowest slide parasite rate and Ghaziabad the highest.

The samples were collected by finger prick on No. 3 Whatman filter paper so as to completely fill a circle of 1.5 cm diameter, by a team of field workers under the supervision of the Malaria Research Centre. Simultaneously, thick and thin blood smears were prepared. The filter papers as well as the smears were labelled, air dried and packed separately. A detailed proforma containing information such as age, sex, socio-economic

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Table 1. Samples collected from different localities around Delhi for seroepidemiology of malaria

Locality	Pretransmission first survey	*P. vivax* peak second survey	*P. falciparum* peak third survey
Sonepat	ELISA (2241)	IHA (4957)	IHA (998) ELISA (1001)
Gurgaon	IHA (2476) ELISA (2498)	IHA (4974)	IHA (1019) ELISA (999)
Ghaziabad	IHA (2465) ELISA (2477)	IHA (4990)	IHA (1019) ELISA (997)

Figures in parentheses indicate the number of samples tested.

status, history of fever etc., pertaining to each case was filled up. The proformas and the slides were transported to MRC, where the slides were stained and examined for the presence of malaria parasites.

Findings were entered in the proforma. The samples were transported to the AIIMS, where they were stored at −20°C until tested. The results of serological test were communicated to the MRC and entered in the proformas. The data was analysed at the computer centre at MRC.

Smear examinations could not be carried out during the third survey. IHA test could not be done on samples collected from Sonepat during the first survey. The IHA test during the second survey was carried out at the CDRI, Lucknow.

The IHA test was done according to the method recommended by Agarwal *et al.* (1982). The antigen was prepared at CDRI, Lucknow from Rhesus monkeys infected with *P. knowlesi*. Glutaraldehyde preserved human O group cells were tanned and sensitized with optimal dilution of the antigen. The test was carried out in microtitre plates. The serum was eluted from the filter papers for 1–2 hrs. at room temperature. It was diluted serially in a row of wells in the microtitre plates (U bottom) and sensitized cells were added. After mixing thoroughly, the plates were left at room temperature for 3 hrs. and then overnight at 4°C. The titre was expressed as the reciprocal of the highest dilution giving a clear 50% agglutination.

ELISA was performed according to the method described by Voller (1975). Briefly, the antigen was an ultrasonic lysate of schizont rich *in vitro* culture of *P. falciparum*. The immunolon II microelisa plates (Dynatech, Singapore) were sensitized with optimal dilution of the antigen overnight at 4°C. Positive and negative control sera as well as test samples (filter paper elutes) at 1/100 dilution were put in duplicate wells and the plates incubated at 4°C overnight. After washing, conjugate (anti-human IgG-HRP conjugate) was added at optimal dilution to all the wells. The plates were incubated at room temperature for 5 hrs. After washing, substrate (orthophenyline-diamine) was added and after 30 min, the reaction was stopped with H_2SO_4. The O.D. readings were taken at 492 nm. The results were expressed in units (The positive standard serum was assumed to have 100 units of antibody per ml).

RESULTS

The mean titre of antibodies, per cent individuals positive for antibodies ($\geq$1/32 titre for IHA test and $\geq$10 units for ELISA), and the slide parasite rate (SPR) for three localities at different surveys is shown in Fig. 1. During the first survey, carried out during the non-transmission period, the mean antibody titre as well as the per cent

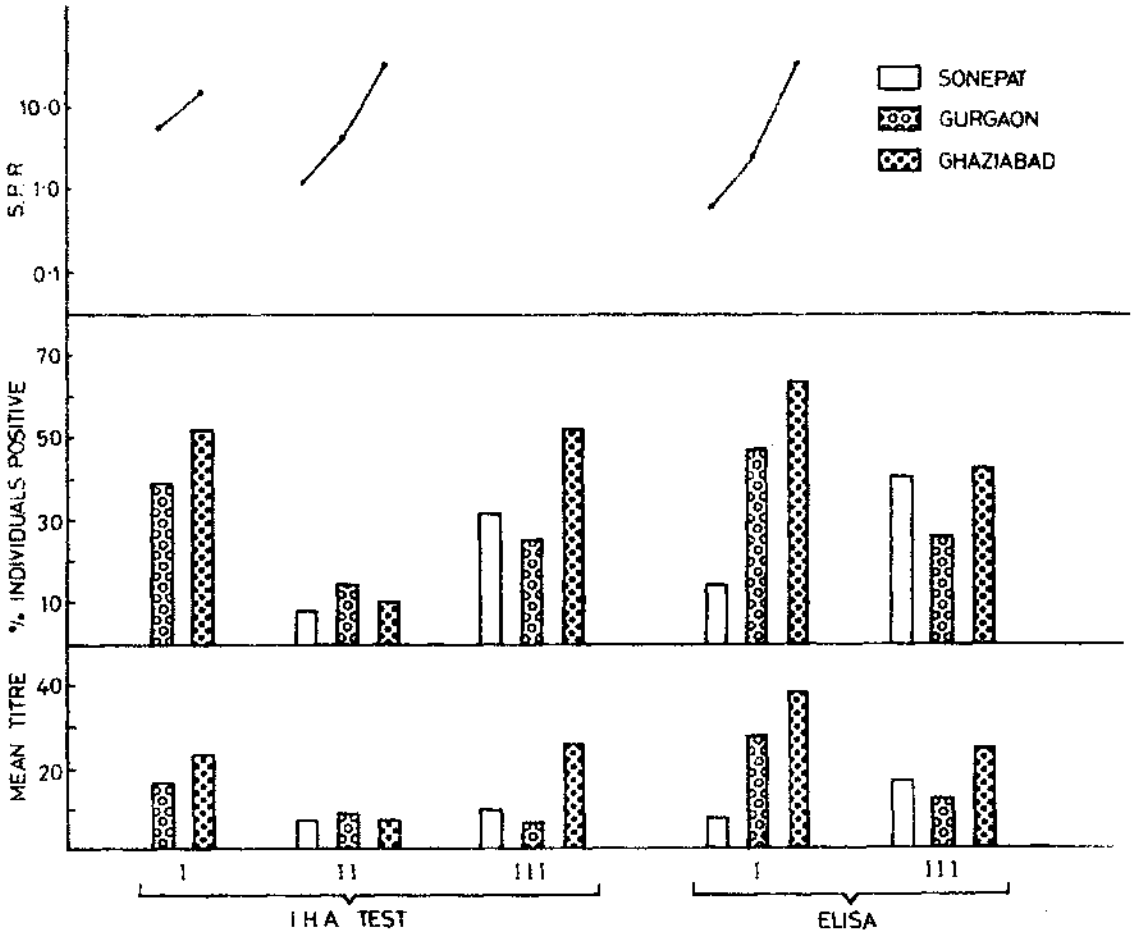

Fig. 1. The mean antibody titres, and the per cent individuals positive by IHA and ELISA tests in the samples collected from Sonepat, Gurgaon and Ghaziabad and their correlation with SPR.
I Samples collected during non transmission season.
II Samples collected during peak transmission of *P. vivax*.
III Samples collected during peak transmission of *P. falciparum*.

individuals positive for antibodies showed a good correlation with SPR, in both the IHA and ELISA. During the second survey carried out during the peak transmission period of *P. vivax*, only the IHA test was done. The titre of antibodies was much lower in comparison to the non-transmission period. Furthermore, the antibody titres had no correlation with the SPR. During the third survey carried out during the peak transmission of *P. falciparum*, in IHA test the antibodies increased over the 2nd survey levels and reached the first survey level in Ghaziabad but remained low in Gurgaon. In ELISA test, during the third survey, the antibodies were higher in Sonepat, and lower in Gurgaon and Ghaziabad in comparison to the first survey. The slides were not examined during the third survey, but there was no correlation between the IHA or

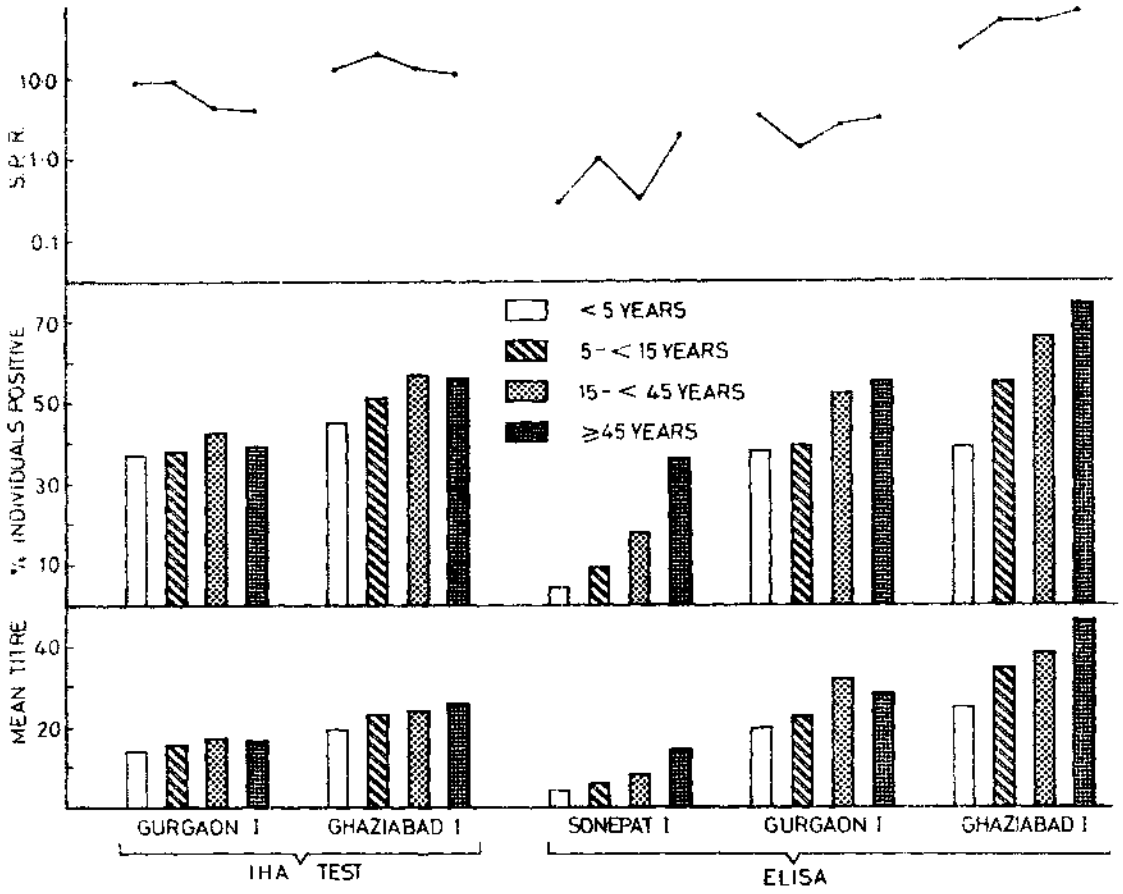

Fig. 2 Age distribution of IHA and ELISA antibodies in samples collected from Sonepat, Gurgaon and Ghaziabad during the non-transmission period and their correlation with SPR.

ELISA antibodies and the known prevalence of malaria in the three localities under investigation.

In Gurgaon and Ghaziabad from where the results of IHA as well as ELISA are available from the first and third surveys, a comparison between the two tests is shown in Table 2. Several more individuals were positive by ELISA than by IHA during the first survey in Gurgaon as well as Ghaziabad, the difference being statistically significant. During the third survey, there was a fall in the proportion of persons positive for antibodies in both the areas in both the tests in comparison to the first survey. However, the extent of the fall was much more marked in ELISA in comparison to IHA test in both the areas. Due to a much higher fall in ELISA, the difference between the two tests (i.e., IHA and ELISA) during the 3rd survey was abolished in Gurgaon and was reversed in Ghaziabad, with several more persons positive for IHA than in ELISA.

The age related antibody titres during the first survey, per cent individuals positive in each age group and the SPR are shown in Fig. 2. As far as

Table 2. Comparison between IHA and ELISA in the serology of malaria

Locality	First survey			Third survey		
	Positive	Negative	X^2	Positive	Negative	X^2
Gurgaon						
IHA	974	1502		253	766	
			31.48			0.042
			p <0.001			NS
ELISA	1180	1318		252	747	
Ghaziabad						
IHA	1288	1167		518	473	
			55.00			20.93
			p <0.001			p <0.001
ELISA	1558	919		419	578	

Figures indicate the number of individuals
Positive: ≥1/32 titre for IHA antibodies
≥10 units for ELISA antibodies
NS Not significant

the IHA test is concerned, the antibodies did not show any age-related variation. In ELISA, however, there was an increase in the antibodies with increasing age in all the three areas. The SPR was not different in different age groups (similar results were obtained during the second and third surveys; unpublished data).

There was no sex-related difference in antibodies in any of the areas by any of the two tests, and in SPR. Similarly no correlation of antibodies was seen with socio-economic status or with history of fever, and SPR was similar in all these subgroups.

DISCUSSION

The present study was designed to assess the feasibility of serological investigations for epidemiological purposes in malaria. Three areas were chosen around Delhi i.e., Sonepat, Gurgaon and Ghaziabad with known low, medium and high prevalence rates of malaria respectively. From each area, samples were collected during the months of February/March (non-transmission season), August/September (peak transmission of *P. vivax*) and November/December (peak transmission of *P. falciparum*). Two serological procedures i.e., (IHA) using antigen prepared from *P. knowlesi* and ELISA using *in vitro* grown *P. falciparum* antigen were evaluated.

The results indicated that, during the non-transmission season, both the IHA and ELISA mean titres correlated well with SPR. However, such a correlation was lacking during the other periods.

The IHA antibodies were equally distributed in different age groups. In contrast, the ELISA antibodies showed a very definite trend towards age-related increase in the titres. Since malaria antibodies in a population reflect a cumulative experience of exposure to the parasite over the previous years, an age-related increase in the titre is to be expected. Lack of such a correlation indicates unsuitability of *P. knowlesi* antigen for such studies. The ELISA results, employing *P. falciparum* antigen, however, gave desired results.

During the second survey conducted at the peak transmission period of *P. vivax* (August–September), only haemagglutination

test was carried out. At this stage, the titres of malarial antibodies were found to be lower in comparison to the titres in non-transmission season and did not correlate with SPR. During the peak transmission of *P. falciparum* also (third survey) the antibody titres were low in comparison to the titres during the first survey, and no correlation was seen with known prevalence of malaria infection (SPR was not determined during the third survey). We believe that, with an ongoing extensive transmission of malarial parasite, the antibodies would be adsorbed at a rate faster than they are produced, which explains these observations. If such be the case, it is expected that a specific antigen would show a more pronounced fall in antibody titre than a non-specific antigen. A reference to Table 2 shows that such was the case. The table also shows that, during the first survey, the per cent individuals positive for antibodies was more with ELISA than IHA test.

It is to be noted that, the absorption of antibodies would occur only in areas with high transmission of malaria, as in Gurgaon and Ghaziabad. With very low transmission, such as in Sonepat, the ELISA antibodies actually increased during the third survey in comparison to the first survey (IHA test was not done in Sonepat during the first survey).

These findings indicate that serological methods can be relied upon for indicating prevalence of malaria in a community. For this purpose, blood samples should be collected during the non-transmission period. A procedure employing a homologous (*P. falciparum*/*P. vivax*) antigen is likely to give more reliable information. Perhaps, even finer distinctions may be feasible after more extensive studies are carried out, particularly in the younger age groups.

Furthermore, the effect of control measures on transmission of malaria is also reflected in the serological parameters. With extensive transmission of the parasite, the antibody levels would fall during the peak transmission season. In other areas, with low prevalence rates of malaria, the antibodies will increase during the peak transmission season. Thus serology, specially ELISA, may be very useful for epidemiological purposes in this country.

There is a need for a serological test to be able to distinguish between *P. falciparum* and *P. vivax* infections and their respective prevalence in a community. This must await availability of the specific antigens of the two parasites at a future date.

It is suggested that ELISA, and if possible Indirect Immunofluorescence tests, should be studied more extensively to evolve reliable serological parameters of malaria endemicity.

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An Adaptation of the Gel Diffusion Technique for Identifying the Source of Mosquito Blood meals

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A modified gel diffusion technique has been designed which only requires a very simple field laboratory for implementation. This method offers a rapid, uncomplicated and inexpensive way to determine locally the origin of mosquito blood meals. Several inherent drawbacks which occur when smears are sent abroad for processing are alleviated or avoided. These include the necessity of attempting to forecast annual serological needs within a prescribed ration of blood smears and delayed feedback of results which often takes several months. Moreover, any error or deficiency in implementing the technique locally becomes a self-contained difficulty which can be addressed and corrected promptly. By this method it is possible to take advantage of even fleeting seasonal phenomena such as limited intervals of intense malaria transmission and abbreviated peaks of population density which characterize some species.

INTRODUCTION

Indoor-resting collections are by far the most productive routine capture techniques being reported by three entomological field units working in Orissa State, India. Typically, in all *10* study villages being surveyed, houses and cattlesheds are in very close proximity, frequently sharing the same roof. This situation provides foraging mosquitoes with ample opportunity to feed on cattle inside their sheds or outdoors and to subsequently enter human dwellings for harbourage, or vice versa. Therefore, regardless of the biotope in which a mosquito has been caught, it is essential to know the host or hosts upon which the mosquito has actually fed before an accurate assessment can be made of the aetiological significance of such catches.

It was decided that it would be preferable if a local means could be found for determining the origin of mosquito blood meals. A subsequent search revealed that the State Forensic Laboratory of the Orissa Police Department, Bhubaneswar, was using a gel diffusion technique to identify the origin of blood stains for criminal investigations. In order to ascertain whether or not their method would satisfy our requirements, the Forensic Laboratory authorities agreed to process several mosquito blood smears which had been made on filter paper by the staff of our field units. The host blood of the several smears submitted were known to us, but not to the laboratory workers. As the results of these known smears tested by the Forensic Labo-

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ratory proved accurate, it was decided to establish our own laboratory in order to adapt the technique specifically for processing mosquito blood smears. Three laboratory technicians were given one week of training in gel diffusion methodology by the Forensic Laboratory staff. Meanwhile supplies of human and cow antisera and of normal rabbit sera were obtained from the Serologist to the Government of India in Calcutta. A small room was set up as a modest laboratory and after completing the training course, one of the laboratory technicians was assigned to perform gel diffusion tests as his primary duty.

MATERIAL AND METHODS

In the gel diffusion technique, antisera and control sera are introduced into certain wells cut into an agarose gel layer which covers the upper surface of a glass slide. Adjacent wells are then filled with saline extracts of antigen to be tested and the fluids from all wells diffuse gradually through the gel towards one another in outwardly extending circles, similar to those seen when a small stone is thrown into the centre of a quiet pool. When an antiserum corresponds and reacts specifically to an antigen against which it is being tested, a white precipitin band forms at the interface. If the titres of the antigen and the antiserum are in balance, the band materializes at or near the halfway point between the two wells involved. If not, the band forms nearer or inside the well which contains weaker titre.

The gel diffusion technique was modified in order to suit the particular requirements. Initially, the smears are screened only against cow and human antisera. Technical justifications for this are mentioned below. Operationally, by confining the tests to two hosts, it was possible to design a testing pattern which is fast, efficient and inexpensive to implement. With this design, saline extracts of at least 16 blood smears can be processed on a 2.5 × 7.6 cm glass slide (currently the number has been increased to 25 smears by employing glass slides 10 cm long). Each antigen extract is exposed to primate and bovine antisera and is also subjected to normal rabbit control sera. In addition to the positive or negative reactions, a side benefit is obtained wherever two adjacent antisera wells are positive to the same antigen. In this case, the double diffusion in two dimensions (Ouchterlony procedure) may reveal reactions of identity or partial identity by the presence or absence of a spur where the positive bands meet (Humphrey & White, 1970).

The materials and procedures used for performing the gel diffusion technique include those required for precoating and layering the gel on the slides, cutting wells in the gel and finally loading the wells with antigen extracts, antisera and control sera. Details of the method used in carrying out these procedures are given below.

Precoating

Precoating the slides ensures that a firm grip will be maintained on the gel overlay so that it is not detached by the suction punching device used to cut out the wells. The precoating material consists of 50% barbiturate buffer (pH 8.6), 49.8% distilled water and 0.2% agarose. This mixture is heated in a water bath until the solution becomes clear. The slides are then precoated by dipping them into this solution and standing them on end to dry. Any unused solution is protected against fungus by adding 1 ml of merthiolate to it, stoppering the container and storing it in a refrigerator at about 4°C. For re-use, the solution is removed from the refrigerator, reheated and applied as before.

Gel layering

A high-grade agar such as agarose which is used for electrophoresis gives much clearer results than agar-agar. Sufficient agarose (0.9 g) is added to 99.1 ml of pH 8.6 barbiturate buffer

solution to provide 100 ml of 0.9% agarose. This liquid is heated in a water bath until it becomes clear. Then 3 ml are taken up in a graduated pipette and are released in the centre of a 1.5 × 7.6 cm glass slide which has been placed on a levelled surface. After each slide has been flooded, it is allowed to cool at ambient temperature for at least five minutes. The gel-layered slides are then placed in a small moist chamber to which a drop or two of merthiolate may be added and the chamber is placed in a refrigerator at 4°C for at least two hours before use. Slides stored in this manner may be held for three weeks or more before use.

