MATERIAL AND METHODS
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1. The study area:

The present study was conducted in 20 villages namely - Pehari, Mirona, Maheba, Dhawani, Narali, Dakwan, Sabri, Moreta, Ghushwan, Gulara, Mod Khurd, Mod Kalan, Sant-Dehra, Hibi, Bajehora, Bangra, Simthiri, Jaryai, Chhizona and Sultantpura, located within the area of Primary Health Centre (P.H.C.) Chirgaon which is the rural health training centre (R.H.T.C.) of the Department of Social and Preventive Medicine, Maharani Laxmi Bai Medical College, Jhansi (U.P.). The centre is being utilized for field training of undergraduate students in community health and for epidemiological researches.

1.1 Topography:

District Jhansi of Bundelkhand region situated in south-west of Uttar Pradesh, is surrounded by districts of Gwalior, Datiya, Shivpuri and Tezhampur of Madhya Pradesh and Lalitpur, Hamirpur and Jalaun of Uttar Pradesh. P.H.C. Chirgaon is situated on Bombay-Kanpur road at a distance of 25 kms. from M.L.B. Medical College, Jhansi, U.P. It renders health care delivery to the population of 120 villages besides Chirgaon town where centre is located. Majority of study villages are
connected by pucca roads with the centre; a few, however are not approachable by easy means.

The geographical area of Community Development Block Chirgaon is 55,233 hectares constituting mainly of Padua soil which is suitable for wheat cultivation.

1.2 **Climate**

Climate of the area is hot and dry. Mean monthly maximum and minimum temperature ranges between 47.1°C to 1.7°C respectively (1986-1987). General and real rainfall was recorded as 879 mm and 506 mm respectively during the calendar year 1986. Mean monthly relative humidity ranges between 15% to 76% at 0730 hrs and 26 to 84% at 1530 hrs (Statistical Diary, U.P., 1987).

1.3 **Population composition**

P.H.C. Chirgaon has a population of 1,61,561, according to 1981 census. The density of population is 1,94/hectare. Male : Female ratio is 1000 : 918. The literacy rate is 1 : 6 higher in comparison to Uttar Pradesh and 7.17% lower in comparison to whole of India (Census, 1981). Majority of them are Hindus followed by Muslims and then others (Govt. of U.P., 1986). Agriculture and labour are main occupations of the area.
1.6 Environmental conditions:

Mostly, houses are either kutcha or semi-pucca with a little or no facility of cross-ventilation. Open and insanitary wells are main source of water supply. There are no sewage and drainage system for disposal of excreta and waste water respectively.

Incidence of malaria was however not uniform throughout the block. These are feel of high and low incidence. The estimated A.F.I. of these villages was over 2.6 per thousand population as reported by District Malaria Office, U.P. (District Malaria Office, Jhansi).

Annual parasite incidence and slide positivity rate in Chiryesn block during the year 1982-83 is as follows:

<table>
<thead>
<tr>
<th>Year</th>
<th>A.F.I.</th>
<th>S.F.R.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1982</td>
<td>12.05</td>
<td>12.17</td>
</tr>
<tr>
<td>1983</td>
<td>13.01</td>
<td>12.16</td>
</tr>
<tr>
<td>1984</td>
<td>7.32</td>
<td>0.39</td>
</tr>
<tr>
<td>1985</td>
<td>7.99</td>
<td>0.16</td>
</tr>
<tr>
<td>1986</td>
<td>6.80</td>
<td>0.17</td>
</tr>
<tr>
<td>1987</td>
<td>2.96</td>
<td>2.09</td>
</tr>
<tr>
<td>1988</td>
<td>2.02</td>
<td>2.28</td>
</tr>
<tr>
<td>1989</td>
<td>1.04</td>
<td>1.19</td>
</tr>
</tbody>
</table>

Source: District Malaria Office, Jhansi (U.P.).
FIG. 3.1
NO. OF SAMPLES COLLECTED FROM EACH VILLAGE (1-20)

