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REVIEW ARTICLE

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Dynamics of Virulence of *Plasmodium falciparum*

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Keywords: Dynamics and virulence, Host-parasite relationship, *P. falciparum*

INTRODUCTION

The system analysis approach deployed in malaria studies allows one to consider an epidemiological process as an entity. It also permits study of those intrinsic mechanisms of the system, like heterogeneity of its elements, adaptation, self-regulation, etc., which determine the stability of the system.[1,2]

This approach can also be used for studying the particular functions of the system at various levels of its organisation.

Attempts to use such an approach have been made by us in order to explain possible mechanism(s) of the modulation of virulence of *Plasmodium falciparum* strains* that cause severe and sometimes — lethal forms of the disease.

*The term strain ≅ connotes here (and elsewhere), the sub-population ≅ of malaria parasites as a unit of transmission from one primary source of infection: the local population of parasites consists of a few such strains ≅, accumulated on this territory as a result of introduction from outside or during the evolutionary process.

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The existence of the populations of *P. falciparum* with a different level of virulence was suspected long ago, which was supported by the results of some studies[3]. However, with the commencement of the Global Malaria Eradication Programme launched in the middle of 1950's by the World Health Organization, the interest in this subject had diminished considerably. This is particularly so during the final stages of many successful programmes of malaria eradication, as the number of severe cases of malaria, and the number of deaths due to malaria were negligibly low.[4]

Adopted by the WHO in 1992, the Global Malaria Control Strategy was aimed at the reduction of malaria morbidity and mortality throughout all malaria endemic countries in the world, including the areas south of the Sahara in Africa. It is there where the number of clinical malaria cases annually constitutes about 80% of total malaria cases in the world, and the number of malaria deaths is more than 90% of total malaria-related deaths, predominantly among the children of young age.[5]

During the course of the implementation of antimalaria activities, many clinicians and epidemiologists came across with facts which did not correspond to the traditional notions of "African malaria" ≅ particularly so in relation to the prevailing belief that the level of malaria mortality is directly related to the level of malaria transmission. It was demonstrated that the risk of severe and complicated malaria, as well as of malaria-related mortality, are lower among the children residing in the territories with a high level of transmission than among children in places with moderate and low malaria transmission.[6,7]

Further observations revealed a few factors which facilitate the occurrence of severe malaria. Among these factors are: (*a*) volume of the inoculum; (*b*) genetic susceptibility of man to infection; (*c*) frequency of previous contacts with the infection; (*d*) health and nutritional status of an infected person; and (*e*) status of health system and other entomological, social and economic factors alone, or during interaction.[6] Many investigators named the virulence of the parasites as one of the important factors directly responsible for cases of death.[8,9] All of these investigators stressed that the phenomenon of malaria virulence is of a complex nature for which there is a need for theoretical and experimental studies and field observations.

DEVELOPMENT OF THE HYPOTHESIS

Virulence of *P. falciparum* strains

Different explanations had been offered as to the nature of the strains of *P. falciparum* with various levels of its virulence. Pampana[10] believed that one of the possible causes of low virulence was the infection of man with a low number of sporozoites in the inoculum. The other possible explanation he proposed was that the pathogenicity of the parasites was lost as a result of the protective action of the immunity of the infected person.

The analysis of vast number of the clinical observations allowed White[9] to make a sugges-

tion that the foci with a high intensity of malaria transmission coexist with genetically different populations of *P. falciparum* with a different level of virulence. He also believed that the populations with high virulence possess characteristics which distinguish them from less virulent populations of the parasite in: (*a*) ability to propagate very rapidly; (*b*) cytoadherence and formation of the rosettes; (*c*) high potential to release cytokines; (*d*) high antigenicity; and (*e*) resistance to anti-malarials. While one can agree with (*b*), (*c*) and (*d*), the existence of (*a*) and (*e*) causes some doubts. Their acceptance might signify that highly virulent populations of *P. falciparum* possess the biological advantages over less virulent populations of the parasite circulating in the same location. If it were the case, then more virulent strains could eventually oust less virulent strains with dire consequences both for parasite and its host. Fortunately, it does not happen.