Any extra gel-layering material to be saved is protected from fungus by adding 1% merthiolate; its container is then stoppered and stored at 4°C in the refrigerator. For re-use it is heated as before in a water bath until it becomes clear and then the same procedure as mentioned above is followed.

Cutting the wells

A plastic guide template cut out of a soft but fairly rigid plate of transparent plastic serves as a spacing and steadying tool for cutting the wells. This tool is 7.6 cm wide, 12.7 cm long and 1 mm thick. Holes slightly larger than 1 mm in diameter are drilled in the proper positions for cutting either 16 or 25 wells. To prepare for cutting, a gel-layered slide is surrounded by four glass pieces, two of which are 12.5 cm long and 2.5 cm wide and two of which are 2.5 cm square. The square pieces abut each end of the gel-layered slide, while the longer pieces are aligned to clasp the sides of it. All the surrounding glass pieces are about 1 mm thicker than the gel-layered slide. The plastic template is placed on top of the surrounding slides so that its bottom rests about 0.5 mm above the surface of the gel layering the standard slide. Next, the holes of the template are oriented in a suitable position above the gel. Finally, all the glass slides surrounding the gel-layered slide, plus the plastic template over it are locked firmly into place. This is done by engaging elastic strips containing small patches of inter-locking mini-hooks which have been glued to the upper surfaces of the glass and plastic with clear epoxy. Now the preparations are completed and cutting can commence.

An eyedropper which has a needle-nose tube of thin glass with an outside diameter of no more than 1.5 mm is used to cut the wells. Each well is made by squeezing the bulb of the eyedropper and inserting its tapered end into a hole of the plastic template. The tip penetrates completely through the gel layering until it touches the precoated surface of the glass slide. The pressure on the bulb is then released and the gel is sucked up into the tube of the eyedropper. when the eyedropper is withdrawn, an empty well has been formed in the gel. Recutting of a few of the wells is sometimes necessary. When all the wells are judged satisfactory, the mini-hooks are disengaged, all surrounding parts are disassembled and the standard slide is removed, after which it is ready for loading. Since the slides are not permanently marked or damaged, they may be re-used indefinitely.

The arrangement of the antigen, serum and antiserum wells

Previous records indicate that most blood meals are taken from cows or humans and since stables as well as houses are included in the spray target of the National Malaria Eradication Programme, it was therefore decided that initial testing would be confined to primate and bovine antisera. This made it possible to arrange the two types of antiserum wells in such a way that they were always separated from one another by an antigen well. With this arrangement for testing 16 smear extracts (in antigen wells) on a standard slide, each of the two antiserum wells at the end of the slide test against two antigen wells, while each of the four wells of bovine antiserum and each of the four

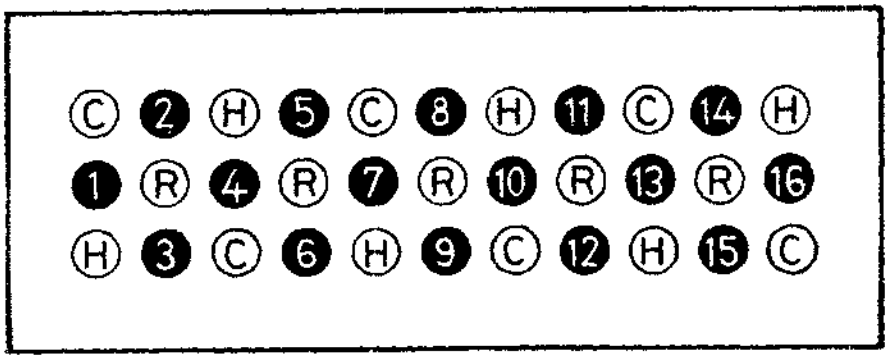

Fig. 1: Pattern of antigen, antiserum and serum wells cut into the gel layer on a standard blood slide. This arrangement tests 16 blood smears against bovine and primate antisera, each with a control.

wells of primate antiserum, located internally, test against three antigen wells. Each normal rabbit serum well serves both as a control for the four antigen wells surrounding it, and as a double check for antigens in positions 4, 7 and 13. Fig. 1 shows the numbered loading positions for 16 antigen and the labelled positions of the serum and the antiserum wells. The same pattern may be extended to cover more smears on a standard slide (i.e., 25 per slide) if the distance between wells is reduced to 4 mm centre-to-centre, or if the 5 mm distance is maintained, but a longer glass slide (i.e., 10 cm rather than 7.6 cm) is used.

Loading the wells

Half of each smear is saved for possible future reference. The half which is processed is snipped off and is allowed to drop into one of the ceramic wells of a botany staining plate. Then 20 μl of saline are added with a 20 μl micropipette and the half-smear is allowed to soak, face down for 4 hours. During this time, the cow antiserum wells, the human antiserum wells and the normal rabbit serum control wells are charged, employing a 5 μl micropipette. The micropipette have disposable plastic tips and these are renewed whenever there is a change from one material to another. Finally the antigen wells are loaded in the same manner, with a new tip utilized for each saline extract being tested.

In order to guide the loading, the gel-layered slide (which is transparent) is oriented over a scale-drawing of a slide in which all wells of the antigen, antisera and sera are colour-coded to illustrate their proper loading positions. The drawing is laminated within sheets of clear plastic. The lamination also includes a thin flat sheet of metal 10 cm square as a base. Magnetic strips are placed all around the glass slide so that once the wells are positioned over their colour-code the slide will remain fixed in place during

the loading process. A deep red colour shows through for all wells to be charged with antigen, a dark blue for those with bovine antiserum, a dark green for those with human antiserum and orange for those with normal rabbit serum. Once all wells have been filled, the slide is placed in a tin box (tiffin) lined with paper towelling. The towelling is moistened by applying distilled water to it with an eyedropper. Then a drop or two of merthiolate may be added to inhibit fungus and the box is stored in a refrigerator at 4°C.

Reading the results

Strong primary positive reactions usually form quite definitely within 4–5 hours after loading the wells. However, as secondary bands sometimes develop several hours later, at least 12 hours must be allowed to lapse between the loading of the wells and the taking of an official reading. Usually the slide is left in the refrigerator overnight and examined the next morning. A slide is examined by holding the edges between the thumb and index finger and tilting it below the glare of a 100 watt bulb until the white bands stand out clearly. An alternative which works well is to hold the slide up to a window which has a solid dark-coloured curtain, as this provides a background against which any white bands are conspicuous. Fig. 2 is a sketch of a gel diffusion test which shows the slide, gel coating, cut wells and precipitin bands of positive reactions).

Antisera and antigen extract

Human and bovine antisera and normal rabbit sera, titered at 1:20,000 are obtained from the Serologist for the Government of India, 3 Kyd Street, Calcutta. These antisera have been subjected to several blind tests, using known blood meals. Also several trials have been conducted in which the amount of saline was increased or decreased or the soaking period was varied in making a saline extract of one-half of a blood smear. From these observations we determined that one-half of a relatively fresh

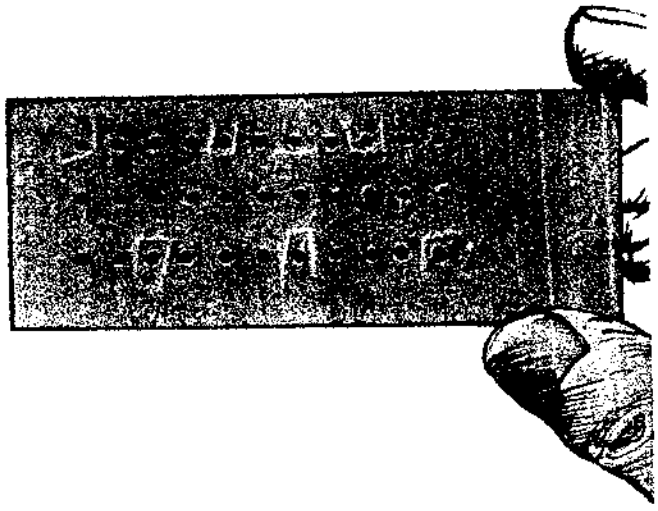

Fig. 2. Sketch showing a gel diffusion test. Primary positive reactions are seen as strong white bands. Smear in position No. 10 shows a strong reaction to both bovine and primate antisera, indicating double (probably interrupted) feeding.

blood smear, soaked at ambient temperatures for four hours, will provide a sharply defined precipitate band in the gel that forms about midway between its well and that of its corresponding antisera. The four-hour soaking period was chosen primarily for operational reasons, so that the smears may be soaked in the morning and loaded in the afternoon.

Spacing the wells

By placing the wells only 4 mm apart from centre-to-centre, it is possible to process 25 rather than 16 blood smears on a standard 2.5×7.6 cm glass slide. However, with this spacing the titre of antigen used sometimes appears to be excessive, in which case the positive band will form right at the edge or partly inside the antiserum well to which it reacts. In this case, the band is usually murky and somewhat difficult to read. Moreover, the wells themselves are slightly cramped together so that additional caution is necessary when cutting and loading the wells.

However, if the distance between wells is 5 mm centre-to-centre, the positive bands are more sharply defined and almost always fall at or near the midpoint between wells. Consequently, 5 mm was selected as the standard centre-to-centre distance between wells, but at this space only 16 smears and labelling can be accommodated comfortably (21 smears is a maximum) on a standard slide. A slide 2.5×10 cm will easily handle 25 smears at a centre-to-centre distance between wells of 5 mm. Recently several slides this size were cut and have started to be used routinely. For the new workload of 25 smears, a filter paper strip (Fig. 3) is cut from rectangular sheets of No. 1 Whatman filter paper, as much filter paper is wasted when using the conventional circular filter-papers and relatively little in the case of the strips.

Average volume of the wells

If the directions for applying gel-layering to a standard slide and for cutting the wells in the gel are followed correctly, approximately 5 μl of liquid are required to fill each well completely. For 1060 wells filled with human antisera, 1053 filled with cow antisera and 873 filled with normal rabbit sera, the average amounts used were 5.38 μl, 5.41 μl and 5.50 μl per well respectively. Consequently, if no 5 μl micropipettes are available, crude micropipettes can be fashioned by drawing out heated glass tubing. If these are used to fill the wells to the brim, then each well should automatically contain slightly more than 5 μl of material.

Use of dyed gel

In other observations, food colouring dyes of red, yellow, green and orange were incorporated into the gel layering to see if this would

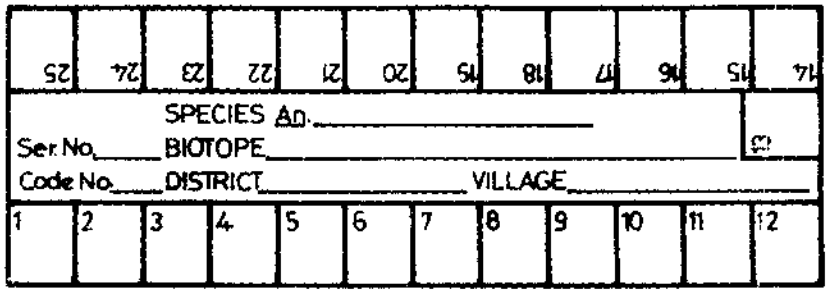

Fig. 3: Copy of a Whatman No. 1 filter-paper strip being used to collect 25 blood smears. The dates the mosquitoes are smeared are written just above the smear numbers. The actual size of the strip is as shown.

increase the clarity of the agglutinate bands formed by positive reactions. The dyes diffused quite uniformly throughout the gel and although the slides were very attractive, they did not make the bands stand out any the more clearly, so the use of dyes is not advocated.

Digestion of antigen

Several general observations were made to see how long after a blood meal has been taken, a smear can be made which will still yield a positive reaction to its antiserum. Generally, it can be said that a relatively "good" smear from *Anopheles culicifacies* will still yield a clear reaction to human blood up to 36 hours after feeding, when the mosquitoes are held in paper cup cages at ambient winter temperatures averaging 27°C.

Storage of slides, field kit

Gel-layered slides placed in a humidity chamber which has been treated with a few drops of merthiolate may be stored under refrigeration for at least one month before use. However, they may be held at room temperature for at least a week under similar conditions, if care is taken to maintain high humidity by administering distilled water once or twice a day; it should therefore be possible to bring gel-layered slides to remote areas as part of a gel diffusion field kit. However, it would probably prove to be much more practical if the filter papers containing the blood smears were brought in from the field to a very simple central laboratory for processing than if all the paraphernalia of a test kit were carried out to more remote areas where the work would have to be done under much more trying conditions. Although a field kit would provide on-the-spot results, these would be available only a few days earlier than those of a field laboratory, and employing a kit would demand a greater effort and probably would be less efficient.

Testing alternative antisera

If an inordinately high proportion of smears do not react to cow or human antisera, this indicates that either the antisera or the smears are deficient or that the mosquitoes are feeding quite often on other hosts. To check on this, the human and cow antisera may easily be tested against known smears. The saved halves of the smears involved should then be examined to see if their quality appears suitable so that if positive to human or cow antisera, they would be expected to react. If so, and if the species, the biotope and/or the number of smears involved seem to indicate a possible aetiological relevance, the saved half-smears may be processed against other antisera. Antisera from other animals known to frequent the biotope(s) concerned should be selected. For this type of follow-up a small supply of fowl, dog, pig and goat antisera are currently on hand. By substituting any two of these in the position routinely used for the human and cow antisera, 16 or 25 smears can be processed on one slide and by using two slides it is possible to test against all 4 of these alternative hosts, using 10 (5 microlitres for each slide) of the 20 μl of saline extract which is prepared for every half-smear tested.

Cost factors

The major material costs include agarose, barbituric acid, sodium barbiturate, normal rabbit serum, and human and cow antisera. On a per smear basis these expenses total 0.07 rupees (approximately US $ 0.01). Labour costs comprise the major expense, but these are much more competitive in India than in many other countries. At an output of 130 smears a day, the total cost (labour plus materials) is about US $ 0.06 per smear.

DISCUSSION

Many authorities (Weitz, 1956; Tempelis and

Lofy, 1963; Crans, 1969; Boreham, 1975; Tempelis, 1975) have compared the advantages and disadvantages of the ring precipitin test and the gel diffusion test. The general consensus seems to be that the ring precipitin test is more sensitive than gel diffusion. However, the quality of agar has been substantially improved since some of these opinions were expressed, and some workers indicate that gel diffusion is 3 to 100 times more sensitive than the precipitation method (Carpenter, 1975). As the ring test procedures have been adapted for mass-processing large numbers of smears, this method might be preferred by a large centralized laboratory which services a considerable number of field units.

Gel diffusion tests, on the other hand, provide the advantage of simultaneous and more permanent readings. Moreover, diffusion through the agarose substrate filters out bits of filter-paper or other debris in the antigen extract. It also filters out the cloudiness which sometimes occurs in a partially denatured antiserum or in antigen extracts made from blood in which ovarian development has begun. This filtration process eliminates the need to centrifuge the extracts and allows the testing of smears from partially engorged as well as fully engorged females (Crans, 1969).

Simultaneous double diffusion readings provide comparisons which may reveal the presence of immunologically identical or partially identical cross-reacting, heterologous antigens. The more permanent results of the gel diffusion method allow for improved supervision or more prolonged follow-up.

A study conducted at the University of North Carolina at Chapel Hill involved comparisons of the capillary tube (flocculation and ring test), the gel diffusion and the microplate methods. This study showed that the three methods were comparable for the titration of antisera and the identification of blood meals. Moreover, the flocculation and ring test methods (conducted in tubes) required excessive amounts of reagents and offered no important advantages over the gel diffusion or microplate method (Eliason, 1971). Microplate methods (Edrissian and Hafizi, 1982) appear to be more complicated, requiring more sophisticated laboratory equipment and support than does gel diffusion.

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We should like to thank the former Director of the National Malaria Eradication Programme, Dr S. Pattanayak, and the former WHO Team Leader, Dr A.V. Kondrashin, for permission to process our blood smears locally. Our thanks go to Mr V.N. Bhatnagar for having initially put us in contact with the Bhubaneswar Police Department Forensic Laboratory. Our sincere gratitude is owed to Mr N. Swain, Director-General of Police, Cuttack, and Dr N. Mohanty, Director of the Forensic Laboratory, Bhubaneswar Police Department, for the excellent interdepartmental cooperation they provided us in arranging for Miss S. Panigrahi and her assistants to train our staff. In addition, we are grateful for the interest and support of the Director of the National Malaria Eradication Programme, Dr S.R. Dwivedi. Finally, we acknowledge the valuable technical assistance and material support received from Dr Andrei E. Beljaev, WHO PfCP Team Leader, and Mr William Rooney, WHO Laboratory Specialist, both of New Delhi.

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In vitro Susceptibility of Chloroquine Resistant *Plasmodium falciparum* to Mefloquine in Delhi

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Comparative susceptibility of 20 *P. falciparum* isolates to chloroquine and mefloquine, was studied using the micro *in vitro* test. Twelve specimens (60%) were resistant to chloroquine (growth at ≥5.7 pmols) and the remaining were sensitive. But all the specimens were inhibited by ≤16 pmols of mefloquine indicating sensitive response.

INTRODUCTION

The problem of chloroquine resistant falciparum malaria in India appears to be more widespread than believed to be confined to the north eastern states (Sharma, 1984). A recent study indicates that there is progressive increase in the incidence of falciparum cases in Delhi (Choudhury, 1984) and here, resistance to chloroquine has been confirmed by both *in vivo* and *in vitro* tests (Choudhury *et al.*, 1983). Operationally useful new drugs are under development for treatment of drug-resistant falciparum malaria cases. Mefloquine, a quinoline methanol, is a new drug under investigation found to be effective against multidrug-resistant strains of *P. falciparum* (WHO, 1981). However, because of sporadic reports of mefloquine resistant strains (Boudreau *et al.*, 1982; Bygberg *et al.*, 1983; Smrkovski *et al.*, 1985), it appears essential to determine the efficacy of the new drug against different geographic strains. Due to paucity of data, we undertook an investigation on the susceptibility of Delhi isolates of *P. falciparum* to mefloquine and compared this with that to chloroquine. Mefloquine being a drug under development, not yet licensed for *in vivo* use in India, only *in vitro* studies were carried out.

MATERIAL AND METHODS

The study was carried out between August and December, 1985. Patients reporting to the National Malaria Eradication Programme Malaria Clinic were selected on the basis of criteria laid down before (WHO, 1981). Informed consent from patients was obtained before collecting the blood specimens. After ensuring the absence of drugs in their urine, about 100 μl of finger prick blood was collected from each patient into heparinized tubes. Age of the patients, included in the tests, ranged from 9–58 years (mean 27) and asexual *P. falciparum*

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parasitaemia ranged from 2000-150000 per mm^3 (mean 10, 195).

The field version (Wernsdorfer, 1980) of Rieckmann's micro *in vitro* technique (Rieckmann *et al.*, 1978) was followed. Tests were conducted in WHO supplied chloroquine-dosed (Batch Nos. C/22, C/25, A/49 & C/50) and mefloquine-dosed (charging dates June 1981, July 1981 & May 1982) microtitre plates. Concentrations of chloroquine ranged from 1-32 pmol/well and those of mefloquine 1-16 pmol/well. RPMI-1640 medium with HEPES buffer (25 mM), glutamine (300 mg/l) and gentamycin (40 mg/l) and pH adjusted to 7.2–7.4 with 5% sodium bicarbonate solution, was used. Tests were set up with 50 μl of blood-medium mixture in each well. Standard procedures for incubation, preparation of post-incubation smears, staining and enumeration of schizonts, were followed (WHO, 1981). A test was considered successful if 10% of schizont maturation was observed in the control well (Draper *et al.*, 1985). Schizont development at 5.7 pmols of chloroquine and 16 pmols of mefloquine indicated a resistant response (Smrkovski *et al.*, 1985).

Schizont counts in drug concentrations were expressed as percentage relating to control and the group data plotted on a log-normal graph. Concentrations required for 50%, 90% and 99% inhibition of schizont formation (EC 50, EC 90 and EC 99) were calculated from the plotted values.