PAHARI 151
MIRONA 91
MAHEBA 27
DHAWANI 84
BARAL 184
BAKUAN 96
BABRI 37
MORETA 35

ANGURI RIVER
TO KANPUR
MOTH
BAMORE
GURSARAI

GHUSHWAN 26
GULARA 46
MOD KHURD 34
MOD KALAN 54
SANT BEHTA 28
NIBI 38

15 BAJHERA 65
16 BANGRA 80
17 SIMTHARI 92
18 JARYAYI 87
19 CHHIRONA 156
20 SULTANPURA 98
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16 BANGRA 80
17 SIMTHARI 92
18 JARYAYI 87
19 CHHIRONA 156
20 SULTANPURA 98
2. **Study design:**

The population survey was carried out once during the transmission period (September and October, 1987). In this district, there was marked increase in the transmission level during the period September to October as observed by Srivastava et al (1975). The maximum prevalence is from August to November in most of the parts of India.

Seeing the paucity of time and limited resources available, it was thought to conduct the study in transmission period only. The number of samples collected from each village is shown in Figure 3.1.

3.1 **Unit of study:**

Households of every tenth house selected by systemic random sampling was the unit of study.

3.2 **Sampling size and sampling:**

In this study every tenth house was the unit of study. All individuals of a household were taken into study irrespective of their age, sex and health status except for infants under six months of age, who were not included to avoid the effect of maternal antibodies on the results of sero-epidemiology.
Twenty percent villages in the area under study were selected using simple random sampling method. This was done to provide a 20% sampling of the population of villages under study with inclusion of all age groups. The selection of villages in the block was done by simple random sampling method using table of random numbers (Fisher & Yates, 1957).

The family records of these selected villages as maintained by respective ANM's and CHV's in Chiragpur block, verified and made up-to-date by making necessary alterations and additions during household listing in the selected villages. The records were re-verified at the time of sampling. The door to door survey was carried out by visiting once during the transmission period (September & October, 1987).

Heads of families of selected household were interviewed on a pre-tested schedule (see Appendix I) to collect information regarding various bio-social characteristics. Thereafter each individual of household was interviewed separately and information were recorded on a separate schedule (Appendix II). Every individual was examined clinically to find out any associated illness and to assess cyanomegaly.

2.3 Collection, transportation and storage of samples:

The blood samples of each individual with family
by door to door visit. By finger prick method, two spots of 2 cm. size were taken on Whatman's No. 3 filter paper strips. A thin and thick smear of individual was also prepared. The filter paper strips were air dried in shade. Dried samples were sealed in polythene bags and were transported to the laboratory in ice. In the laboratory, the filter paper strips were stored at -20°C until final analysis. The slides were fixed in methanol on the same day and stained with Giemsa stain. Later, they were examined under oil immersion lens of binocular compound microscope.

The collection and staining of glass slides were performed in usual manner (W.H.O., 1961). The blood films were stained with Giemsa stain and examined for malarial parasites.

3. Performance of Serological Test:

3.1 Antigen:

*P. falciparum* antigen was prepared from in-vitro culture of *P. falciparum* maintained at National Institute of Communicable Diseases (NICD), Delhi. Test was essentially performed as described by Wall et al (1978) and some modifications suggested by Ray et al (1983). The parasite was at a sub-culture level of 251 and contained approximately 8-9 percent parasitaemia with mainly schizonts. The antigen was prepared by saponin
treatment of the culture followed by sonication. Antigen was schizont antigen and was more than 90% pure.

3.2 Reference serum:

The positive reference serum was obtained from a person having heavy malaria infection. The negative reference serum was a pool from slide negative apparently healthy human beings. These had previously been tested by the IIF & ELISA.

4. ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

a) The Micro-ELISA test was performed in the 96-well flat bottom polystyrene Micro-ELISA plate (Nunc, Micro titre No. 1 were used as carrier surface for antigen.

b) Enzyme Conjugate: Anti-human IgG (heavy and light chains) labelled with horse radish peroxidase was obtained from Cappel Laboratories (Cochran ville, U.S.A.)

c) Substrate: Enzyme substrate ortho-phenylene diamine (O.P.D.) was obtained from Sigma Chemical, U.S.A. for measuring peroxidase activity.

d) A reference positive and reference negative serum were used for determining optimal dilution of antigens, serum and conjugate using in-vitro culture P.falcipareum antigen dilution from 1:3000 and serum dilution ranging from 1:400 in PBS Tween-20.
The optimum antigen dilution using in vitro culture *P. falciparum* antigen that gave a strong reading with positive serum and low reading with similar dilution of negative serum was found 1 : 3000 and serum dilution was 1 : 400. The optimal conjugate dilution was determined by chequer board titration. Conjugate dilution (1 : 1500) was found to be optimum.

**Micro-ELISA Procedure** :- Test was performed according to method described by Ray et al (1983).

(i) **Sensitisation of Micro-titre plate** :

Antigen was diluted to optimum concentration in coating carbonate buffer (0.05 M, PH 9.6). 200 ul each of the optimally diluted antigen was added into the wells of a micro-titre plate. The plates were covered and kept in plastic box to incubate at 4°C for 18 hrs.

Dilutions (1 : 200 ul) of the test and control sera were made in PBS, pH 7.2 containing 0.05% Tween-20 (see Appendix III). The antigen sensitized plates were took out from the refrigerator and the excess liquid was removed from the plate. The plate was washed twice in same buffer for 5 minutes each time and then dried.

(ii) **Incubation with sera** :

The antigen coated wells were filled with test and control sera of 200 ul on the washed antigen sensitized plate. Antigen control was kept adding PBS, Tween-20 only.
The plates were covered and incubated at room temperature inside the wet plastic box for one hour. The wells were washed for 5 minutes thrice with P.B.S./7 to remove unbound serum.

(iii) Incubation with Conjugate: Each of the well was then filled with 200 ul vol. of optimal diluted (1 : 1500) conjugate and then incubated in a humid chamber at room temperature for one hour. After incubation unbound conjugate was removed with washing three times P.B.S./7 for five minutes each.

(iv) Substrate reaction: Peroxidase bound to the wells was finally estimated by adding 200 ul of substrate solution in each well and incubating the plate at room temperature in the dark. The reaction is allowed to proceed for 10-15 minutes. The reaction was stopped with the addition of 50 ul of SN H₂SO₄ in each well.

For expression of results, the reading at a dilution of 1 : 400 was used since at this dilution, the positive-negative differentiation was best. 93.6 percent of the control sera from Delhi and all the sera from Kashmir gave a negative (\leq 0.4) reading. Taking this as the point of differentiation between the positive and negative sera, 13.30 percent of our study individuals showed reaction upto 0 - 0.4 O.D. (E₄₉₃); 26.01 percent showed 0.4 - 0.6 O.D. reaction (E₄₉₂); 14.81 percent
individual showed 0.6 - 0.8 O.D. (E492) and 6.05 percent showed 0.1 - 1.0 O.D. (E492) reaction. All the slide positive individual showed more than 1.0 O.D. (E492) reaction.

Reading was taken at optical density (OD) at wave length of 492 n.m. using spectronic 20 spectrophotometer.

5. **Indirect Immunofluorescence Test**

A local strain of *P. falciparum* (PAN-5) was adopted to continuous culture and maintained in HICD laboratory since 1976 by the method of Rai Chowdhuri et al. At sub-culture level of 122 when the parasitaemia was 8% with rings (20 percent), trophozoites (35 percent) and schizont (45%), the culture was washed five times in P.B.S. pH 7.2. After the final washing, it was suspended in the same buffer in such a way so as to contain about 20-30 plasmodesm per high power field in a thick smear (Sulzer et al. 1969). While preparing the smears, care was taken that the cells did not settle out of the antigen suspension in the pasteur pipettes. After drying, the smears were stored at -70°C, wrapped in wax papers.