Genetic character of the virulence of malaria parasites was demonstrated by some investigators under laboratory conditions, who, however, consider this only as one of the possible ways of its mechanism(s).[11] In some of these observations, virulence was determined by mutation with subsequent selection for this phenomenon.[12, 13] In others, it was shown that non-virulent forms of *P. yoeli* were established as a result of crossing of two virulent lines.[13] This phenomenon, under natural conditions, probably takes place in the residual foci of *P. falciparum* malaria in the Fayume Governorate in Egypt (A. Beljaev, personal communication). Nevertheless, the mechanisms of the mutations resulting in the establishment of virulent forms of *P. falciparum* still remain obscure.

Another approach to explain the nature of the virulence of *P. falciparum* was used by Gupta and Day.[14] They developed a mathematical model based on the assumption that the virulence of each strain is predetermined. Thus, cases of cerebral malaria may be caused by strains with well expressed virulence, while strains with less expressed virulence result in a clinically mild form of the disease, although they are instrumental in the occurrence of a number of cases with well expressed anaemia. Changes in the ratio of strains with different levels of the virulence were attributed to variations of the level of cross-immunity. This hypothesis could not explain, however, the reason for low malaria mortality among children in the foci with a high level of malaria transmission.[6, 7, 15–18]

Further development of the mathematical model by Gupta and Shill[19] and Gupta *et al.*[8] resulted in the appreciation of a very important fact, i.e. that the spectrum of the severity of the clinical manifestations depends not only on the virulence of the parasites of *P. falciparum* but also on the immunity status of the infected person. Heterogeneity of the immunity status of the human host may, under certain circumstances, determine heterogeneity of the virulence of the parasite and vice versa. Such an approach makes a first step towards identification of the mecha-nism(s) of self-regulation of the malaria system at the organism level (man-parasite); however, it does not describe as to how this mechanism operates.

Very important results of the observations on the mechanism of interaction of malaria parasites with different virulence with the host were obtained by Smith *et al.*,[20] who in many respects are in agreement with the major points of the hypothesis suggested by White.[9] They believe that highly virulent clones* of *P. falciparum* are capable of propagating very quickly and that they are highly immunogenic in the non-immune hosts (usually children of young age). They also concluded that it is in the organisms of the latter that a selection of highly virulent strains takes place.

Taking results of the reviewed literature as a basis for further development of the hypothesis, the existence of other mechanisms of variations of parasite virulence may be looked for. One can assume, for example, that different forms of clinical manifestations of *P. falciparum* are the result of the modifications of parasite virulence in the course of the passage through the hosts with different immunity status.

Such a possibility in principle was demonstrated both in animal models as well as in volunteers. James *et al.*[21] demonstrated increased virulence of the Madagascar strain of *P. vivax* through its passage in volunteers. Severe manifestations of the disease caused by that strain were accompanied by very high parasitaemia due to its high reproductive ability and viability. Ciuca *et al.*,[22] by way of consequent passages among volunteers, managed to increase the virulence of *P. knowlesi*, and the virulence increased with each passage. Carrescia and Areolo (as quoted by Pampana)[10] showed the increased virulence of *P. berghei* through passages in white mice. Sergent (as quoted by Pampana)[10] confirmed the results of the studies of Carrescia and Areolo, and demonstrated that the severity of the disease caused by *P. berghei* was directly related to the number of passages.

Based on these observations, one can assume that the passage of *P. falciparum* under natural conditions through non-immune persons may result in increased virulence of the parasite. Conversely, passage of highly virulent parasites through an immune or semi-immune person does not result in further increased virulence, but it may even be reduced. This assumption is in good agreement with the results of the studies by Carlson,[23] who showed that the process of rosetting in the capillaries of the infected man (which is a good indicator of high virulence of parasite) is directly related to the immunity status of the host.