RESULTS AND DISCUSSION

Comparative tests on 30 isolates yielded interpretable results for 20. Results are summarized in Fig. 1. The minimum inhibitory concentrations (MIC) for chloroquine ranged from 4 to 32 pmols. 12 specimens (60%) showed schizont formation at $\geq$5.7 pmols of the drug

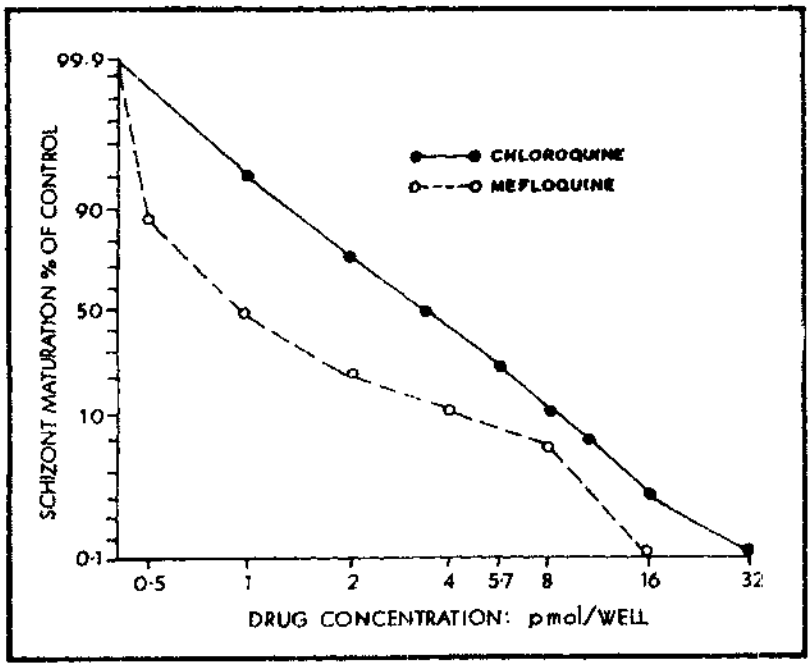

Fig. 1. Responses of 20 isolates of *P. falciparum* to Mefloquine and Chloroquine in Delhi.

indicating resistant response. The remaining were sensitive. On the other hand all the successful specimens were inhibited by ≤ 16 pmols of mefloquine indicating sensitive response; the MIC being 1.0 to 16 pmols. The EC 50, 90 and 99 values for chloroquine were 3.5, 8.4 and 16 and for mefloquine 0.98, 4.4 and 10.2 pmols respectively. This suggested that mefloquine was about 3.6, 2 and 1.6 times more effective than chloroquine at these levels.

The above results indicate that *in vitro* there is no cross resistance between chloroquine and mefloquine in Delhi isolates of *P. falciparum*. In Gadchiroli (Maharashtra State) also Dutta *et al.* (1984) found that all isolates, including chloroquine resistant ones, were sensitive to mefloquine, being inhibited by ≤ 16 pmol. These observations are similar to those obtained *in vitro* in Brazil and Colombia (Lopez Antunano and Wernsdorfer, 1979) but unlike those of Smrkovski *et al.* (1985) in Philippines, where they found cross-resistance between the two drugs *in vitro*. An interesting observation, however, is that the EC values for mefloquine sensitivity of Delhi isolates appear to be much higher than those of the corresponding values available for strains from 9 countries presented in the Annex 5(h) of a document by Payne (1984). The significance of this may become clear if periodical monitoring of sensitivity to mefloquine is carried out in this area.

The results of the present *in vitro* study show that mefloquine is fully effective against chloroquine sensitive and resistant *P. falciparum* in Delhi.

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Bio-environmental Control of Malaria in Nadiad, Kheda district, Gujarat

V.P. SHARMA[1], R.C. SHARMA[1] and A.S. GAUTAM[1]

A study on the bio-environmental control of malaria was launched in Nadiad taluka villages (Gujarat) in 1983. The area was endemic for malaria with high incidence of falciparum malaria. Insecticidal spraying to interrupt transmission did not produce desired results. Simple and age old methods of source reduction and biological control with the active involvement of communities through health education, and prompt case detection and radical treatment were used to combat malaria in 21 villages with 60,000 population. In 2 years malaria incidence was reduced to low levels. Mosquito densities in general and that of *A. culicifacies* in particular were greatly reduced. Reduction in the incidence of malaria was substantiated by reduction of spleen enlargement in children and low seropositivity rate. Fish culture, forestry schemes, improved chulahs, and soak pits etc., were introduced to improve the environment and village economy. The feasibility of a holistic approach to malaria control was demonstrated.

INTRODUCTION

Large parts of India are endemic for malaria. In rural areas about 350 million people are being protected by spraying of residual insecticides and chemotherapy by the National Malaria Eradication Programme (NMEP). The main strategy of malaria control in rural areas is the spraying of residual insecticides to interrupt transmission.

Insecticidal spraying as a means to interrupt malaria transmission is beset with many problems such as vector resistance to insecticides and gradual evolution of multiple resistance, apart from the high cost and misuse of insecticides, large-scale refusals by the inhabitants to allow their dwellings to be sprayed and environmental contamination. In many rural areas the spraying is not accepted due to sericulture and bee keeping and in some areas sprayed walls are plastered with mud soon after spraying.

Environmental scientists are seriously concerned about the massive use of insecticides which is taking place in public health almost on a permanent basis. As a result there is a demand for major cuts or total ban in the use of DDT in malaria control. Therefore, spraying as a means to control malaria would have to be given up in due course of time and during this period alternate strategies should be developed to combat malaria.

One such possibility was the bio-environmental control of malaria. Therefore, a demonstration

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cum feasibility study on this alternate strategy was launched in Nadiad taluka, Kheda distt., Gujarat in 1983. The field study was started with the following objectives; (i) to demonstrate malaria control by bio-environmental methods without the use of insecticides, and (ii) to develop a cost effective model for its extension to other parts of the country. Results of a 3 year study from 1983 to 1985 are reported in this paper.

MATERIAL AND METHODS

Study area

Kheda district has a total area of 7194 sq. kms. Its adjoining districts are Baroda, Panch Mahals, Sabarkanta and Ahmedabad. Kheda district is a plain area with the exception of small hilly regions in Balasinor and Kapadwanj talukas. The district has two perennial rivers;

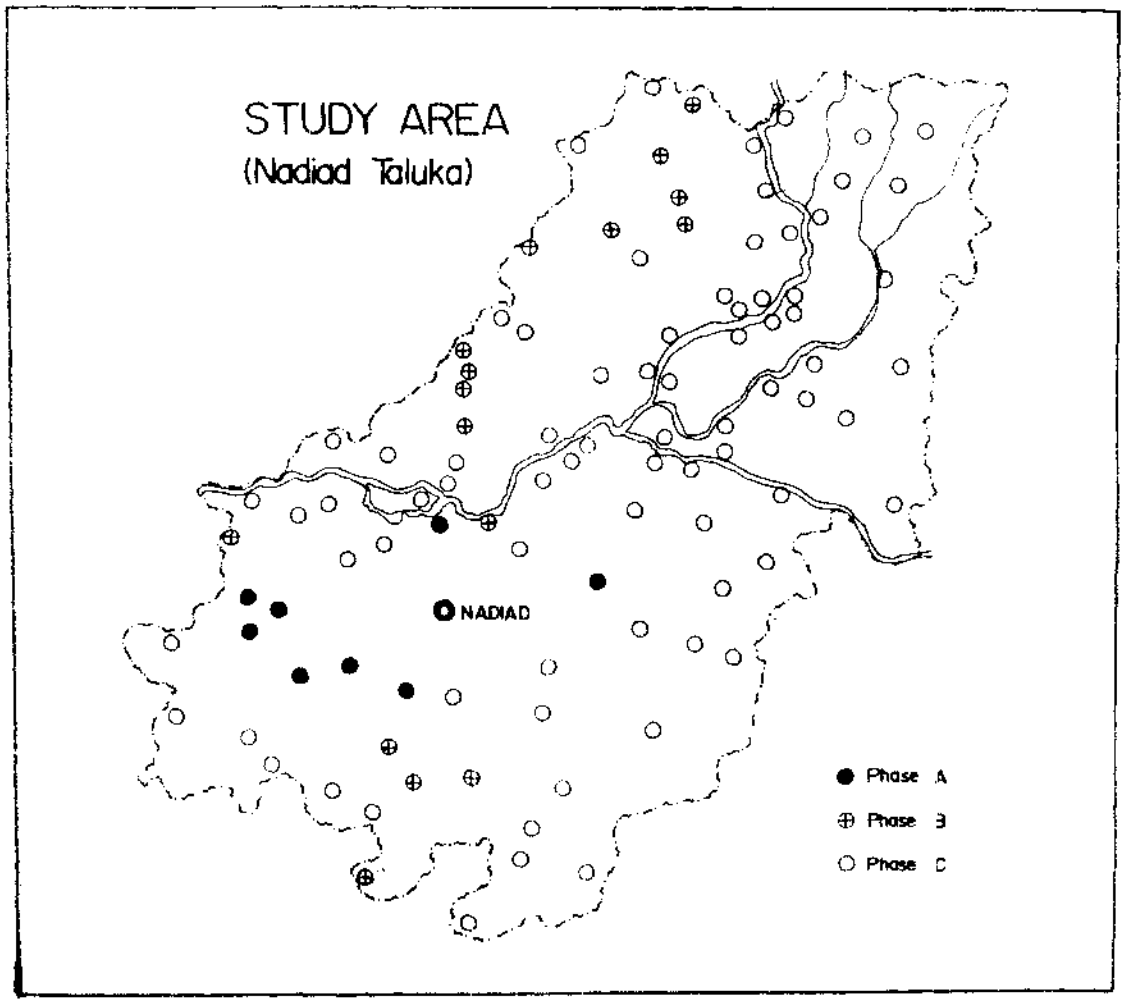

Fig. 1: Map of Nadiad taluka.

Mahisagar and Sabarmati whereas the rivers: Vatrak, Shedhi, Meshavo and Khari are seasonal, flowing only during the monsoons. The rainfall of the district is between 60 to 75 cms. Except for occasional showers in winter, annual rainfall is received between June and October. The distribution of rainfall over the monsoon is itself uneven necessitating irrigation for certain crops. The district has a good network of canals. All the talukas are well connected by road and rail, except Balasinor and Matar talukas. The main crops are paddy, wheat, cotton, Bajra (millet), tobacco, ground nut and til.

According to the census of 1982, the total population of the district is 20,69,942 living in 972 villages and 7 towns. People are employed in agriculture, industry and business. About 46% of the total population is literate which is considerably higher than the other districts of the state. Nadiad town is the headquarters of the district. The study villages are within 20 kms. of Nadiad. State records revealed that malaria incidence was highest in Kheda district as compared to other districts of Gujarat in the preceding years. Kheda district has 10 talukas. Nadiad taluka comprises of 100 villages. The total population of the taluka is 3,50,000. In 1983, bio-environmental control of malaria was started in 26,000 population residing in 7 villages (designated as complex A). In 1984, the experimental areas were extended to cover 14 more villages comprising of 34,000 population (designated as complex B) and the remaining 79 villages were designated as complex C where intervention work started in late 1985 (Fig. 1).

Control strategy

In the bio-environmental methodology of malaria control the following anti-vector and anti-parasitic measures were integrated with the objective to control malaria and reduce/eliminate mosquito nuisance.

(i) *Source reduction* was carried out on a weekly basis. All mosquito breeding sites such as pitchers, discarded tins, and other water receptacles were searched and emptied. Weekly surveys also helped in monitoring the intradomestic breeding. Well breeding was controlled by the application of expanded polystyrene (EPS) beads (Sharma, 1984; Sharma *et al.*, 1985 a). In each well about 1 to 2 kgs of EPS beads were applied.

(ii) *Minor engineering work* was carried out to fill and level pits, depressions and roadside ditches which may otherwise hold water. Earth was transported in trolleys pulled by tractors. Flowing water on the streets was eliminated by the introduction of soak pits. Leakages in taps and pipe lines were repaired.

(iii) *Biological Control.* There were a large number of abandoned ponds and cement tanks which were cleaned and converted to village hatcheries for the production of larvivorous fishes. Many ponds infested with water hyacinth were also cleaned manually for this purpose. Some ponds were also seeded with food fishes like the Chinese or common carp. These hatcheries were protected from predators and given fish food. All our requirement of larvivorous fishes was met by these hatcheries. In addition to this the Inland Fisheries Department supplied Guppy and *Aplocheilus* fishes. Margins of the ponds were kept clean mechanically to enable the fishes to feed on the larvae in the margins.

(iv) *Health Education and Community involvement.* Villagers were informed about malaria and the methods of its control. They were also informed of the intervention methods in which they could participate and eliminate mosquito breeding. Health education was also used in eliciting community participation in various anti-malaria activities and in bringing about awareness in the community. *Shram Dan* or free labour camps were organized to carry

out earthwork in which men, women and children participated. In addition to constant interaction with villagers health camps were also organized periodically.

(v) *Environmental improvement.* There were many marshy areas in the villages. One site was cleaned and converted to a playground. In many other areas Eucalyptus was planted to convert marshy areas into dry land. Large-scale plantation of a variety of trees was carried out in the waste land with the objective of making the land productive.

(vi) *Surveillance and treatment.* Weekly surveillance was carried out by workers hired on daily wage basis belonging to the same village. Initially, once a week, all surveillance workers were called for discussion and training. Slides were collected daily and sent to the laboratory for examination. The slides were fixed, stained with JSB and examined on the same day and radical treatment started within 24 hours. All cases of fever were given presumptive treatment of 600 mg chloroquine adult dose followed by radical treatment. *P. vivax* cases were given 15 mg primaquine daily for 5 days. *P. falciparum* cases were given a total of 1500 mg chloroquine adult dose (i.e., 300 mg daily for 3 days plus 45 mg primaquine on day 3). Children were given proportionately low dosages. In cases where radical treatment was delayed for reasons beyond our control 600 mg chloroquine was given on day 0 followed by radical treatment as described above.

(vii) *Mosquito densities* were monitored by hand catch method using suction tubes. Man Hour Density (MHD) was collected from study villages from 6 fixed and 6 random catching stations. Fixed catching stations included human dwellings, mixed dwellings, and cattlesheds (2 each). At each catching station anophelines were collected for 15 min. duration.

RESULTS

Intervention measures and their impact on disease transmission are described below.

Intervention measures

Source reduction. Routine surveys revealed a large number of water collection sites. Elimination of these sites which were potential mosquito breeding areas was given first priority (Fig. 2a,b). Borrow pits and puddles were filled with soil. Drains were cleaned periodically and water channelized into pits which were emptied on weekly basis. Surveys revealed that intradomestic breeding in discarded earthenware pots, tins and other containers was an important source of mosquito breeding including malaria vectors. All breeding in the containers was destroyed on weekly basis. During this operation, villagers (more particularly women and children) were shown mosquito breeding in these containers and also shown the simple method of destruction by turning the pots upside down. As a result of these demonstrations villagers became conscious of the mosquito breeding in their houses and in a few months they started to destroy it on their own. Intradomestic breeding was controlled in all study villages throughout the year.

Well surveys in the agricultural fields revealed that there may be about 1000 wells in Nadiad taluka. Most of these wells were disused and abandoned. Random checks showed that almost all the unused wells supported moderate to heavy *Culex* and occasionally *Anopheles* breeding. *A. culicifacies* and *A. stephensi* were found breeding in these wells. Well breeding was controlled by the application of expanded polystyrene (EPS) beads (Sharma *et al.*, 1985a). The introduction of EPS beads sealed the wells completely and there was no breeding in any well for an observation period of 2 years. In some wells which were used rather infrequently Guppy

Fig. 2(a): Waste water flowing on street.

Fig. 2(b): Source reduction.

Fig. 3: Soakage pit.

Fig. 4: Ditches eliminated by earthwork.

fishes were introduced. There was no breeding of mosquitoes in wells used regularly.

Minor engineering works. Nadiad villages have piped water supply. However, one could often see leaking pipes, taps and discharge of water from tube wells which created mosquito breeding sites. All these sources of water stagnation were repaired and breeding sites levelled with earthwork. These are now being checked routinely. In addition to this soak pits are being encouraged to eliminate flowing water on the streets and there is a great demand for this innovation in all the villages. During 1985, 80 soak pits were constructed in 6 villages (Fig. 3).

There are also many borrow pits and low-lying areas in the villages and fields which were common sites for the collection of rain water and at times they supported mosquito breeding. All these sites were levelled by earthwork. Many seepage areas were eliminated by earthwork (Fig. 4). During 1985, in 13 villages, 876 trolleys of soil in complex A and 900 trolleys of soil in complex B villages were transported for levelling the pits and other low-lying areas. This work is now continuing at a much faster speed and the number of tractors have been increased from 2 to 4.

Biological control. Introduction of Guppy fishes in all ponds, water troughs, rice fields, wells etc., was the only biological control method used in the experimental villages (Fig. 5a, b). Initially fish fauna surveys were carried out in all water bodies in the experimental villages. Identification of the indigenous fishes revealed that there were 21 types of fishes commonly encountered in the study villages. Laboratory tests showed that at least 14 species were larvivorous (Sharma *et al.*, in press). Local fish fauna also consisted of two well-known larvivorous fishes, i.e., Guppy and *Aplocheilus*. Guppies are being mass produced in hatcheries maintained by the Centre. Almost all village hatcheries were established in abandoned cement tanks and ponds. It was estimated that hatcheries established in 7 villages were enough to supply Guppies to another group of 20 to 25 villages.

Since Guppies, *Aplocheilus* and other larvivorous fishes were commonly found in village ponds, it was difficult to explain the presence of mosquito larvae in almost all ponds. However, discussions with the villagers and fishermen revealed that the ponds were being auctioned for fishing and the fishermen were collecting even the spawns and fries with the help of fine mesh nets; thus depleting the ponds of all the developmental stages of the fishes. These ponds supported heavy mosquito breeding. As a result of health education it has now been agreed that the size of fishing nets should be at least 2.5 to 3 sq cm mesh.

Another factor that contributed to mosquito breeding was the grassy margins where fishes were not able to feed because of shallow water. These margins are now cleaned periodically to enable the fishes to negotiate better (Fig. 6). During 1984 and 1985 a total of 4.27 million fishes, mainly Guppies, were introduced in the ponds of Nadiad village. Guppy and *Aplocheilus* are now found in all ponds and there is no mosquito breeding in the ponds, wells and cattle water troughs.

Health education. Educating the villagers about our programme and its lasting benefits was the main task of health educators. This was achieved by brief talks, discussions, demonstrations, video shows and exhibitions. Constant interaction with the community has dispelled ignorance and has brought about awareness and scientific temper. The demonstrations included fishes eating the mosquito larvae, microscopic view of the malaria parasite in the red blood cells, mosquitoes and their developmental stages and methods of protection from malaria. Frequently malaria clinics and health education camps were organized (Fig. 7). Dur-

ing 1984, a total of 27 clinics and health education camps were held. Each camp was attended by 100–200 people of all age groups. A large number of villagers also visited the MRC field station laboratories.

Occasionally, video shows on the ongoing activities of the Centre were organized which were very well attended. During 1985, a total of 93 demonstrations were held in the experimental villages and an estimated number of 18,000 villagers attended these demonstrations. Brief talks in schools and panchayats on the biology of mosquitoes, methods of malaria and mosquito control, how malaria is transmitted, what to do in case of fever, and how one can help control mosquito breeding were very useful in soliciting the help of the villagers in vector control activities. As a result of these demonstrations and health education activities the people's enthusiasm was awakened and their participation in all our activities was more widely accepted. During 1985, 517 visitors from 38 villages visited the laboratory to familiarise themselves with the research activities of the field station. Four video films were prepared by professionals and shown on the Ahmedabad TV network.

Community Involvement. This was an important component of the vector control strategy. Initially the staff was given indifferent treatment in the villages. In spite of this indifference, vector control work continued in the villages and emphasis was given to health education and treatment of malaria cases. Prompt detection and treatment infused confidence in the villagers and our acceptance in the community started to increase at a steady pace. Panchayat meetings were called to discuss MRC activities. This followed a closer interaction with almost all age groups of the village community. Women were particularly interested in cleanliness and obtaining treatment for themselves and protection for their children. So they invariably came forward for blood tests on their own.

Occasionally *Shram Dans* or free labour camps were organized. Men, women and children came out in large numbers to participate in *Shram Dan* (Fig. 8). *Shram Dan* was very helpful in converting our work into a movement which was the key to success. Those who could not participate in *Shram Dan* made cash donations. During 1985, cash donations made by the individuals, milk cooperatives and other agencies were Rs. 24,592 for the intervention work from 20 villages. Money was handled by the panchayats and donations are used to hire daily wage workers for vector control work by the panchayats. This spontaneous response of the people has become a very encouraging feature of community participation and a positive step towards self help in improving the environment.

For community involvement through health education, discussions were the turning point in motivating people. The villagers were advised to keep their surroundings clean and empty pits at least once a week. Women and children helped in the control of intradomestic breeding by making sure that no pot or discarded tin was left unturned. Over a period of time there was such a deep impact that MRC became a household name. In the first year a proposal made by the MRC to the village youth, (Yuvak Mandal) to clean the dumping ground and convert it into a playground was accepted as a challenge. Young boys came out in large numbers and converted the dumping ground into a beautiful cricket field. Similarly, a large number of young men cleaned ponds infested with hyacinth, helped in the introduction of larvivorous fishes and earthwork and many other activities related to vector control (Figs. 9 and 10).