5.1 **Reference sera**

The reference malaria positive sera from malaria cases and negative sera from non-malarious area were received from C.D.C. Atlanta and stored as above.
5.2 **Fluorescent Conjugate:**

Anti-human IgG (heavy and light chains) labelled with fluorescein isothiocyanate was obtained from immuno-diagnostic limited. Different conjugate dilutions were tested for finding the optimum dilution to be used. It was found that conjugate dilution 1:10 was giving highest titre with reference positive and lowest with reference negative sera.

5.3 **Performance of the Test:**

The test was carried out essentially by the method of Bulzer *et al.* (1969) with some modifications suggested by Ray *et al.* (1982). The antigen slides (stored at -70°C) were taken out and kept on racks made in glass petridishes and were labelled and allowed to dry. The test sera along with positive and one negative control sera were diluted in two fold dilution starting from 1:32 to 64 in P.B.S. pH 7.2. A drop of each dilution of test sera was placed covering each antigen smear. A control smear was kept receiving P.B.S. pH 7.2 instead of serum. The slides were placed inside humid petri dishes and incubated at 70°C for 20 minutes. Next the slides were washed thrice (each time for 10 minutes with P.B.S. pH 7.2) with manual stirring and dried quickly under the fans.
Optimal dilutions (1 : 40) of commercial anti-human IgG, A and M (M and L) conjugated with fluorescin isothiocyanate (Institute Pasteur Production) was added to cover the smear fully.

Incubation, washing and drying in the above manner followed. The slides were mounted with buffered glycerol (pH 7.2) and examined under a fluorescent microscope.

5.4 Reading and interpretation of Results:

Fluorescence was subjectively graded from negative to + and ++ and above were considered positive. The fluorescence of the parasites were seen against a background of faintly visible erythrocytes (Ray et al. 1982).

6. Compilation, Tabulation and Interpretation of Data:

Data so obtained from the study was subjected to critical statistical analysis which consist of estimation of the prevalence of antibody titre in random population and to find out, correlating it with various bio-social characteristics of the population.

The usual tests of significance such as Chi square test was used to determine the significance of the association between the two variables and difference between two parametric values.
7. **Limitation of study**

The study had been carried out in partial fulfilment of the requirements of M.D. (Social & Preventive Medicine) examination and therefore suffers from limitations of time and resources. Many of the informations sought, are based on the capacity to recall, the limitations of which do not need any emphasis. The reluctance on the part of individuals in giving the blood samples proved a great difficulty in the course of study. Inspite of the best efforts made, such samples of all individuals could not be obtained.

To show seasonal variations in the transmission of the disease, the non-transmission survey could not be conducted due to paucity of time and resources available.

Due to unavoidable reasons and paucity of time, the IIF test could be performed only in ELISA positive proven samples of blood.

8. **Different criteria adopted**:  

8.1 **Family type**:  

Any family with husband, wife and their offsprings was considered as nuclear and rest were considered as joint.
8.2 Family size:

A family up to 5 members was considered as small, whereas one with 6 or more members was taken as large.

8.3 Social class:

Social classification of families used in this study was as given by Srivastava et al. (1982). Criterion of social classification brought forth by Srivastava et al. (1982) is given below:

<table>
<thead>
<tr>
<th>Mean monthly per capita income</th>
<th>Social Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rs. 600/- and above</td>
<td>I</td>
</tr>
<tr>
<td>Rs. 300/- to Rs. 599/-</td>
<td>II</td>
</tr>
<tr>
<td>Rs. 140/- to Rs. 299/-</td>
<td>III</td>
</tr>
<tr>
<td>Rs. 60/- to Rs. 139/-</td>
<td>IV</td>
</tr>
<tr>
<td>Rs. 60/-</td>
<td>V</td>
</tr>
</tbody>
</table>