Every village needed a lot of earthwork to fill the borrow pits and uneven/low-lying land. MRC had no resources for such massive development work which was possible only with community involvement. As an experiment, we hired a tractor with trolley and started to do the earthwork by hiring daily wage workers. This

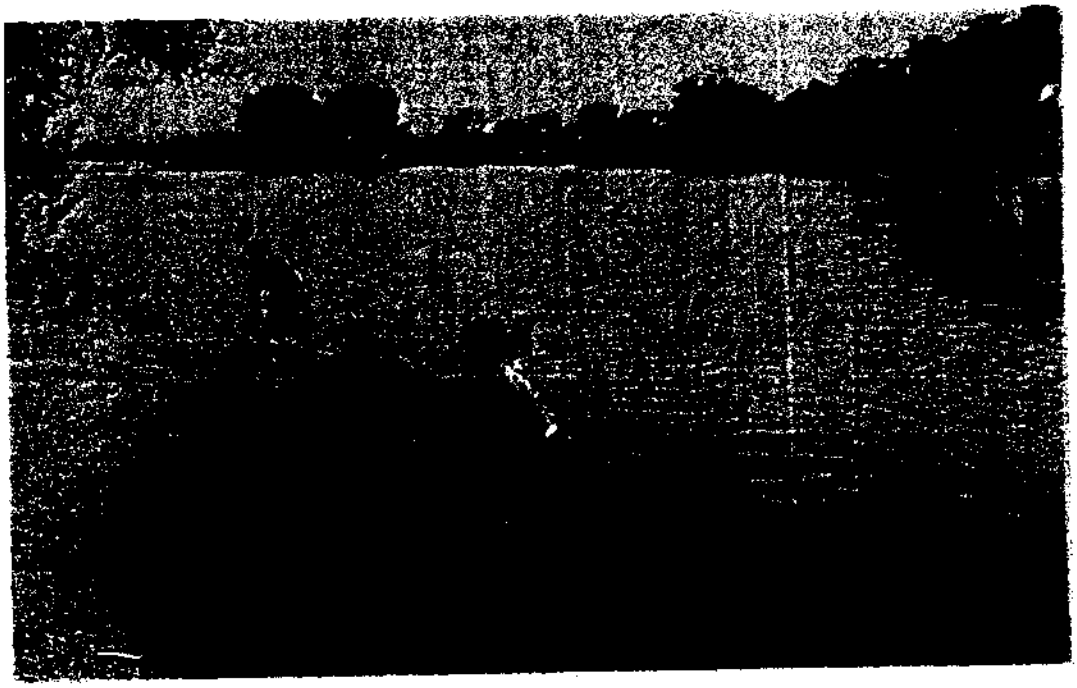

Fig. 5(a): Collection of Guppy fishes

Fig. 5(b): Larvivorous fishes being released in abandoned pond

Fig. 6: Margins of pond being cleaned.

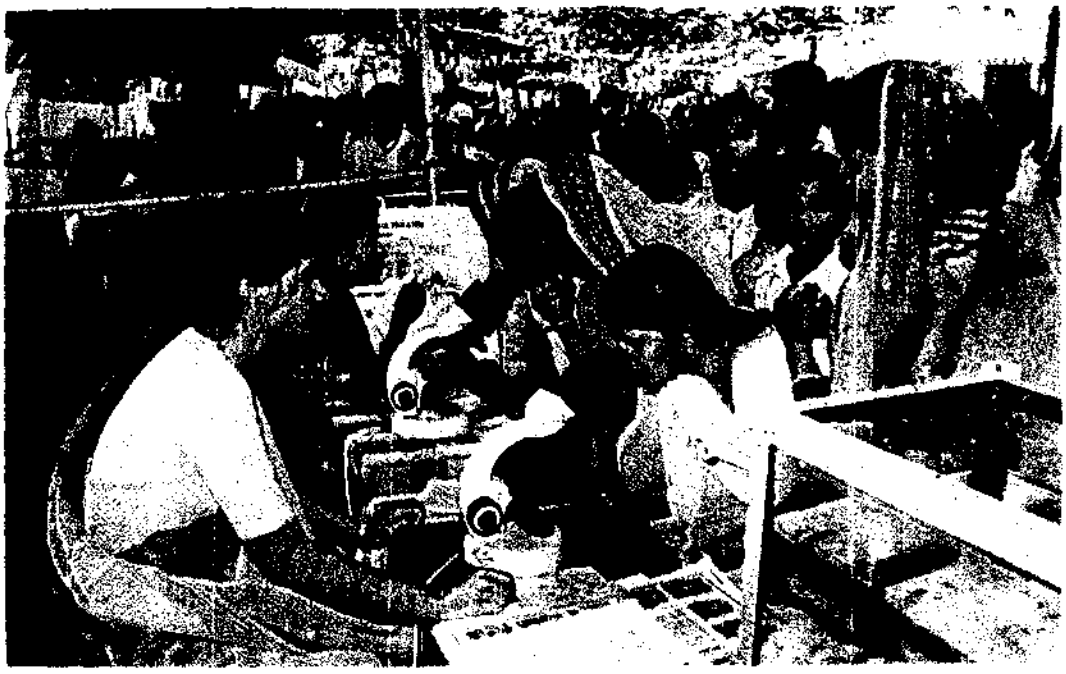

Fig. 7: Health education camp.

Fig. 8: Shram Dan — transporting earth to fill borrow pits.

Fig. 9: Water hyacinth being removed.

Fig. 10: NCC cadets participating in plantation.

Fig. 11: Tractor and trolley being used for earthwork.

gave the villagers an insight into what could be achieved by simple earthwork. Since labour was quite expensive, the villagers were encouraged to come forward for participation in field work. The village panchayats had no tractor and therefore a second breakthrough was unlikely to come unless this facility was made available to them. We, therefore, bought a tractor and a trolley. The tractor with driver and diesel was made available without charge to the villagers, provided they did the earthwork as per our advice. Expenditure on labour was incurred by the village panchayats. This turned out to be a very attractive proposal and demand for tractor started to increase. Now there are 4 tractors with at least 4 weeks of booking for each (Fig. 11). The village community can see the advantage of their own work and they do not need any more health education or any other kind of persuasion.

Impact assessment

Epidemiological observations

Parasitological surveys. As a result of bio-environmental control there was drastic reduction in the incidence of malaria in complex A and complex B villages as shown in Tables 1 and 2 and also by the epidemiological indices i.e., SPR and API (Table 3). In contrast the incidence of malaria remained extremely high even when the villages were sprayed with HCH/Malathion. There was, however, a considerable reduction in the incidence of malaria after DDT spraying. This may be due to the fact that DDT proved more effective as it was not sprayed in this area for a very long time. In addition to this poor surveillance may have also contributed to low recording of malaria cases.

Table 1: Incidence of malaria in complex A villages (7 Villages: Population 26,000)

Year	History of spray	Quarterly record of malaria cases				Total
		Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	
1981	HCH/Mal. sprayed	374	955	914	1797	4040
1982	HCH/Mal. sprayed	982	849	417	107	2355
1983	DDT sprayed	64	75	187	85	411
1984	Bioenvironmental control*	26	36	61	18	141
1985	Bioenvironmental control*	6	8	44	10	68

Table 2: Incidence of malaria in complex B villages (14 Villages: Population 34,000)

Year	History Of Spray	Quarterly record of malaria cases				Total
		Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	
1981	HCH/Mal. sprayed	214	586	754	477	2031
1982	HCH/Mal. sprayed	396	908	549	115	1968
1983	DDT sprayed	86	149	72	52	472
1984	DDT sprayed	17	33	72	21	143
1985	Bioenvironmental control*	4	29	48	5	86

*Without spraying.

Table 3: Epidemiological Indices
(Complex A & B Villages)

Year	Villages (Nos.)	Population	Malaria cases	ABER	SPR	API	SFR
Complex-A							
1984	Experimental (7)	26022	141 (64 Pf)	45.8	1.23 (−60%)	5.6 (−56.8%)	0.56 (−45.1%)
	Control (1)	5152	72 (23 Pf)	47.7	3.2	13.9	1.02
1985	Experimental (7)	26022	68 (28 Pf)	34.2	0.8	2.6 (−25.7%)	0.3 (−25.0%)
	Control (1)	5152	18 (9 Pf)	28.3	0.8	3.5	0.4
Complex-B							
1985	Experimental (14)	32697	86 (24 Pf)	34.6	0.7 (−50%)	2.6 (−27.8%)	0.2 (−33.4%)
	Control (2)	9357	34 (7 Pf)	25.9	1.4	3.6	0.3

Note: Figures in parentheses indicate % reduction compared to control villages.

Table 4: Results of spleen surveys

Year	Area	No. of children examined	No. of children with palpable spleen					Total	Spleen rate
			Grade I	Grade II	Grade III	Grade IV	Grade V		
1983	E	3933	200	115	11	1	-	327	8.3
	C	985	40	25	5	1		71	7.2
1984	E	2508		61	4	—	—	122	4.86
	C	607	10	12	—	—	—	22	3.62
1985	E	1334	15	8	4	—	—	27	2.02
	C*	498	4	—	—			4	0.8

1983 : Pre-intervention data
E : Experimental
C : Control
C* : included in the experimental areas
— : denotes not found

Table 5: Results of seroepidemiological surveys using IHA test

Year	Area	No. of samples tested	Titres									Sero positivity rate*
			<1:8	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	
1984	E (7 (villages)	3,767	2535	585	440	120	42	18	17	9	1	5.5
	C (22 villages)	10,017	5598	2061	1554	418	167	63	53	60	43	8.0
1985	E (7 villages)	2,540	1666	462	337	60	12	3	—	—	—	2.9
	C (22 villages)	3,190	1958	544	425	116	54	58	30	3	2	8.2

* Cut off titre 1:32

A study of the epidemiological indices also showed improvement in malaria situation in the experimental villages (Table 3). It may be noted that the annual blood examination rate (ABER) was very high in all the study villages (range 25.9 to 47.7%). ABER is generally about 10% in the normal activities of the NMEP, suggesting that MRC surveillance in Kheda was unlikely to have missed malaria cases in the study villages.

Spleen surveys. A study of children upto 10 years of age was carried out for spleen enlargement by Hackett's method. Results of the survey are given in Table 4. During 1983 survey, baseline data was collected by examining 3933 children from experimental and 985 from the control villages. The spleen rate was 8.3 and 7.2 with cases showing spleen enlargement upto grade IV indicating prolonged or repeated malaria infection. In 1984 spleen rate was low as compared to previous year, in the experimental and control villages. Also no case of grade IV spleen enlargement was found. This was perhaps the result of intensive weekly surveillance, prompt examination and radical treatment of all positive cases which was similar in the experimental and control villages. Spleen examinations during 1985 demonstrated a further decrease in splenomegaly in experimental villages and the spleen rate came down to 2.02. There was, therefore, a definite improvement in the malaria situation in the experimental villages.

Seroepidemiological surveys. Further confirmation of the impact of intervention measures was provided by the results of serological surveys given in Table 5. In 1984 samples collected from experimental and control villages for IHA test had titres upto 1:1024. During 1985 no sample had titres above 1:128 in the experimental villages whereas in the control villages titres upto 1:1024 were found. Seropositivity rate in 1985 in the experimental villages came down from 5.5 to 2.9 in one year at a cut-off titre of 1:32. At the same cut-off titre seropositivity rate in control villages remained constant in both years. Results clearly indicated a drop in the antibody titre in populations living in experimental villages due to low or lack of transmission.

Entomological observations

Monitoring of mosquito densities at the immature and adult stages continued on weekly basis. During 1985 mosquito breeding was controlled by source reduction in 7324 sites in complex A and 9114 sites in complex B villages. A study revealed that in complex A villages 1,23,193 containers were checked and 5196 were found positive (4.21%) whereas in the control village 45,286 were checked and 9748 were found positive (21.52%). In complex B villages 1,57,469 containers were checked and 2435 were found positive (1.5%) and in the control villages 19451 containers were checked and 826 were found positive (4.2%). There was therefore a definite reduction in the mosquito breeding sites as a result of the control of intradomestic breeding (Table 6).

Table 6: Results of intradomestic breeding surveys carried out on weekly basis during 1985

Villages	Sites checked		
	Total no.	Positive	% Range
Complex A			
Experimental	123193	5196	4.2(2.97-7.54)
Control	54286	9748	21.5(9.85-35.74)
Complex B			
Experimental	157469	2435	1.5(0.49-5.3)
Control	19451	826	4.2(0.81-11.2)

Average man hour densities of anophelines in 21 experimental and 3 control villages are shown in Table 7. There was great reduction in the densities of all anophelines in the experimental villages for the entire duration of 1985. A study of the densities in different structures showed that highest densities were found in cattlesheds followed by mixed dwellings and lowest den-

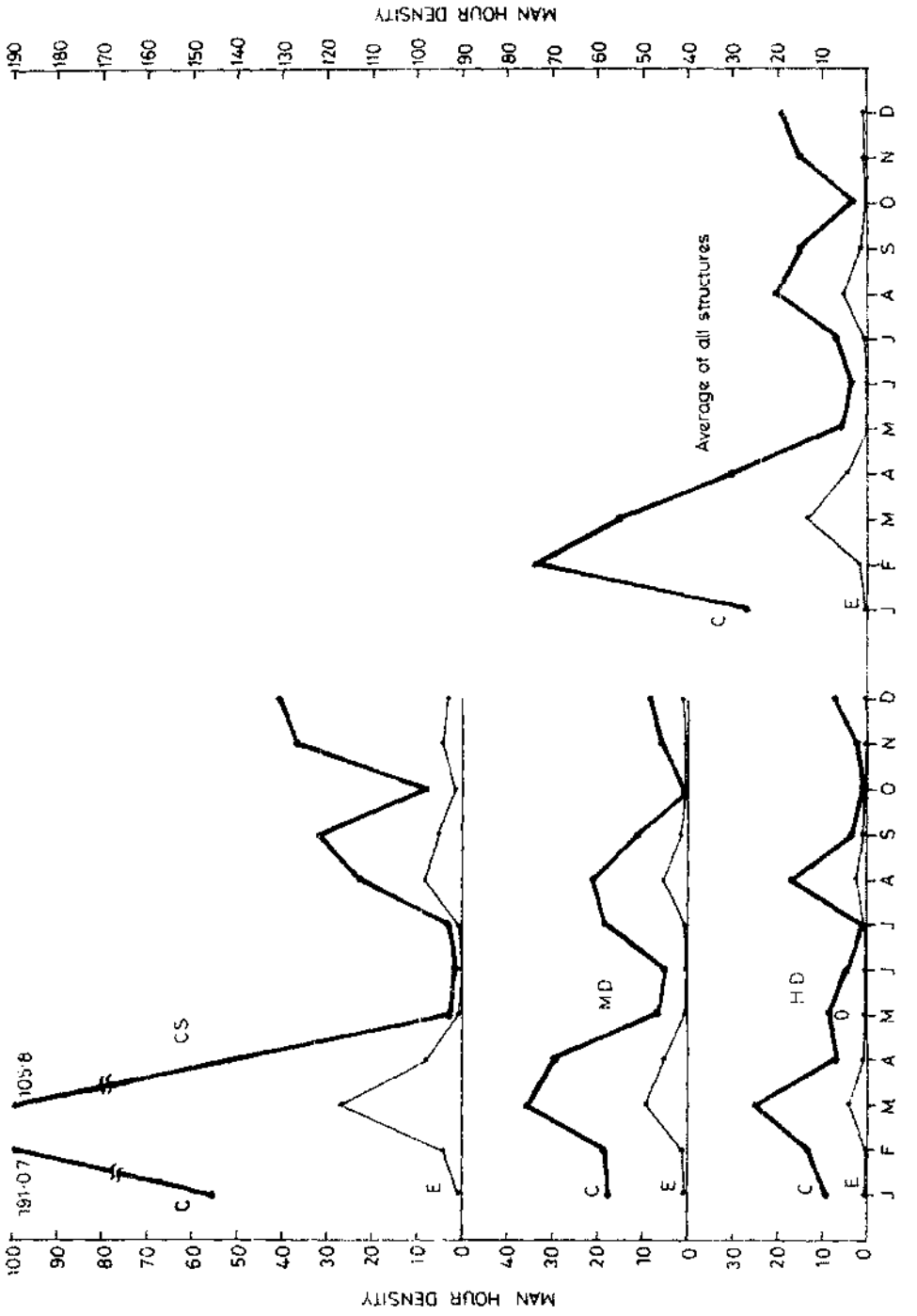

Fig. 12 Density of *A. culicifacies* in 21 experimental and 3 control villages in Nadiad taluka (1985)

Table 7: Man hour density of anopheline mosquitoes

Species	Area	1985											
		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
A. culicifacies	E	0.19	2.06	13.57	5.26	0.64	0.18	0.53	5.71	2.71	0.74	1.79	1.61
	C	27.83	74.41	55.61	30.21	6.44	4.33	7.66	20.1	15.55	3.55	15.33	19.0
A. stephensi	E	0.04	0.12	0.12	0.2	0.04	0.04	0.17	0.11	0.079	0.047	0.03	0.05
	C	1.66	2.76	1.72	0.44	0.11	0.33	0.44	0	0.11	0.33	0.11	0.33
A. annularis	E	2.81	1.84	3.35	4.06	1.76	1.38	0.41	2.02	1.76	0.66	4.51	5.0
	C	15.11	11.07	11.61	8.55	2.28	1.44	0.77	0.77	0.66	0.88	1.1	2.0
A. subpictus	E	4.6	6.822	75.49	80.12	42.76	26.6	12.57	455.4	110.41	53.52	89.88	24.74
	C	13.22	15.02	59.88	95.11	47.88	54.44	19.44	534.11	101.33	61.65	89.88	23.0
Others	E	0.09	0.037	0.014		0.01	0.01	0.03	0.031		0.03	0.15	0.0
	C	0.65	0.78	0.38			0.22					0.22	0.22
Total Density	E	7.73	10.88	92.54	89.64	45.21	28.21	13.71	463.27	114.96	55.00	96.36	31.41
	C	57.47	104.04	129.20	134.31	56.71	60.76	28.31	554.98	117.65	66.41	106.64	44.55

E = Experimental villages (21)
C = Control villages (3)

sities were found in human dwellings (Fig. 12). The densities of *A. culicifacies* in human dwellings were almost negligible in the experimental villages.

A. culicifacies is basically a zoophilic species and due to fall in population density the vector's host preference probably shifted to cattle, resulting in reduced disease transmission. The impact of intervention measures was also reflected in the change in proportion of different species of anophelines in the experimental villages. A total of 73,156 specimens were collected from 21 experimental villages and 15,954 from 3 control villages. Analysis of the data revealed that the proportion of *A. subpictus* has increased from 67.9 to 92.2% whereas there was a great reduction in the proportion of other anophelines, the most noticeable change being in *A. culicifacies* from 25.4 to 4.4% (Table 8). It was observed that the breeding sites of *A. culicifacies* were very extensive and therefore it was not possible to bring about a major reduction in its populations. However, it was possible to control the breeding of other anophelines. For this reason the proportion of *A. culicifacies* showed considerable increase.

Species specific breeding was studied to elicit information on the breeding potential of vectors and non-vectors inside and outside the houses. Immatures of anophelines were collected from

Table 8: Percentage composition of anophelines in Nadiad*

Mosquito species	Experimental villages (21) (specimens–73156)	Control villages (3) (specimens- 15954)
A. subpictus	92.2	67.9
A. culicifacies	4.4	25.4
A. annularis	3.3	5.4
A. stephensi	0.1	0.8
Other Anophelines	0.05	0.4

*Analysis of adults collected during 1985.

Table 9: Species specific breeding sites in Nadiad*

Breeding sites	Percentage (%) breeding				Others
	A. culicifacies	*A. stephensi*	*A. annularis*	*A. subpictus*	
PERIDOMESTIC					
Ponds	0.9	0.9	5.0	91.7	+
Ditches	0.2	0.8	0.3	98.2	-
Rivers	53.6	1.6	0.3	44.1	+
Wells	1.9	39.2	0.3	51.0	+
Canals	4.2	0	5.4	87.7	+
Rice fields	2.5	0	20.6	95.1	+
Hoof prints	5.0	1.4	0	94.6	-
INTRADOMESTIC					
Overhead tanks	1.9	69.0	0	29.1	
Underground tanks	13.5	38.2	0	48.3	
Outside tanks	0.3	62.0	0	37.7	
Inside tanks	0	95.6	0	4.4	
Pots etc.	4.3	78.8	0	16.9	
Barrels etc.	3.8	7.2	0	89.3	

* Duration of study April to December 1985.

breeding sites. Field collected specimens were held in the laboratory for identification at the adult stage. Results of this study are given in Table 9. It was interesting to note that no anopheline except *A. culicifacies*, *A. stephensi* and *A. subpictus* was found breeding inside houses. Of all the breeding sites rivers and tributaries supported maximum *A. culicifacies* breeding. *A. stephensi* prefers to breed inside houses in various types of receptacles. Tanks and wells are the common peridomestic breeding habitats. Therefore, for effective vector control based on anti-larval methods, control of mosquito breeding in the river and its tributaries, wells and intradomestic containers should receive priority.

Income generating and environmental improvement schemes

Social forestry has been introduced in the experimental villages with the aim to plant Eucalyptus in the marshy areas and other productive trees in waste land (Fig. 13). In 1984, 70,000 and in 1985, 1,14,000 trees were planted. The demand for trees has increased manifold. A compilation of demands from individuals and panchayats showed that in 1986, 6 million saplings would be required for Nadiad taluka. It was not possible to arrange such large requirements from the forest department or any other source. Therefore, in-house and panchayat nurseries are being encouraged (Fig. 14). The village communities can carry out plantations of their choice independently.

Experiments in the laboratory and outdoor ponds showed that Guppy, common carp, major carp (Rohu, Katla and Mrigal) and prawns were compatible and can be grown together (Sharma *et al.*, in press). Ponds which were a source of mosquito breeding could therefore be used for the production of food fishes and prawns, and at the same time mosquito breeding could be eliminated. The villagers were given this proposal which was immediately accepted. The village panchayats gave 8 ponds (1 to 3 hectare each) to MRC on 5 year lease without any condition or payment. These ponds are now being experimented on for optimizing fish production. The ponds were cleaned; 2,25,560 fries and 10,250 fingerlings of major carp and 40,000 juvenile prawns were introduced. These fishes and prawns developed

Fig. 13: Productive trees planted in wasteland

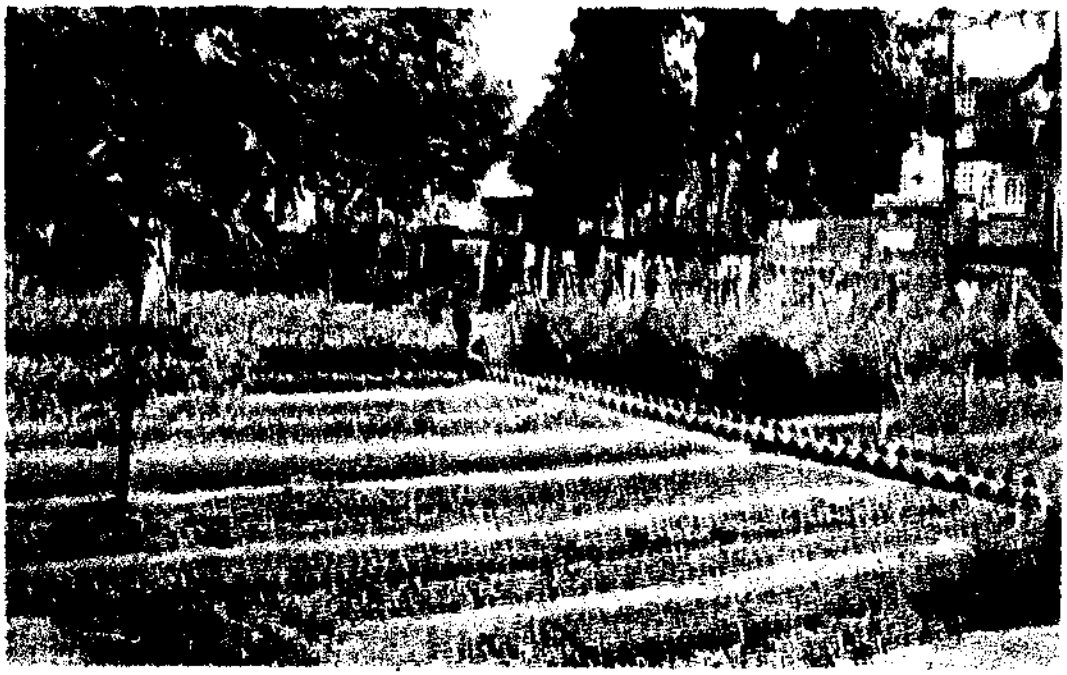

Fig. 14: Village nursery.

Fig. 15: Partial fish yield of one pond.

Fig. 16: Application of EPS beads on slurry of Gobar gas plant.

extremely well (Fig. 15). Despite large-scale thefts fishes were auctioned for about Rs. 1,00,000 of which one pond above was auctioned for Rs 40,000 for a one year period (Sharma *et al.*, in press). The economic development of villages should have a synergistic effect on health programmes and human development (Ramalingaswami, 1984).

Beside social forestry, the Department of Non-conventional Energy Sources is collaborating with the Gujarat Energy Development Agency for assistance in the popularisation of smokeless chulahs, biogas plants, solar cooker etc. It is envisaged that in the coming years these alternate sources of energy would become popular in the countryside (Fig. 16).

DISCUSSION

The strategy of malaria control in rural areas is to achieve interruption of transmission by spraying residual insecticides. Insecticidal spraying is losing its impact for a variety of reasons viz., development of insecticide resistance, lack of collateral benefits of spraying, exophilic and exophagic behaviour of vectors, environmental contamination and adverse effect on beneficial fauna. At present the problem of insecticide resistance is being tackled by using replacement insecticides. DDT is used in areas where the vector is still susceptible to DDT but in 112 million population HCH is used to overcome DDT resistance, similarly in 22 million population malathion is used to overcome resistance to DDT and HCH. Unfortunately some degree of resistance has also developed to malathion thereby making it ineffective, largely in parts of Gujarat and Maharashtra. The strategy of the use of replacement insecticides is very expensive e.g., in 1986 cost of spraying insecticides to cover 1 million population with DDT, HCH and malathion is Rs. 34, 37 and 199 lakhs respectively.

Therefore, increase in the cost of insecticides alone would make residual spraying prohibitive. It may also be noted that the eradication of malaria was not possible because of many epidemiological and environmental reasons and it was scrapped in favour of control programme under the Modified Plan of Operation in 1977 (Pattanayak and Roy, 1980). Malaria control, therefore, is a long-term commitment and methods would have to be evolved to combat malaria within the available fund allocations. Malaria cases have gradually stabilized to about 2 million during the mid 1980s. 30% of these are due to *P. falciparum*. Several studies have revealed that if spraying is not effective or a good spraying is not done, malaria strikes back with vengeance (Ansari *et al.*, 1984; Sharma *et al.*, 1985a,b; Malhotra *et al.*, 1985a,b). There is therefore an urgent need to minimize our dependence on insecticides and work out an alternate strategy that would be environmentally sound. With this background importance of the bio-environmental control of malaria in Kheda comes out strongly.

The alternate strategy combines simple and inexpensive methods of the control of mosquito breeding supported by chemotherapy. Thus, the transmission chain is broken by attack on the mosquito and the parasite with the involvement of the community. Health education is therefore an important component of the programme. The method brings about semi-permanent to permanent changes in the environment thus making the areas non-conducive to disease multiplication. The elimination of mosquito breeding sites also reduces mosquito nuisance which may transmit other diseases such as Japanese encephalitis, dengue, filaria etc.

The Kheda experiment has demonstrated that confidence generated in the communities could be used as an entry point for other developmental schemes in a holistic approach for overall development and a healthy environment. The introduction of environmental improvement schemes of social forestry, improved chulahs (stoves), soak pits, playgrounds, parks

etc., have been of great value in improving the environment and sustaining the interest of the community. Simultaneously, efforts were made to launch schemes to improve the village economy. Village ponds that were one of the main sources of mosquito breeding were utilized for the production of food fishes and prawns. Large-scale plantations under the social forestry scheme were taken up to utilize the waste land. It was demonstrated that one scheme alone if carried out on scientific lines can pick up the entire cost of malaria control for Nadiad taluka, and there would be enough savings to provide support to other developmental activities (Sharma and Sharma, 1986). As an example during 1986 money earned from fish production was diverted to lay an underground sewage system in one village.

The strategy of bio-environmental control of malaria as developed could be extended to many areas with similar terrain and agroclimatic conditions but may not be universally applicable. The strategy would have to be tailored to each malariogenic stratum depending upon agro-climatic, entomological and epidemiological considerations. In some areas it may be necessary to include a limited role of insecticides, while spraying strategy may be held in reserve for situations which are not amenable to control by alternate strategy or where malaria outbreaks are imminent. The study has clearly shown that our philosophy of vector control should change in favour of alternate strategy which should be the first line of attack followed by bio-environmental control in which insecticides would have a limited role to play.

Such a change would not require any additional financial commitments since the cost-effectiveness of alternate strategy is comparable to the expenditure being incurred on malaria control using DDT and HCH. It is noteworthy to mention that the alternate strategy would cost about 15% of the expenditure being incurred in areas under malathion spraying. Therefore, areas under malathion spraying (about 22 million population) should be tackled in the first phase. This would need large trained manpower at all levels and training facilities should be developed for such a change, while research should be simultaneously intensified to develop methods for other malaria endemic zones. This would also provide an opportunity to test the large-scale operational feasibility of the alternate strategy under the primary health care system.

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Host Preference of *Anopheles annularis* in Different Biotopes

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Blood meal analysis of *Anopheles annularis* in two different biotopes was performed by capturing mosquitoes from cowsheds and human habitations. The human blood index of *An. annularis* collected from human habitations is as high as 21.4%, indicating that a large number of this species have got human contact.

INTRODUCTION

In view of the resurgence of malaria in rural West Bengal, there is a renewed interest on host preference of *An. annularis*, the mosquito species known to be responsible for transmission of malaria in rural West Bengal in recent years (Ghosh *et al.*, 1985). Hence a study on blood meal analysis of *An. annularis* in two different biotopes was conducted by capturing mosquitoes from cowsheds and human dwellings.

MATERIAL AND METHODS

Anopheles annularis mosquitoes were caught regularly during January '81 to June '82 with the help of test tubes and battery operated torches from cowsheds and human dwellings (mud huts), situated side by side in Gurap village of Hooghly district 70 km away from the central laboratory situated at School of Tropical Medicine, Calcutta. Collection methods were selected from those suggested by the WHO Expert Committee on Malaria (1962, 1964) and Holstein (1954). No insecticide had been sprayed there for the last 20 years. Fully fed females of *An. annularis* were selected for blood meal analysis so that no difficulty would be encountered in conducting the test. The stomach contents of the females were dissected with a pair of needles and the abdominal contents were squeezed on to the filter paper. The remaining carcass of the mosquito was discarded. A fresh pair of dissecting needles was used for each dissection. The blood smears of these mosquitoes were separated by cutting the blood spots stamped on filter papers and these were arranged in serology tubes to which a small quantity of normal saline (0.05 ml) was added.

The blood meal analysis was performed in wells prepared on glass slide with agar gel by modified Ouchterlony gel diffusion method (Ouchterlony *et al.*, 1973) as adapted by Collins *et al.* (1983). The above mentioned method was employed for

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its distinctive advantages; (i) the assurance of equal consideration to all the host species, as the tests of all the blood meals were set up before any results could be known, (ii) even a haemolysed or cloudy antiserum does not interfere visually with the reading, (iii) an unfiltered, uncentrifuged crushed saline extract of the mosquito does not hamper the clarity of precipitating band at the zone of equilibrium, and (iv) its ability to preserve the slides for permanent recording of the result. Further, agar gel diffusion technique, when carried out over a bigger slide, can accomodate a larger number of samples giving an opportunity to compare the results of the blood meal analysis from different hosts in different mosquitoes as well as mixed sources. The antisera used in the analysis were prepared by the laboratory of the Serologist and Chemical Examiner to the Government of India, Calcutta. The samples were run against antihuman, antibovine, antiporcine and antiavian sera.

RESULTS

Blood meal analysis of 469 *An. annularis* was performed, of which 240 were taken from cowsheds and 229 from human habitations. In the overall analysis of blood meal of *An. annularis* caught irrespective of biotopes, bovine blood and human blood indices were 76.3% and 14.5% respectively (Table 1), the difference was highly significant. But when blood meal analysis was done according to different biotopes, the results were quite different. In cowsheds the human blood index of *An. annularis* was only 7.9%, while it was 21.4% in human dwellings (Table 1), the difference was highly significant. The same was true in the case of bovine blood index (83.3% in cowsheds and 69% in human dwellings). A total of 4.3% of *An. annularis* took porcine blood and 0.6% of this species took avian blood. Taken as a whole, altogether 2.5% of *An. annularis* fed on both man and cattle, 1.5% on cattle and pigs and 0.2% on pigs and birds.

Statistical significance of the differences between the two biotope classes with respect to blood meal was tested through Z (normal deviate) test and the results show that there is marked difference in case of human and bovine blood while no difference was observed in porcine blood.

DISCUSSION

The blood meal tested included 51.2% of the total from cowsheds and 48.8% from human habitations, showing the bias in favour of the available host, which of course represents the population to a certain degree.

An. annularis has been previously reported to

Table 1: Blood meal analysis of *Anopheles annularis* in different biotopes

Blood smears	Cowsheds		Human habitations		Total	Percentage
	No.	%	No.	%		
Bovine	200	83.3	158	69.0	358	76.3
Human	19	7.9	49	21.4	68	14.5
Porcine	10	4.2	10	4.4	20	4.3
Avian	2	0.8	1	0.4	3	0.6
Bovine and human	1	0.4	11	4.8	12	2.5
Bovine and porcine	7	2.9	--	--	7	1.5
Bovine and avian	1	0.4	-	—	1	0.2
Total	240	100.0	229	100.0	469	100.0

have an anthropophilic index of 0% (Afridi *et al.*, 1939). Senior White (1947) found anthropophilic indices of 1.3% and 1.8% in Orissa and Madhya Pradesh respectively, while in the present study attraction to man has been significantly increased. The data presented at the V International Parasitology Congress in Toronto (Ghosh and Hati, 1982) revealed the HBI of *An. annularis* as 19% in human habitations. In another study in West Bengal (Bhattacharya *et al.*, 1982), 27.5% HBI was obtained when the mosquitoes were collected from human dwellings. The important part in the context of the resurgence of malaria in West Bengal is that in recent years high anthropophilic indices are being seen for this species, while in the pre-control era very low indices had been reported. However, none of the earlier reports is from West Bengal. Bruce-Chwatt *et al.* (1966) report its high HBI (25.1%) when collected from human habitations from Andhra Pradesh, Assam, Bihar, Madhya Pradesh, Mysore and Uttar Pradesh in India. It seems that the choice of host of *An. annularis* has changed considerably in recent years.

The HBI of *An. annularis collected from human* habitations is as high as 21.4%, indicating that a large number of this species have got human contact. In other words man-vector contact regarding this species has definitely increased in recent years, which may reasonably influence the epidemiology of transmission of malaria in rural West Bengal.

Another interesting finding is the presence of mixed blood meal in a percentage of the *An. annularis* population. It is probable that some of them were driven out while feeding and later came to suck the blood of other host/s after the first feeding. This study points out that the host preference of *An. annularis* varies widely in different biotopes. In human habitations the chance of getting human blood is more than that in cowsheds. This indicates a greater facility to transmit disease in such surroundings.

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Cell-mediated Immune Responses in Drug-suppressed Simian *Plasmodium knowlesi* Infection

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Rhesus monkeys (*Macaca mulatta*) were immunized by repeated drug-suppressed *Plasmodium knowlesi* infection. Leucocyte migration inhibition (LMI) test was performed to assess the specific T-cell mediated immune response at recovery phase of infection. The soluble extract of the whole parasite was used as antigen for LMI test. The animals which recovered from infection showed *in vitro* inhibition of leucocyte migration. Antigens eliciting the protective immune response would probably improve the accuracy and sensitivity of LMI test as an indicator of protective immunity.

INTRODUCTION

Acquired immune response is an assemblance of a series of immunological changes in a previously susceptible host, associated with parasitaemia and represent the host's defensive mechanism to eliminate parasites or to restrict the clinical effects of parasitaemia. Several studies on experimental models exhibited that cell mediated immune responses are involved in protective immunity to malaria, particularly to the asexual erythrocytic stages. Experimental evidence indicates that T-lymphocytes are required for the development of effective immunity. Macrophages are activated during malaria infection and release some products which are capable of killing intra-erythrocytic parasites (Ockenhouse and Shear, 1983). The activation of macrophages is controlled by T-cells through the mechanism involving a cascade of lymphokines. There is good evidence from several studies that CMI may correlate more closely with immune status than do antibody levels (Brown *et al.*, 1970; Fugui and Allison, 1979). Studies on lymphocytes from 15 patients who had been infected with *P. falciparum* from one month to 15 years have shown a significant proliferation *in vitro* in response to *P. falciparum* antigen prepared from *Aotus* erythrocytes (Wyler and Oppenheim, 1974). In immune individuals blastogenic transformation of T-cells was maintained for a long period and high levels of γ interferon were secreted. The importance of cellular mechanisms of immunity is demonstrable in the present study on experimental *P. knowlesi* infection by using LMI test as a tool for measurement of specific T-cell mediated response.

MATERIAL AND METHODS

Animals

Male rhesus monkeys (*Macaca mulatta*) weighing 3–4 kg were used for the study. Before starting the experiment the animals were kept

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under quarantine and were screened for tuberculosis. A natural diet consisting of fresh fruit, vegetables, salts, minerals, vitamins, nuts and soaked gram was given during the day. Water was provided *ad libitum*.

Strain of parasite

Plasmodium knowlesi W1 variant was obtained from Guy's Hospital Medical School, London, U.K. The parasite was stored in cryopreservative at −70°C. The frozen parasite stabilate was revived by inoculation of the parasite material intravenously into normal rhesus monkeys.

Infection and collection of sera

Rhesus monkeys were injected intravenously with 1×10^4 parasitized erythrocytes collected from donors in sterile 0.15 M sodium citrate. The course of infection was monitored by the examination of thick and thin smears of blood. When the parasitaemia reached around 15 to 25 per cent, the animals were cured with chloroquine phosphate, first dose 20 mg per kg and second dose 15 mg per kg body weight on the next day. These animals were infected after 30 days with the same strain and cured until self recovery at each recovery phase of infection for the study.

Preparation of antigen

Rhesus monkeys were infected with *P. knowlesi* as described. When parasitaemia reached about 60 to 70 per cent, the animals were anaesthetized by intravenously injecting sodium pentobarbitone (30 mg per kg body weight) and blood was collected in 0.15 M sodium citrate. The plasma and buffy coat were removed by centrifugation, erythrocytes were washed with normal saline till the supernate was clear. Erythrocyte pellet was then mixed with 0.15 per cent saponin solution (10 times over the volume of RBC's) and the mixture was incubated in a 37°C water bath for 20 minutes with occasional stirring. The suspension was then centrifuged in cold at 10,000 rpm (8,000 g). The supernate was discarded and the pellet was washed repeatedly with phosphate buffered saline to remove haemoglobin and excess saponin.

The saponin released parasites were sonicated at 10 μA for 30 seconds in MSE Soniprep 150 and then centrifuged in cold at 10,000 rpm (8,000 g). The soluble extract was used for the study.

Leucocyte Migration Inhibition (LMI) test for assessment of cell-mediated immune response

The test was performed under sterile conditions. About 4 ml of peripheral blood was drawn from each monkey in 200 units of heparin and poured in a tube containing 1 ml of 3 per cent dextran in normal saline. The mixture was allowed to stand at 37°C for 40 minutes. Erythrocytes were gradually sedimented by keeping the tube in a slanting position. Leucocyte rich plasma was aspirated and spun at 900 rpm. for 5 minutes at room temperature. The pellet was washed thrice in medium RPMI 1640. Leucocytes were suspended in the enriched media (RPMI 1640 with 5 per cent foetal calf serum) containing gentamycin at a concentration of 40 μg per ml. The final concentration of leucocytes for the test was fixed at 15×10^6 cells per ml. Capillaries of volume 80 μl were filled with this leucocyte suspension and one end of the capillaries was sealed with plasticine, then these were centrifuged at 900 rpm for 5 minutes. The capillaries were then cut at the cell-medium interface and mounted in perspex chambers of capacity 1.5 ml each. Enriched medium with antibiotics was added in control chambers and medium mixed with antigen (protein concentration 50 μg per ml) was used for test chambers. The chambers were made airtight with cover-slips and incubated at 37°C for 18 hours. The area of migration of leucocytes was recorded with the help of camera lucida. The percentage of migration-inhibition in antigen incorporated chambers was calculated by the formula:

Percentage of migration inhibition = 100

$$\frac{\text{Area of migration in test chambers (with antigen)}}{\text{Area of migration in control chambers}} \times 100$$

RESULTS

After repeated challenges with *P. knowlesi*, Rhesus monkeys developed protective immunity. The animals were grouped in three according to their protective immune responses. First group of animals (Nos. 432, 436 and 502) survived the second bout of infection. The animals which belonged to the second group (Nos. 125, 127, 128, 504, 505, 507 and 509) recovered naturally on third bout of infection and two animals (Nos. 126 and 129) of the third group had three bouts of drug-suppressed infections, then on fourth challenge they developed immunity.

The specific T-cell mediated response in these animals has been performed by leucocyte migration inhibition test. Blood was collected from these animals just prior to infection. Table 1 shows the bouts of infection, protection obtained on the challenge bout, cumulative parasitaemia in total exposure and percentage of leucocyte migration inhibition in twelve rhesus monkeys. *P. knowlesi* antigen having a protein concentration of 50 μg per ml was found to be non-toxic to normal leucocytes by comparing with the areas of migration in control and antigen chambers. The test has been performed on zero day before giving the first infection and at each recovery phase. An inhibition in leucocyte migration has been observed which may prove that in malaria immunity, the role played by T-cells is important.

DISCUSSION

The immune system produces both humoral and cell mediated responses to malaria as a result of immunization with malarial antigen or by infection. Both the types of responses may be required for a fully expressed immune response

Table 1. T-cell mediated immune response in 12 monkeys on which leucocyte migration inhibition test was performed

Monkey Number	Number of bouts given	Showed protection bout	Cumulative parasitaemia	Percentage of Leucocyte Migration Inhibition				
				Base-line, Day-0	Before giving 2nd bout, Day-30	Before giving 3rd bout, Day-60	Before giving 4th bout, Day-90	After 4th exposure, Day-120
432			10.090	1.2	10.5	15.6		
436	3	2nd	6.188	0.5	9.5	18.0	--	--
502			8.735	1.4	10.0	16.0		
125			5.170	0	2.7	10.6	13.4	
127			5.900	1.1	3.4	7.2	12.1	
128			6.228	0	2.2	6.2	8.8	
504	3	3rd	6.699	0.3	3.0	11.0	12.2	-
505			10.895	0	6.7	15.5	16.5	
507			12.770	1	2.0	11.2	10.2	
509			8.050	0	4.4	13.2	17.0	
126	4	4th	11.005	1.2	3.4	10.4	12.5	13.5
129			9.390	0.5	3.3	8.9	9.2	11.2

Note: Non-toxicity of the antigen at the protein concentration of 50 μg per ml was tested in a group of 15 monkeys. An average of 5 per cent migration inhibition was taken as cut-off point. Percentage migration inhibition of leucocytes in experimental animals was recorded after subtracting from the cut-off value on day '0' to subsequent experimenting days.

(Brown *et al.*, 1970). The participation of thymus derived lymphocytes in the development of protective immunity to malaria has been shown by various workers (Spitalny *et al.*, 1977; Brown, 1976; Cohen and Langhorne, 1981) in rodent, avian and human malarias. T-cell responses to homologous parasite antigens have been demonstrated by delayed skin reactions and by *in vitro* blastogenesis in simian, human and rodent malarias (Cottrell *et al.*, 1978; Phillips *et al.*, 1970; Wyler and Oppenheim, 1974; Weissburger and Spira, 1978). In the present study leucocyte migration inhibition test was performed to assess the specific T-cell mediated immune response in animals immunized by drug suppressed infection. The animals that recovered or with a partial protective immunity to infection showed migration inhibition of leucocytes *in vitro* when tested with homologous antigen preparations . The soluble extract of the whole parasite contains a wide variety of antigens and include mitogenic components. After the exposure to parasites and drug recovery a certain amount of inhibition has been observed in the animals. Animals which survived the challenge infection showed inhibition in migration of leucocytes but percentage inhibition was low. Antigen used for the test consists of a mixture or battery of antigens, the particular antigens which elicit the protective immune response may be masked by other antigens. Ravindran *et al.* (1983) have shown that LMI test can be a parameter to monitor the cell mediated immune response against human *P. vivax* infection. From the present study it can be cited that use of the antigens which elicit protective response would probably improve the accuracy of the test as an indicator of protective immunity.

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Village Scale Trial of the Impact of Deltamethrin (K-othrine) Spraying in Areas with DDT and HCH Resistant *Anopheles culicifacies*

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A synthetic pyrethroid Deltamethrin (trade name K-othrine) with residual toxicity was sprayed in malaria endemic village with vector *A. culicifacies* resistant to DDT and HCH. The spraying was carried out @ 12.5 mg/m² and compared with HCH spraying @ 200 mg/m². Results of the 3 rounds sprayed in both the villages revealed that K-othrine spraying suppressed the vector populations and greatly reduced malaria transmission. In contrast spraying of HCH did not produce satisfactory results in either reducing the vector densities or the incidence of malaria.

INTRODUCTION

Anopheles culicifacies is a principal vector of malaria and responsible for bulk of malaria transmission in rural and semi-urban areas of the country. The vector has become resistant to DDT and HCH in most parts of the country, while in Gujarat and Maharashtra it has also become resistant to malathion (Sharma *et al.*, 1982, 1986). There is therefore an urgent need in our armamentorium of a replacement insecticide to control the double or multiresistant *A. culicifacies* populations. We report the results of a field trial with synthetic pyrethroid Deltamethrin marketed by the trade name of K-othrine by Roussel Uclaf. The study was carried out in Bhanera village of Ghaziabad district (U.P.) on *A. culicifacies* populations resistant to DDT and HCH. Results of this study are reported in this paper.

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MATERIAL AND METHODS

Deltamethrin, (s)-α-cyanon-phenoxybenzyl (1R, 3R)-3 (2,2-dibromovinyl)-2, 2-dimethylcyclopropane carboxylate is a synthetic pyrethroid with residual toxicity. The water dispersible powder (wdp) formulation containing 2.5% active ingredient (ai) was obtained gratis through the courtesy of M/s. Roussel Pharmaceuticals (India) Ltd. The trial was carried out in a relatively isolated village with high vector population and intense malaria transmission. Experimental village Bhanera is situated on the bank of Hindon river in Loni PHC, District Ghaziabad (U.P.). The village has 972 human population, 128 houses and 65 cattlesheds. Most of the houses are made of bricks while cattlesheds are made of mud with thatched roofs. The pre-monsoon period (April to June) was extremely hot and dry, while the

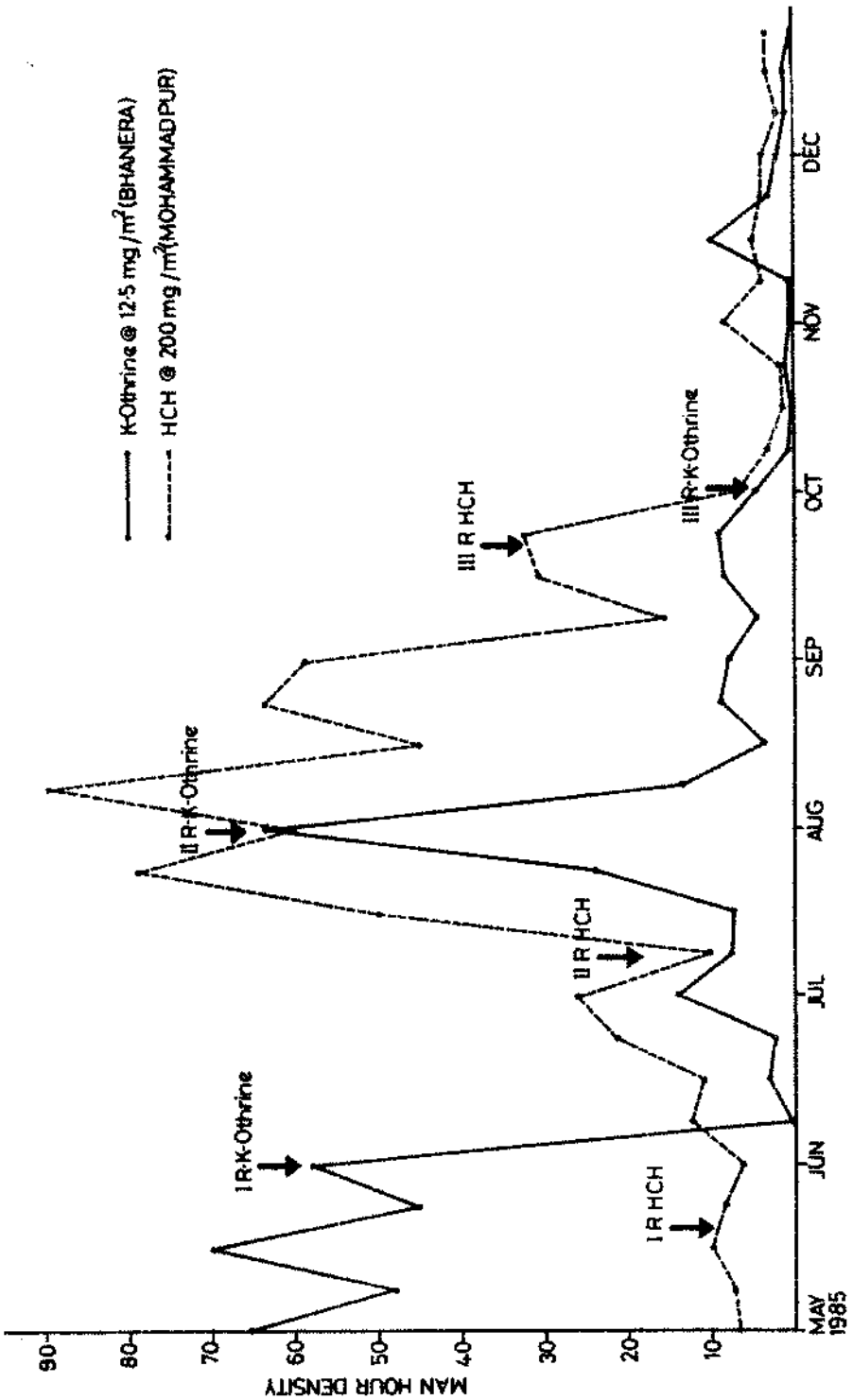

Fig. 1: Density of *A. culicifacies* in Bhanera and Mohammadpur villages.

post-monsoon period (October to November) was moderately hot and humid. Major crops cultivated in the area are wheat, rice, maize and vegetables.

The impact of spraying was compared with a control village Mohammadpur. It is located on the same river at a distance of about 20 kms from Muradnagar PHC. This village is small in size, isolated and with comparable agro-climatic conditions. In this village HCH was sprayed by the National Malaria Eradication Programme (NMEP) @ 200 mg/m^2. Both the experimental and control villages were being sprayed with HCH for about 8 years before the start of field trials with deltamethrin.

Residual spraying of deltamethrin 2.5% (wdp) was started in June 1985. Spraying was carried out with the help of stirrup pumps at a dose of 12.5 mg/m^2. Three rounds were sprayed i.e., one each in the month of June, August and October 1985. Efforts were made to maximize coverage and all houses and cattlesheds were sprayed in each round. A total of 18.5 kg 2.5% wdp K-othrine was used in each round to cover 36649/m^2 surface area. The discharge rate of the spray was maintained at 780 mg/40 stroke min.

The density of indoor resting *A. culicifacies* was monitored on weekly basis in the control and sprayed villages. Active surveillance was carried out daily by visiting every house throughout the study period. Every fever case was given presumptive treatment (600 mg chloroquine) followed by radical treatment to the parasite positive cases as per the anti-malaria drug policy of the NMEP (Sharma, 1984).

RESULTS AND DISCUSSION

Results revealed (Fig. 1) that pre-spray densities of *A. culicifacies* were extremely high in the experimental village (7 to 8 times more) as compared to the control village. Soon after K-othrine spraying there was a dramatic reduction in *A. culicifacies* densities. The pre-spray *A. culicifacies* per man hour densities were reduced to 0.5 from 58.0 within 1 week of spraying. No such reduction was observed in the control village sprayed with HCH. *A. culicifacies* populations remained at very low level upto 3rd week and gradually started to increase in the 4th week and reached high numbers by the end of July. The 2nd round of spraying in August again produced a similar impact on vector densities. The impact of spraying lasted till the end of September. As compared to the K-othrine sprayed village, vector densities in the control village continued to show an upward trend despite the 1st and 2nd round of HCH spraying and attained a peak in the month of August. The third round of spraying reduced vector densities to very low levels in the K-othrine and HCH sprayed villages. In the third round HCH spraying produced better impact compared to the first and second rounds. This may be due to a natural decline in vector densities, and this trend was common in both HCH and K-othrine sprayed villages.

The epidemiological indices of HCH sprayed (control) and K-othrine sprayed (treated) villages are given in Table I. There was reduction in malaria incidence as revealed by the epidemiological indices. Total malaria cases were reduced by 62.7% (50% Pv and 70.4% Pf). The SPR and SfR was also reduced by 29.8% and 44.9% respectively. It is noteworthy to mention that during the peak transmission season of falciparum malaria (August to October) there were 91 cases of falciparum malaria in the control village as against 26 in the K-othrine sprayed village. Malaria cases started to decline in the control and experimental villages with the onset of winters. It was however not possible to achieve complete interruption of malaria transmission even in the experimental village which may require an extended spraying schedule during the subsequent years. It may be noted

Table 1. Epidemiological indices of Mohammadpur and Bhanera villages

Month (1985)	Mohammadpur* HCH @ 200 mg/m² (Population 860)							Bhanera** K-othrine @ 12.5 mg/m² (Population 972)						
	B.S. coll.	BER	Pv	Pf	Total	SPR	SFR	B.S. coll.	BER	Pv	Pf	Total	SPR	SFR
May	60	6.9	10	1	11	18.3	1.6	38	3.9	5	0	5	13.1	0.0
June	37	4.3	10	0	10	27.0	0.0	24	2.4	5	0	5	20.8	0.0
July	43	5.0	9	0	9	20.9	0.0	42	4.3	12	1	13	30.9	2.3
August	163	18.9	24	36	60	36.8	22.0	67	6.8	6	9	15	22.3	13.4
September	83	9.6	7	29	36	43.3	34.9	65	6.6	6	11	17	26.1	16.9
October	53	6.1	0	26	26	49.0	49.0	30	3.0	0	6	6	20.0	20.0
November	22	2.5	0	6	6	27.2	27.2	11	1.1	0	3	3	27.2	27.2
December	18	2.1	0	1	1	5.5	5.5	10	1.0	0	3	3	30.0	30.0
Total	479	55.6	60	99	159	33.2	20.7	287	29.5	34	33	67	23.3	11.4
Cases/1000	—	—	70	115	185			—	—	35	34	69	—	—
% reduction	—	—	—	—	—			—	—	50	70.4	62.7	29.8	44.9

Note: Spraying dates

*Rounds	**Rounds
1. 24 May	1. 6 June
2. 17 July	2. 6 Aug.
3. 27 Sept.	3. 7 Oct.

that the results achieved were of a comparative study of HCH vs. K-othrine as it was not possible to leave any areas unsprayed for control purposes.

Residual application of K-othrine @ 12.5 mg/m² was found successful in reducing the *A. culicifacies* densities to a very large extent after each round of spray as compared to the control (HCH sprayed) village. The residual effect of K-othrine produced satisfactory results in the control of vector populations upto 4 weeks and then it started to decline and populations returned to original densities after about 8 weeks.

Earlier trials of Deltamethrin in Nigeria showed that the compound at a dosage of 0.05 gm/m² was found safe for inhabitants and spraymen. The spraying also proved very effective in the control of *A. gambiae* and *A. funestus* for up to 20 weeks on mud wall and thatched structures (Rishikesh *et al.*, 1979). However, the residual effect against *A. stephensi* on cement and mud wall structure @ 50 mg/m² was negligible although spraying was effective upto 20 weeks on thatched surface. On cement surface K-othrine was effective upto 3 weeks against *Cx. quinquefasciatus* and *A. aegypti*. On mud surface it was effective for 14 weeks against *A. aegypti* and for 3 weeks against *Cx. quinquefasciatus*. Both normal and malathion resistant strain of *Cx. quinquefasciatus* were found equally susceptible to K-othrine (Das and Kalyanasundram, 1984).

During the present study K-othrine was found effective on mud and cement surface from 4-7 weeks respectively while on brick wall surface the effect lasted upto at least 8 weeks as observations were discontinued after 2nd round of spray.

In view of this it may be concluded that K-othrine 2.5% wdp is a promising residual insecticide when sprayed @ 12.5 mg/m². The acceptability of K-othrine was overwhelmingly

high because of its effectiveness against a variety of household pests viz., houseflies, cockroaches, crickets and other crawling insects. The spraying also did not spoil the wall surface. Occasionally burning sensation was observed by the spraymen not using the face mask. These symptoms were transitory and disappeared after a thorough wash. In view of the possible toxicity, precautionary measures such as using a mask, and washing hands and face with soap soon after spraying must be followed. Because of the significant reduction in vector densities and reduction in malaria transmission including several collateral advantages the study is being extended to about 1.75,000 population for a period of 3 years.

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Preliminary Observations on Development of Quinine sulphate Resistance in *Plasmodium berghei*

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A sensitive strain of *P. berghei* was subjected to interrupted subcurative doses of quinine followed by increasing drug selection pressure for 11 passages in Swiss mice which resulted in the selection of a strain resistant to 400 mg/kg daily dose of quinine (for 4 days, from Day 0–3). The quinine resistant strain had shown 16-fold cross-resistance to mefloquine and nearly 2-fold resistance to chloroquine, amodiaquine and dapsone. Its sensitivity to other drugs was not significantly altered. Sulphadoxin showed highest activity against quinine resistant strain (ED_{50} 0.066 mg/kg).

The results of the present study suggest that in the areas where quinine resistance in *P. falciparum* is becoming established, there is potential danger of the emergence of mefloquine cross-resistance in the field.

INTRODUCTION

Experimental studies on the induction of drug resistance in *P. berghei* have shown that a high level of resistance to conventional antimalarials can be built up in this parasite by interrupted sub-curative therapy. Agarwal, Puri and Dutta (1979) reported the selection of a pyrimethamine resistant strain of *P. berghei* with 128-fold resistance to this drug; Puri, Agarwal, Kazim and Dutta (1979) developed a strain with 32-fold resistance to chloroquine, Puri and Dutta (1979) selected a strain with 4-fold resistance to primaquine; Kazim and Dutta (1980) obtained a strain with 8-fold resistance to sulphanilamide and Kazim, Puri and Dutta (1979) selected another strain with 64-fold resistance to mefloquine. Besides these a strain of *P. berghei* has been developed in which pyrimethamine resistance has been superimposed on a mefloquine resistant line (Puri and Dutta, 1982). This strain shows resistance to both mefloquine (256 mg/kg) and pyrimethamine (128 mg/kg). Earlier a quinine resistant strain of *P. berghei* (400 mg/kg × 4 days) was developed by Jacobs (1965). The cross-sensitivity studies of this strain, as well as its chemotherapeutic response to other drugs based on ED_{50} and ED_{90} data, had not been studied by these workers. The present communication reports the selection of quinine resistant strain of *P. berghei*. The drug sensitivity studies on this line have further shown cross-resistance to mefloquine to which the strain had never been exposed.

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MATERIAL AND METHODS

The present strain of *P. berghei*, procured from NICD in 1976, was maintained at CDRI by

weekly blood passages in random bred Swiss mice, weighing 20 ± 1 gm. These mice were also used for selection of resistant strain by interrupted subcurative oral therapy. The mice were infected with 2.5×10^7 parasitized RBC and daily parasitaemia per 10,000 red blood cells was recorded. The stability of resistance was determined by a 4-day test after maintaining the resistant strain in the absence of the drug. The cross-sensitivity/resistance to other antimalarials was determined in Swiss mice. The data on ED_{50} and ED_{90} values with various antimalarials were obtained by log-probit activity graph method (Peters, 1965). The parasitaemia of the mice inoculated with 2.5×10^7 parasitized RBC was recorded on day 4 (after 4 oral doses of drug from day 0 to + 3).

The following drugs were used in the study: Quinine sulphate (IDPL), Mefloquine HCl (Roche Products, Basel), Chloroquine (Bayer India Ltd., Bombay), Amodiaquine (Parke-Davis, India, Ltd., Bombay), Mepacrine (British Pharmaceutical Lab., Bombay), Primaquine (SIGMA), Pyrimethamine (Burroughs Wellcome & Co., (I), Pvt., Ltd., Bombay), sulphanilamide (IDPL), Sulphadoxin (Roche), Dapsone (Bengal Immunity Company, Calcutta) and DADDS (CDRI, Lucknow).

RESULTS

Selection of the quinine resistant strain: The sensitive (normal) strain of *P. berghei* maintained by serial blood passage (2.5×10^7 parasitized RBC, i/p) in Swiss mice was subjected to interrupted subcurative oral doses of quinine starting from 37.5 mg/kg dose in the Ist passage. The dose was gradually raised in successive passages and finally the strain in 11th passage was found to exhibit resistance to 400 mg/kg dose (for 5 days) of quinine (Table 1). In the 14th passage, the strain showed resistance to this dose of quinine when given for 4 days from the day of infection i.e., day 0.

Stability of resistance: The resistance to quinine upto a dose of 400 mg/kg × 4 days, was found to be stable after 8th drug free passage i.e., after 43 days.

Table 1. Selection of quinine resistant strain of *P. berghei* in Swiss mice by increasing drug selection pressure and subcurative therapy.

Serial passage No.	Duration of serial passage (days)	Daily dose (mg/kg) 20 gm mouse	No. of doses (On days)	Total drug administered per mouse mg/kg	Parasitaemia/10^4 red cells Days							
					2	3	4	5	6	7	8	9
1.	0–6	375	4(0-3)	150	90	—	250	210	—	380	—	—
2.	7–13	75	5(1-4, 6)	375	60	—	225	—	300	—	250	—
3.	14–20	75	3(0–2)	225	58	56	131	—	130	—	52	—
4.	21–23	75	7(3, 6-11)	525	140	—	239	—	310	—	211	—
5.	34–42	150	5(2–6)	750	363	—	105	—	336	—	340	—
6.	43–55	150	4(2–5)	600	288	364	—	110	—	350	—	—
7.	56–65	150	7(2, 3, 5-9)	600	367	—	268	278	—	485	—	434
8.	66–76	300	5(4–8)	1500	95	—	602	—	188	—	200	—
9.	77–86	300	6(3, 5–9)	1800	70	—	422	317	—	264	—	—
10.	87–95	300	4(4–6, 8)	1200	65	—	274	—	150	—	235	—
11.	96–102	400	5(2–6)	2000	198	—	164	—	400	—	—	—
14.	114–122	400	4(0–3)	1600	158	—	205	—	218	—	200	—

— Parasitaemia not recorded.

Table 2. Sensitivity cross-resistance of quinine resistant and sensitive strain of *P. berghei*

Drug	No. of doses*	Quinine resistant strain		Sensitive strain	
		ED_{50} (mg/kg)	ED_{90} (mg/kg)	ED_{50} (mg/kg)	ED_{90} (mg/kg)
Quinine	4	225.75	412.5	116.25	262.50
Mefloquine	4	19.2	34	1.2	2.0
Chloroquine	4	4.1	7.2	1.7	3.3
Amodiaquine	4	2.5	4.55	0.9	2.5
Mepacrine	4	2.75	4.4	3.3	6.0
Primaquine	4	3.7	7.2	2.9	5.6
Pyrimethamine	4	0.25	0.52	0.23	0.49
Sulphanilamide	4	22.0	43.0	22.0	38.0
Sulphadoxin	4	0.066	0.129	0.06	0.11
Dapsone	4	0.43	0.93	0.22	0.57
DADDS	4	2.1	3.5	—	4.80

*Treatment was given for 4 consecutive days (from Day 0 to Day 3) and blood parasitaemia was recorded on Day 4.

Chemotherapeutic response to drugs: The sensitivity/cross-resistance pattern of the quinine resistant strain has been studied using ED_{50}/ED_{90} as criteria (Table 2). The strain has developed 16-fold cross-resistance to mefloquine on ED_{50} basis although the strain was not exposed to this drug earlier. The strain had developed a low level i.e., nearly 2-fold resistance to chloroquine, amodiaquine, and dapsone. There was no change in its sensitivity to other drugs. Out of all the drugs screened, sulphadoxin showed highest activity against the quinine resistant strains, its ED_{50} was 0.066 and ED_{90} 0.129 mg/kg.

DISCUSSION

The emergence of drug resistant strains of malaria particularly of *P. falciparum* is posing problems and its spread into other areas is causing great concern among public health workers. Review of literature in this area by Dutta *et al.* (1977), Puri and Dutta (1982), Das *et al.* (1981), Barkakaty *et al.* (1984) and Sharma (1984) shows that foci of chloroquine resistance and resistance to drug combinations like sulfalene-pyrimethamine, and sulfadoxin-pyrimethamine are enlarging day by day, though no resistance to quinine has been reported from India. Upto 1973, quinine was considered the drug of choice for the treatment of chloroquine/multiple drug resistant strains of *P. falciparum* (WHO, 1973). However, during the last decade multiple drug resistant strains have appeared widely in southeast Asia which are showing decreased sensitivity or even partial resistance to quinine besides showing complete resistance to chloroquine and other drugs. Thus Vietnam I (SV-I) strain of *P. falciparum* was reported to have developed resistance for chloroquine, proguanil and quinine (Chin *et al.*, 1966); Thailand (Man) strain from Bangkok had shown resistance to chloroquine, pyrimethamine and quinine; Malaya (Poo) and Malaya (Tay) strains from West Malaysia had developed resistance to chloroquine, proguanil, pyrimethamine and quinine; Vietnam (Brai) strain from Vietnam had developed resistance to chloroquine and quinine (Clyde *et al.*, 1969); Vietnam (Smith and Marks) strains from Vietnam and Burma (Thau) strain from Burma had acquired resistance to chloroquine, amodiaquine, proguanil, pyrimethamine and quinine (Clyde *et al.*, 1970; 1972; Rieckmann, 1971; Willerson *et al.*, (1974), Phillipines (Per) strain from Phillipines had developed resistance to chloroquine, pyrimethamine and quinine (Clyde *et al.*, 1971); Cambodia (Buch) strain from Cambodia had shown

resistance to chloroquine, amodiaquine and quinine (Rieckmann *et al.*, 1972) and Indonesia (Whit) strain from Jaya was resistant to chloroquine, amodiaquine, pyrimethamine and quinine (Clyde *et al.*, 1976). Fortunately, no reports of quinine resistance among *P. falciparum* strains are available in India.

Experimental studies on drug resistance in rodent malaria carried out in this Institute (Dutta *et al.*, 1977; Agarwal *et al.*, 1979; Puri *et al.*, 1979; Puri and Dutta, 1979; 1982; Kazim and Dutta, 1980; Kazim *et al.*, 1979) and elsewhere (Peters, 1970) clearly showed that strains of *P. berghei* resistant to pyrimethamine, chloroquine, primaquine, sulphanilamide, mefloquine and a combination of mefloquine and pyrimethamine, could be readily selected. The present study further shows that *P. berghei* can also acquire resistance to quinine if the drug is given in subcurative or suppressive doses. Surprisingly, this quinine resistant strain although not exposed earlier to mefloquine, has developed 16-fold cross-resistance to 4-day mefloquine treatment on the basis of ED_{50} level as compared to the sensitive strain (Kazim *et al.*, 1979). Out of all the drugs screened against the quinine resistant strain, sulphadoxin has shown the highest activity. The results of the present study strongly suggest that in southeast Asia where quinine resistance is gradually becoming established, there is potential danger of the emergence of mefloquine cross-resistance in the field and the large-scale use of mefloquine alone for the control of resistant cases in such areas should be made with caution. Our study also shows that sulphadoxin is the only drug which possesses high activity against the quinine resistant strain of malaria.

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SHORT NOTES

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Cost Effectiveness of the Bio-environmental Control of Malaria in Kheda district, Gujarat

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The strategy of malaria control has been the interruption of transmission by spraying residual insecticides in rural areas and by source reduction and larviciding in urban areas. Chemotherapy is an important anti-malaria supplemental measure to reduce morbidity and mortality and eliminate parasite reservoir from the community. The National Malaria Eradication Programme (NMEP) is providing protection to 351.80 million people living in rural areas by spraying insecticides. During 1985–86 NMEP's target was to spray 21629 MT DDT (75%) in 216.29 million population, 38149 MT HCH (50%) in 113 million population and 20259 MT malathion (25%) in 22.51 million population. The estimated cost of insecticides alone comes to Rs. 112 crores at the 1985 price line. In addition to this, 133 towns are under the urban malaria scheme (UMS), and in these towns an additional 62.1 million population is being protected.

Environmentalists are not in favour of the continued use of residual insecticides. Their main concern relates to ecological problems which may result due to accumulation of insecticides specially DDT in the environment. In this context it is noteworthy to mention that in tropical countries DDT is rapidly degraded and its half life is about 3 months (Agnihotri, 1978). Although huge quantities of DDT have been used in malaria control, fears of its presence in human body fat in large quantities could not be substantiated and in fact, the concentration of DDT in human body fat of Indians has shown a declining trend in the last 2 decades (Dale *et al.*, 1965; Ramachandran *et al.*, 1973; Bhaskaran *et al.*, 1979). It may also be noted that DDT or HCH used in agriculture directly comes into contact with the biosphere, which is not true for spraying under public health programmes as the sprayed insecticides stay on the inside walls of the structures. However, due to continuous spraying of insecticides for over 2 decades, the dramatic and sustained reduction in malaria incidence or mosquito populations including the collateral benefits of spraying, as perceived by the community in early years has gradually diminished. As a result, the strategy of residual spraying of insecticides is being met with increasing resistance in rural areas.

Villagers are unwilling to allow spraying as side benefits such as relief from nuisance insects no longer results after indoor residual insecticide spray leading to high refusal rates. In order to increase coverage, more cattlesheds than houses are sprayed, which has much less impact on malaria transmission. Consequently, the co-

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verage under residual insecticides irrespective of the type of insecticide used is not adequate and has failed to provide the desired impact on transmission of malaria. The main vector, *Anopheles culicifacies* has become resistant to DDT and HCH in almost all parts of the country, and to malathion in Gujarat and Maharashtra further adding to the problem of insecticide efficacy as a control measure. The high cost of insecticides and operational costs are adding to the already acute problem of resource mobilization. It has repeatedly been observed that operational failure or poor implementation of the programme results in flare ups of malaria, and in many areas the disease recurs with virulence such as the recent epidemics in Shahjahanpur (Chandrahas and Sharma 1983; Sharma *et al.*, 1985).

A more notable example is that of the areas outside the *Plasmodium falciparum* Containment Programme (PfCP). In 66 districts with 98 million population having high *P. falciparum* malaria, the Swedish International Development Authority (SIDA) supported programme is receiving special attention (Ray, 1979). A study of the impact of PfCP in 1984 showed that in 1981 there were 589591 cases of falciparum malaria in the country, and of these 443185 or 75% were in PfCP areas. In 1984, total *P. falciparum* cases in India increased to 655454 but in PfCP areas they were reduced to 367378 cases (A. P. Ray, personal communication). Therefore under the normal NMEP operations falciparum malaria is increasing as the total cases in non-PfCP areas increased from 146406 to 288076 i.e., from 25% to 46% or about 100% increase. This increasing trend of *P. falciparum* malaria is seen in spite of massive investments in malaria control. Unfortunately it is more likely that this situation would further deteriorate because of logistic and operational problems, as also the problems of insecticide resistance and drug resistance which are getting aggravated with the passage of time (Sharma 1984, a, b).

The effectiveness of an alternate strategy of bio-environmental malaria control without the use of insecticides has been demonstrated on long-term basis (Sharma and Sharma, 1986). This method, although widely accepted as the best means of malaria control was thought to be prohibitively expensive. A study currently in progress in Gujarat provides an ideal setting for comparing the cost of malaria control by the two methods i.e., bio-environmental control vs. insecticidal spraying. Results of this study are reported in this paper.

The study is in progress in Nadiad taluka, district Kheda (Gujarat). Studies on malaria endemicity revealed that the annual parasite incidence (API) since the implementation of the Modified Plan of Operation (MPO) was very high in some parts of the country, Gujarat being one of them. In Gujarat State, the highest API was recorded from Kheda district. In Kheda district Nadiad taluka villages were worst affected. In 1981, in Bamroli village there were at least 32 unusual deaths and majority of these deaths were attributed to falciparum malaria. There were innumerable mosquito breeding sites. *Anopheles culicifacies*, the main vector showed varying degrees of resistance to DDT, HCH and malathion. The spraying of insecticides did not interrupt transmission.

The study on the bio-environmental control of malaria was launched in June 1983 in seven villages (26 thousand population) and the impact of intervention measures was monitored from January 1984. In August 1984, the study was extended to another 14 villages (34 thousand population) and monitoring started in January 1985. In August 1985 study was further extended to cover the entire Nadiad taluka (0.35 million population) comprising of a total of 100 villages. Monitoring of the impact of intervention measures was started in April, 1986. In these villages malaria control is being achieved by integrating all methods except the use of insecticides. The NMEP has withdrawn all activities related to

surveillance, blood smear examination, drug distribution, spraying etc. and all work related to malaria is being done by the project staff. In addition to malaria control, several environmental improvement and income generating schemes have also been introduced.

A cost analysis of malaria control by insecticide vs. the bio-environmental methods is given in Table 1. The cost has been calculated for 3 population groups i.e., (i) Nadiad taluka which is the smallest administrative unit, and is currently under bio-environmental control, (ii) per million population, and (iii) also for the entire district (2.7 million rural population) an area which is targeted for spraying by insecticides (DDT and malathion). The last would provide realistic estimates of the two systems of control. Cost of malaria control by insecticidal spraying is the actual cost being incurred by the NMEP at the 1985 price line. For bio-environmental control, the cost has been calculated from the actual expenditure incurred during 1985–86 and extrapolated for 1 million and 2.7 million population. The actual expenditure to control malaria is higher due to the establishment cost which has not been included in Table 1.

Table 1. Malaria control by spraying residual insecticides vs. bio-environmental control

Method of control	Estimate in rupees (lakhs) Nadiad taluka (0.35 m Pop.)	Three talukas (1 m Pop.)	Kheda distt. (2.7 m Pop.)
DDT*	12.0	34.0	92.0
HCH*	13.0	37.0	100.0
Malathion*	70.0	199.0	537.0
Bio-environmental control**	18.0	37.0	85.0

Sources:
*NMEP
**From Table 2

It may be pointed out that in the bio-environmental control project considerable expenditure is being incurred on monitoring the impact of intervention measures. Table 2 shows the actual expenditure incurred during 1985–86 in the control of malaria in Nadiad taluka (0.35 million population). Based on the expenditure incurred in Nadiad, malaria control costs have been calculated per million population and also for the entire rural population of Kheda distt., (2.7 million). The expenditure has also been divided into intervention and monitoring. It may be noted that the expenditure to control malaria per million or 2.7 million population would not increase proportionately to the initial expenditure being incurred on Nadiad. This is because with the increase in population size, expenditure on impact assessment or monitoring would remain the same, since it is envisaged that monitoring would be carried out by sampling procedures to reduce the cost. As the size of experimental areas increases there is optimum utilization of manpower, the operational cost related to vector control by source reduction and minor engineering works would go down because relatively less work would be required in subsequent years. Since careful monitoring is absolutely essential, because of the scientific nature of the project, it has substantially increased the cost.

Table 2. Estimated cost of bio-environmental control

	Cost in rupees (lakhs) Nadia taluka (0.35 m Pop.)	Three talukas (1 m Pop.)	Kheda distt. (2.7 m Pop.)
(i) Intervention			
Staff	4.8	10.0	25.0
Daily wages	6.0	16.0	40.0
Contingency	2.4	6.0	15.0
Sub total	13.2	32.0	80.0
(ii) Monitoring			
Staff	3.6	3.7	3.7
Contingency	1.2	1.3	1.3
Sub total	4.8	5.0	5.0
Total	18.0	37.0	85.0

Note: Calculations are based on actual expenditure incurred during 1985–86.

It can be seen from Table 1 that the bio-environmental control of malaria is expensive if the target population is small. This is because of the monitoring cost and the fact that the same staff has the capacity to cover more areas. Obviously as the target population increases bio-environmental control expenditure is reduced mainly because of the reduced monitoring and overhead costs. The cost of malaria control in Kheda district by bio-environmental control is Rs. 85 lakhs compared to Rs. 92 lakhs with DDT.

In addition to the above costs both systems of control would have to incur establishment costs. At present this cost is about Rs. 7 lakh per million population for spraying. In the bio-environmental control of malaria; purchase of vehicles, tractors, microscopes and furnishing of laboratories and depreciation would cost about Rs. 60 lakh per million population. Replacement cost of these items would be about the same as is being currently incurred on managing malaria by insecticidal spraying.

The study clearly brought out that the bio-environmental control of malaria strategy adopted in Kheda is cheaper than DDT spraying which is the cheapest insecticide available for malaria control in the country. It costs about 1/6th as much as malathion spraying.

There are several other benefits of the bio-environmental control methodology. Some of these are listed below:

1. The alternate strategy prevents pollution by harmful chemicals and preserves the ecological integrity of the area. It has also established a base of science awareness in the rural areas.
2. Since the strategy brings about semi-permanent to permanent changes in mosquito breeding habitats, malaria control is achieved for a much longer period and only sustaining efforts are required in subsequent years contrary to ever-escalating operations required for the spraying of insecticides.
3. The cost of insecticide increases every year, and at times it may show a steep rise. Besides spraying operations always have to be carried out by external agencies unlike bio-environmental control which becomes even more feasible due to community participation and interdepartmental coordination.
4. It was demonstrated that malaria control by bio-environmental methodology provided an entry point for other developmental activities in the villages. The staff employed in malaria control also took up additional responsibilities of rural development and can also be used in the delivery of other health services.
5. The sociological implications of the bio-environmental control methodology are too many and cannot be quantified in terms of money. For example, how does one value the lives saved, better health conditions generated, general social awareness and the spirit of self-help that is awakened. However, some of these benefits can be measured in terms of profit and loss, e.g., social forestry and fisheries.

In Nadiad taluka 2 million fast growing/fruit bearing trees were planted in marshy areas and waste land. If we calculate the returns from 1 million (i.e., 50% only) surviving trees @ Rs. 10 per tree per year either at the time of felling or during its life time, the returns from social forestry in one year alone would be Rs. 10 million. This amount can support the control of malaria in Nadiad taluka by bio-environmental methods for more than 5 years.

A second profitable scheme introduced in villages is fish and prawn culture. The size of ponds in Nadiad vary from 1 to 5 hectares and there are 100 such ponds in that area. During 1985 the villagers allowed 8 ponds to be used by the MRC for experimental work. All the ponds were first cleaned of predators and fish culture was carried out on scientific lines with the technical assistance of the Inland Fisheries Department. Nearly 2.5 lakh fries of the common carp, a food fish,

were introduced. In addition to this 40,000 freshwater prawns were also introduced. The margins of these ponds were cleaned routinely, and none of the ponds supported mosquito breeding. Some guppies were also introduced to control mosquito breeding.

Our estimates based on discussions with the Inland Fisheries Department and the contractors etc., showed that 8 ponds would provide a net return of at least Rs. 3.0 lakh in one year. Therefore, fish and prawn culture alone, if carried out in all ponds of Nadiad taluka can generate more money than required for the bio-environmental control of malaria. This movement is spreading and more village panchayats have come forward with offers of giving ponds to MRC for food fish production. We envisage that eventually village cooperatives will manage the entire programme and the income from the ponds/forestry etc. would go to village panchayats. These funds in turn would be used in the control of mosquito breeding and other developmental activities of the villages with the technical assistance of MRC or any other government agency.

6. The study also envisages the promotion of other developmental activities such as the biogas plants, improved "chulahs", solar cookers, and the introduction of modern agricultural practices within the existing infrastructure. These schemes would benefit the villagers directly.

7. The expenditure on bio-environmental control is generating employment in rural areas. This money would have otherwise gone in the purchase of insecticides resulting in environmental pollution. It is also noteworthy to mention that this innovative approach to malaria control automatically tackles the intractable problem of insecticide resistance which has otherwise become a serious obstacle in the successful control of malaria.

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Malaria Situation in Meerut district Villages (U.P.)

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Following news of deaths due to malaria, an investigation into the causes was carried out in Rohta and Jani PHCs. Results of the study are reported below.

Blood smears from fever cases and contacts of deceased patients were collected. Children between 2–12 years were examined for spleen enlargement. Anopheline mosquitoes were dissected for gut or gland infection. Susceptibility of *A. culicifacies* adults to insecticides was tested following the WHO procedure.

Parasitological surveys were carried out for a few days in each village and prevalence of malaria was compared with the data collected by the DMO for that month (Table 1). The incidence of falciparum malaria was extremely high in all the affected villages. In November 1983, of the 311 blood smears collected from fever cases, 237 were positive for malaria parasite. The slide positivity rate was 76.2% and parasite formula was 4.21% *P. vivax*, 95.3% *P. falciparum* and 0.4% mixed infection. During the next transmission season in July and August 1984, 687 slides were collected from fever cases and 335 were positive for malaria. The slide positivity rate was 48.7% and parasite formula was 38.5% *P. vivax*, 60.2% *P. falciparum* and 1.19% mixed infection. Falciparum malaria was 100 per cent in Jangethi village and 93.02%, 92.1% and 94.5% in Dayampur, Dobka and Zakhera villages respectively. Malaria cases recorded by the District Malaria Officer based on active case detection (ACD) at an interval of 15 days were negligible because of poor surveillance and/or laboratory services. It may also be noted that ACD would miss afebrile cases and half of the cases detected were afebrile and asymptomatic.

Further proof of high endemicity to malaria was obtained by examination of enlarged spleen and blood tests of children between 2 - 12 years. Of the 305 children examined from Jangethi, Dayampur and Zakhera villages, 56 had enlarged spleen. The average spleen (AS) was 0.26, 1.15 and 0.84 in Jangethi, Dayampur and Zakhera villages respectively. Similarly the proportion of afebrile cases found positive for malaria parasite was 22.2, 53.8 and 55.0 per cent in Jangethi, Dayampur and Zakhera villages respectively.

During investigations 32 deaths were investigated from Jangethi, Dayampur and Zakhera villages. Results of blood smears collected from the houses of the deceased revealed that out of 113 slides collected, 51 were positive for *P. falciparum*. The most likely cause of these deaths was probably malaria, a situation similar to Shahjahanpur (Chandrahas and Sharma, 1983; Sharma *et al.*, 1985) and Bareilly (Ansari *et al.*, 1984).

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Table 1. Results of parasitological surveys in villages of Meerut district (U.P.)

Village	Month/year	MRC data (prevalence)							Corresponding NMEP data (monthly incidence)*			
		B.S. Coll.	Pv	Pf	Mix	Total +ve	Pf %	SPR	Pv	Pf	Mix	Total +ve
Jangethi, Rohta PHC (Pop. 4600)	Nov. 1983	87	0	66	0	66	100	75.8	6	7	0	13
	Jul. 1984	34	3	9	0	12	75	35.2	3	0	0	3
	Aug. 1984	167	39	76	4	119	63.86	71.25	6	3	0	9
Dayampur, Rohta PHC (Pop. 2085)	Nov. 1983	70	2	40	1	43	93.02	61.4	0	0	0	0
	Aug. 1984	155	33	37	0	70	52.85	45.16	0	1	0	1
Dobka, Rohta PHC (Pop. 2200)	Nov. 1983	61	4	47	0	51	92.15	83.6	0	0	0	0
	Aug. 1984	200	30	55	0	85	64.7	42.5	0	0	0	0
Zakhera, Jani PHC (Pop. 1284)	Nov. 1983	93	4	73	0	77	94.8	82.7	0	0	0	0
	Aug. 1984	131	24	25	0	49	51.02	37.4	0	1	0	1

*Source: MO, Rohta and Jani PHCs

Table 2. Density of anophelines in villages of Meerut district U.P.

Village/ PHC	Month/ Year	*A. culicifacies*			*A. stephensi*			*A. annularis*			*A. subpictus*		
		Total catch	PMH	Proportion (%)	Total catch	PMH	Proportion (%)	Total catch	PMH	Proportion (%)	Total catch	PMH	Proportion (%)
Jangethi/ Rohta	Nov. 83	71	8.8	61.2	0	0	0	17	2.1	14.6	28	3.5	24.1
	Jul. 84	226	113	43.9	3	1.5	0.5	15	7.5	2.9	270	135	52.5
	Aug. 84	266	44.3	50.1	1	0.1	0.1	1	0.1	0.1	262	43.6	49.4
Dayampur/ Rohta	Nov. 83	34	11.3	53.1	2	0.6	3.1	0	0	0	28	9.3	43.7
	Aug. 84	178	48.5	68.7	1	0.2	0.3	3	0.8	0.1	77	21	29.7
Zakhera/ Jani	Nov. 83	74	24.6	60.0	0	0	0	15	5	12.2	33	11	27
	Aug. 84	144	18	27	31	3.8	5.8	1	0.1	0.1	356	44.5	66.9

Entomological observations revealed that among the anopheline mosquitoes *A. culicifacies, A. stephensi, A. annularis, A. pallidus* and *A. subpictus* were the commonly encountered species (Table 2). During November 1983, *A. culicifacies* was the dominant species and the per man hour (PMH) densities ranged between 8.8 to 24.6, whereas *A. stephensi* populations were negligible. During July-August 1984 very high *A. culicifacies* densities ranging between 18.0 to 113.0 were encountered. Dissection of *A. culicifacies* revealed four positive mosquitoes out of 816 dissected from Jangethi village. *A. culicifacies* was therefore responsible for maintaining high malaria transmission.

Susceptibility tests revealed that *A. culicifacies* was resistant to DDT and HCH (Table 3). There was

Table 3. Results of insecticide susceptibility tests in Jangethi, Rohta PHC, Meerut District (U.P.)

Insecticide		Exposure time*	Nos. exposed	Nos. dead**	Mortality (%)
		A. culicifacies adults			
DDT	4.0%	1 hour	75	9	12.0
Dieldrin	4.0%	2 hours	75	4	5.3
Malathion	5.0%	1 hour	75	75	100.0
Control	...	—	75	1	1.3
		A. culicifacies larvae			
DDT	2.5 ppm	24 hours	60	19	31.6
Dieldrin	0.1 ppm	24 hours	60	0	0.0
HCH	0.5 ppm	24 hours	60	13	21.6
Temephos	0.125 ppm	24 hours	60	40	100.0
Malathion	3.125 ppm	24 hours	40	40	100.0
Control	—	24 hours	100	2	2.0

*Adults were given 24 hours recovery period

**Mosquitoes were collected from Jangethi village, Rohta PHC

12.0% and 5.3% mortality of *A. culicifacies* adults on 1 hour exposure of adults on 4% DDT and 2 hour exposure on 4% dieldrin impregnated papers respectively. Similarly 31.6% and 0 and 21.6% mortality was recorded of the III and early IV instar larvae to discriminatory dosages of DDT. dieldrin and HCH respectively. Adults were susceptible to malathion and larvae to malathion and temephos.

In the study villages DDT spraying was discontinued in 1980 in favour of HCH, and 2 rounds of HCH @ 200 mg/m^2 are sprayed every year. Despite regular HCH spraying, malaria transmission had remained uninterrupted. The situation did not improve during 1985 as the SPR in Jangethi and Dayampur villages was 71.2% and 45.16% respectively. There was, however, 50% reduction in SPR in Dobka and Zakhera villages as a result of mass radical treatment and HCH spray.

The failure of HCH spraying in interrupting the transmission is due to high degree of resistance in *A. culicifacies* and poor coverage. DDT spraying with improved coverage can provide better results as has been demonstrated by Sharma *et al.* (1982; 1986a) in Haryana and U. P. It may also be pointed out that complete interruption of transmission is only possible by the use of replacement insecticides like malathion as was done in Haryana villages (Subbarao *et al.*, 1984). Alternatively, the implementation of the bio-environmental control of malaria as being developed in Nadiad may be a viable alternative to the problem (Sharma *et al.*, 1986b).

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High Performance Liquid Chromatographic Determination of Chloroquine in Finger Tip Blood dried on Filter Paper: Sample Handling Problems

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The use of HPLC with fluorescence detection appears to be the best combination of convenience, sensitivity and specificity for the determination of chloroquine in plasma, serum and whole blood (Rombo *et al.*, 1985; Bergqvist, 1983). Strictly standardized handling of the blood samples is necessary but this is almost impossible in tropical field work. One way of avoiding this problem is to use finger tip blood dried on filter paper. This type of blood sampling is practical and more acceptable for persons in the tropical countries. However, there is considerable risk of contamination of the filter papers with chloroquine from tablets during blood sampling and handling of the papers (Bergqvist *et al.*, 1986). This report discusses the method of sampling and storing of the filer paper samples to avoid chloroquine contamination.

The liquid chromatograph consisted of a Consta Metric III pump (LDC, Riviera Beach, USA), a WISP 710 B automatic injector (Waters Associates, Milford, USA) and a RF 530 fluorescence detector (Shimadzu, Kyoto, Japan). The excitation and emission wave lengths were set at 335 and 380 nm respectively. The column (150 × 4.6 mm) was an Ultrasphere-Si 5 μm (Beckman, Berkeley USA). The mobile phase was a mixture of acetonitrile and methanol (2:1) containing 0.8% v/v of 25% ammonia. The mobile phase was filtered before use. The standard curves were linear within the concentration range of interest in this study. The internal standard used was 4-(4-dimethyl-amino-1-methylbutyl-amino)-7-chloroquinoline. Desethyl-chloroquine was a gift from Sterling-Winthrop, and chloroquine phosphate was supplied by Kabi Vitrum, Stockholm, Sweden. Acetonitrile and methanol were of HPLC grade and other reagents were of analytical grade.

The subjects in the study had been given chloroquine (600 mg free base) as a single dose 2, 3, 4 or 5 days prior to blood sampling. The subjects had not taken chloroquine during two weeks before sampling.

Whole blood (75 μl) was obtained in heparinized capillary tubes filled up to the red mark (Drummond Scientific Co., USA) and applied on the centre of the filter paper (Whatman No. 1). The

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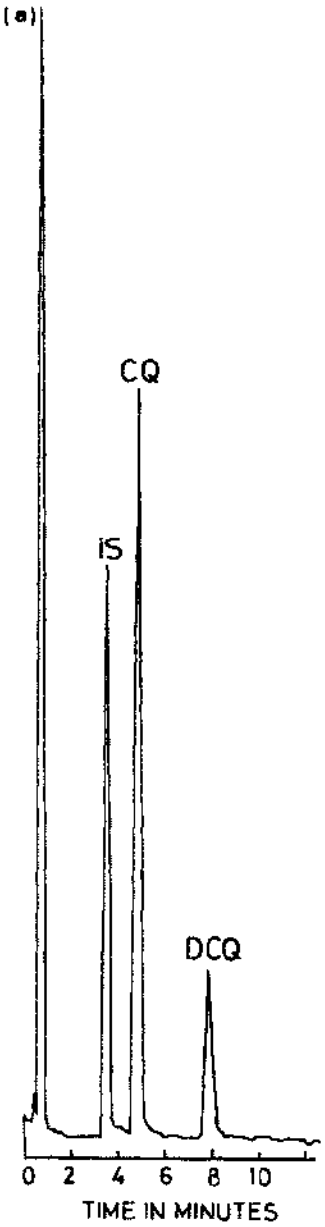

Fig. 1: HPLC Chromatogram of reference substance

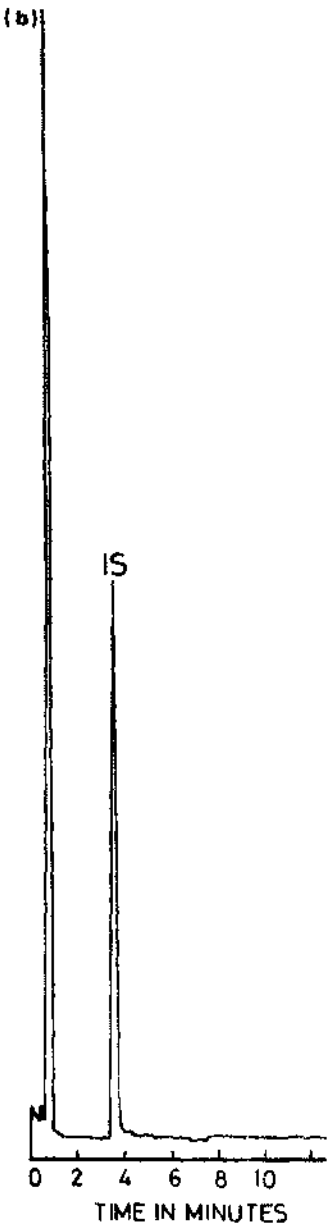

Fig. 2: HPLC Chromatogram of blank filter paper (control)

HPLC Conditions:

Column	: Ultrasphere-Si 5
Solvent	: Acetonitril: Methanol (2.1) containing 0.8% v/v of 25% Ammonia
Detection	: Fluorescence Ex 335, Em 380 nm.
Flow	: 2.5 ml./min.

spot of blood on the filter paper should not be too small. The filter paper is dried for half an hour in a hanging position and protected from insects. After drying, each filter paper was placed between two pieces of waxed paper and stapled. Proper care was taken to avoid cross contamination during packing. Each day samples were packed in one lot. The package of filter papers was kept in the refrigerator. It is worth pointing out that there should be no exposure to free chloroquine or chloroquine solution in the refrigerator.

The extraction of the dried samples was according to the method of Lindstrom *et al.* (1985). Blank samples were prepared by cutting out a piece of the unused section of filter paper, identical in size to that of the dried blood sample and processing it in the same way as the blood sample. Chloroquine and desethylchloroquine were determined in 200 dried blood samples and in order to detect contamination due to chloroquine 100 blank samples were also analyzed.

Fig. 1 and 2 show HPLC chromatograms of a dried blood sample and a blank filter paper sample respectively, processed under identical conditions. It is clear from the comparison of the two figures that there is no chloroquine or desethylchloroquine in the blank sample. Of 100 blank samples, only 5 showed contamination due to chloroquine, and the level of chloroquine contamination was below 10% of the amount in the corresponding blood samples. Table 1 shows the mean chloroquine concentrations 2, 3, 4, and 5 days after dosing. The ratio of chloroquine and desethylchloroquine is between 3 and 4 in most cases which is the normal ratio found by others (Bergqvist, 1983).

Table 1. Mean chloroquine concentration in subjects taking a single dose of 600 mg. chloroquine base

Blood samples in relation to chloroquine treatment	No. of samples	Chloroquine mean (in μ mol/l)
Before treatment	50	0
2 days after dosing	60	1.20
3 days after dosing	40	0.80
4 days after dosing	30	0.68
5 days after dosing	10	0.32

When chloroquine resistance is monitored with *in vivo* and *in vitro* tests, determination of drug concentration in blood might be helpful to distinguish between true resistance and therapeutic failure due to insufficient dosage or poor compliance (Brohult *et al.*, 1979). In tropical field work, venous punctures are often not accepted by the people. Besides that, the strictly standarized handling of plasma and serum samples which is required is not possible in tropical field work (Rombo *et al.*, 1985). The storing of plasma or serum will often also be a problem. The use of dried finger tip blood samples have three big advantages: (i) They are accepted by the people, (ii) the handling of these samples is not too complicated and (iii) dried samples can be stored for considerable periods of time (Lindstrom *et al.*, 1985). However, if handled in the wrong way contamination of chloroquine will make this procedure impossible to use. Before taking blood samples on filter papers, one should ensure that (i) there is no chloroquine handling in the room or nearby, (ii) the persons taking the blood samples are not dealing with the distribution of drugs, (iii) the person from which the sample is taken has not recently been in contact with chloroquine tablets, (iv) the packages of unsampled filter papers are separated from the sampled ones, and (v) a finger on the left hand should be pricked for the capillary blood samples.

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Some Ecological Observations on Anophelines collected from Manipur

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Manipur state is located between 23.83° and 25.68° N latitude and 93.05° and 94.78° E longitude, bounded on the north by Nagaland, on the south by the Lushai hills and Burma, on the west by Cachar district of Assam and on the east by Burma. Out of an area of 22,356 km², 20,736 km² (92%) is hilly. Topographically, the state can be divided into two parts, centrally situated valleys (1,765 km²) and hilly region having parallel north to south running hill ranges, with a maximum altitude of 3,050 m and rich riverine system. Most of the hilly region is covered with tropical moist deciduous and tropical evergreen forests with prominent bamboo groves. Paddy is the main crop.

The earlier records of anophelines from Manipur by Covell (1927, 1931), Barraud (1933) and Puri (1936) present only five species from the state. Mortimer (1946) raised the fauna of the state upto 16 species. Again, a survey by Malhotra *et al.* (1983) reports only seven anophelines.

In view of the above, the present study was conducted during September 1983 to October 1985, on the anopheline fauna of the state and the possible change in fauna with changing ecological conditions.

During the study 2,076 adult mosquitoes were collected and 365 were reared from larval collections. Out of 45 localities surveyed, 32 were found positive for anophelines. Mosquitoes collected were identified by the keys of Christophers (1933), Puri (1955), Wattal and Kalra (1961), Harrison and Scanlon (1975) and Rao (1984). The nomenclature is after Knight and Stone (1977).

The prevalent species in order of majority were found to be *Anopheles vagus*, *An. nigerrimus*, *An. annularis*, *An. barbirostris* and *An. subpictus*. *An. crawfordi*, *An. gigas*, *An. jeyporiensis* var. *candidiensis*, *An. kochi*, *An. maculatus* and its variety *willmori*, *An. minimus*, *An. nitidus*, *An. philippinensis*, *An. peditaeniatus*, *An. sinensis*, *An. splendidus* and *An. tessellatus* were also recorded.

Anopheles crawfordi, *An. kochi*, *An. nitidus*, *An. peditaeniatus*, *An. splendidus*, *An. subpictus*, *An. tessellatus* and *An. minimus* were recorded for the first time from the state. *Anopheles ahomi*, *An. aitkenii*, *An. bengalensis*, *An. culicifacies*, *An. gigas* var. *baileyi* and *An. pallidus* reported by earlier workers were found absent.

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Notes on the species collected

Anopheles annularis was recorded from four localities. The species was found to be prevalent in the plains. The maximum number of adults were collected from cattlesheds and bovine bait. Breeding was mainly observed in ponds with clear water having submerged, emergent and mixed vegetation.

Anopheles barbirostris was collected from both hilly and plains regions of the state. The species was recorded from seven localities. Adults were collected mainly from shrubby forests of *Quercus acutissima* Carruthers and bovine bait. The species was reported to rest inside jungles in Burma (Khin-Maung-Kyi, 1971) also. Some day-biting behaviour, as reported earlier from shaded forests of Andaman Islands (Christophers, 1933) and Burma (Khin-Maung-Kyi, 1971), was also noticed.

One female specimen of *Anopheles crawfordi* was collected from human bait from a high altitude locality Mao (1,850 m.).

Anopheles gigas was reported earlier from high altitude streams of Burma (Khin-Maung-Kyi, 1971) from freshwater springs, ponds with vegetation, spring seepages and pools along the shallow hill streams from India (Puri, 1936). Mortimer (1946) collected the larvae from shaded hilly pools containing *Spirogyra*, in association with *Culex bitaeniorhynchus* and *Cx. mimeticus*. During the present survey *An. gigas* was recorded only from a high altitude locality, Ukhrul (Viewland area, 2,000 m) breeding in discarded motor tyres. Out of eight motor tyres searched during the month of September 1984, three were found positive for larvae.

Anopheles jeyporiensis var. *candidiensis* was recorded from a single locality Zaphou (1,000 m.). The species was reported by Mortimer (1946) to be rare, but during the present survey a considerable number of mosquitoes were collected during dusk-biting.

Anopheles kochi was recorded from two localities of the state showing its distribution in both hilly and plains regions. Most of the adult specimens were collected from herbaceous undergrowth in forests, though a single specimen was also collected from a nullah margin in the dense forest of the Indo-Burma area. The species was reported to be exophilic in nature by earlier workers including record of Khin-Maung-Kyi (1971) from Burma. The breeding of the species was recorded from a turbid rain pool in a forest of the Indo-Burma area. Shallow muddy collections of water, ground pools with or without grass, hoof marks and fallow rice fields were reported to be its breeding places from other parts of India (Rao, 1984).

Breeding of *Anopheles lindesayi* was noticed in a stream margin pool with shaded and clear water at an altitude of 1,550 m. High altitude, clear water and shady conditions seem to be important for breeding of the species. Mortimer (1946) reported the breeding of the species at an altitude of 915 to 990 m. in the lightly shaded pools of rocky streams in association with *An. aitkenii*.

Anopheles maculatus was collected from eight localities of the state, mainly from the plains. The adults were collected from herbs, shrubby vegetation and bovine bait. Studies in Southeast Asian countries revealed it to be an outdoor rester. Outdoor resting on forest undergrowth has been reported from Malaysia, Indonesia and Burma. Breeding was mainly recorded from roadside drains, rocky streams and their marginal pits. The species is a clear-water breeder but can breed in slightly turbid water also. No vegetation was observed in these breeding places. All the larvae were collected from sunlit areas. The breeding of the species in similar conditions has been reported from Southeast Asian countries including Burma (Khin-Maung-Kyi, 1971). It can be mentioned that both *An. maculatus* and var. *willmorei* share the same breeding sites with high percentage of var. *willmorei* (reported to be about 73% by Mortimer, 1946). *An maculatus*

var. *willmorei* was also recorded from the plains.

Anopheles minimus, an important malaria vector in certain parts of the country, the neighbouring state of Nagaland and in adjoining Burma, was recorded for the first time from the state. One adult was collected from a nullah margin groove in a dense shrubby forest. From Burma, Macan (1949) reported that, in the forested Kabaw Valley it is an outdoor rester but in open cultivated plains it is an indoor rester. It was also found breeding in roadside pools with turbid water.

Anopheles nigerrimus was found to be the most widely distributedspecies in the state. The adults prefer bovine bait, and the main resting places during the day were seen to be shrubby forests of *Quercus acutissima* Carruthers, and cattlesheds. A considerable number of mosquitoes were also collected from electric lights at night in the rainy season. The main breeding places were pond margins, pits, paddy fields and roadside pools. The species prefers clear to slightly turbid water with emergent and submerged vegetation.

One male specimen of *Anopheles nitidus* was reared from larvae collected at a pond margin with clear water in the plains area.

Anopheles philippinensis larvae were recorded in the plains region from a pond with clear water and emergent vegetation. The breeding preference for tanks and ponds has been observed earlier by Covell (1927). Mortimer (1946) reported breeding of the species from paddy fields with *An. pallidus* during November-December 1943 from Manipur.

Anopheles peditaeniatus was collected from five localities and was prevalent in the plains area. The maximum number were collected from lights at night, followed by collection from herb vegetation, cattlesheds and human bait. The specimens were also reared from a pond with prevalent *Hydrilla* and clear water.

Anopheles sinensis reported earlier from locality Ukhrul (2,000 m.) was also collected from other localities of the state viz., Chingmeirong, Gwaltabi, Kwakta, Langolhill, Mao, Mantripukhri and Zaphou. Adults were collected from human bait, herb vegetation, cattlesheds, bovine bait and electric lights at night. Breeding of the species was noticed from the pits in harvested paddy fields (Hundung, 2,000 m.) which serve as domestic water reservoirs for the local Naga tribes in winter. Collections were made during the month of November 1984 and February 1985. The species breeds mainly in paddy fields and weedy algal pools.

One specimen each of *Anopheles splendidus* was recorded from two localities of the state. The specimens were collected from human bait and herbaceous growth.

Anopheles subpictus was recorded for the first time from Manipur. Most of the adults were collected from bovine bait and cattlesheds. Breeding was noticed in a pit with greenish turbid water without vegetation and from a dried pond during the month of April 1984.

Anopheles tessellatus was collected from electric lights at night from a locality in the plains. The species was not recorded earlier from the state.

Anopheles vagus was the most prevalent species, representing 45% of the total collection. Adults were collected mainly from bovine bait, cattlesheds and herb vegetation. Breeding was recorded from roadside pools, muddy pits in dried ponds, and from cart-tracks. Turbid water without any vegetation (pH 8) was noted for breeding. Preference for muddy-water for breeding has also been reported by other workers (Rao, 1984).

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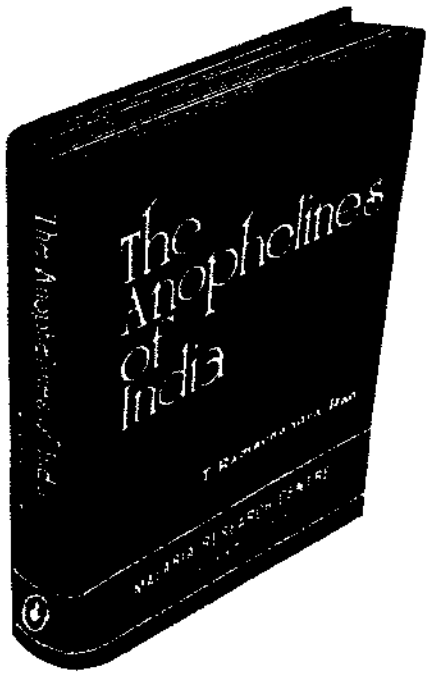

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