Similar results were obtained in the studies on virulence in other infections. The passage of the *Leishmania major* strain with a low virulence through highly susceptible laboratory animals resulted in a considerable increase of the virulent strains capable of infecting man.[24]

In epidemics of meningitis caused by *Neisseria meningitidis*, the moderation of the clinical manifestations during its course is attributed to the reduction of virulence of circulating strains as a result of the passage through immune or partially immune persons.[25]

Anderson and May[26] demonstrated in their mathematical model of HIV infection that the

*Same, p. 1.

existence of its population is determined by the necessity to change the virulence of the agent in time either chaotically or cyclically. Mathematical models developed by Koella and Doebeli[27] demonstrated an inevitability of periodic rise of the sub-populations of parasites with low and high virulence in order to keep the parasitic system in a state of dynamic stability.

Interaction of parasite virulence and the level of immunity of the host

Based on data derived from the review of the literature given in the previous section, we assume that similar principles of the dynamics of virulence of *P. falciparum* along with some other factors, facilitate the existence of this parasite in the community. If it is further assumed that in the non-immune organism, initial virulence of the parasite increases in the course of the epidemiological process, and that on the contrary it diminishes in the immune organism, then various situations may exist under natural conditions as follows.

Source of infection—Non-immune: A weak non-specific response of a non-immune organism as a result of development of a micro-population of *P. falciparum* leads to the development of gametocytes with high virulence. If a modified variant of the parasite is thus transmitted to an immune person, it probably will not cause clinical manifestations of the disease, the reason being a very rapid elimination of parasites through the mechanism of specific immunity. However, under certain circumstances, when, for example, there is some reduction of the level of malaria immunity in an otherwise immune organism, highly virulent parasites of *P. falciparum* can overcome the barrier of the immune response. This will result in parasitaemia without clinical manifestation of the disease, although capable of producing gametocytes. That is probably a mechanism of the phenomenon of gametocyte carriers among the immunes residing in the foci with high levels of malaria transmission. If highly virulent parasites of *P. falciparum* are transmitted to a non-immune host, further increase of virulence may occur. Such a situation may lead to a severe form of the disease, and eventual death due to malaria in case of other unfavourable factors (low nutritional status, other infections, etc.).

Source of Infection—Immune: Transmission of low-virulent strains of *P. falciparum* from one immune to another immune does not result in the clinical manifestations of the disease, unless an alien strain for this place was introduced into the community. Other exclusions are pregnant women with their reduced immunity status during the pregnancy. If the low-virulent strains are transmitted to a non-immune person, then increase of virulence takes place. Transmission of low-virulent strains from immune to partially immune persons (e.g. children above 5 years of age in the hyper-endemic malaria foci) may result in some increase of virulence due to a partial elimination of the parasites with a high level of virulence.

The role of various population groups

Adults in the foci with a high level of malaria transmission in endemic areas were considered as malaria immune persons. It was believed that

children, particularly of young age were more attractive to mosquitoes as a source of their feeding than adults.[3] Therefore, it was thought that the role of adults in the maintenance of malaria transmission was negligible.

At present, however, this point of view is being challenged. It was demonstrated that in the rural malaria endemic areas of tropical Africa, contribution of adults to the circulation of malaria parasites is considerably higher than it was thought.[28] It appears that the adult host is more attractive and accessible to mosquitoes than children. It was shown that in Garki, Nigeria[29] that about 10% of adults were carriers of malaria gametocytes. Similar data were obtained in holo-endemic areas in western Kenya[30] and in hyper-endemic areas outside tropical Africa, like Papua New Guinea.[31]

If the situation is more or less the same in other areas of tropical Africa, then about 10% of malaria immune adults are the source of infection with low virulence of malaria parasites. Non-immune children of young age constitute not more than 10% of the total number of the population; thus, some parts (when infected) are the reservoir of highly virulent parasites of *P. falciparum*. Children older than five years of age and pregnant women may be considered as a group harbouring parasites with a moderate level of virulence.

Some evidence supporting the hypothesis

Appreciable reduction of malaria-related child mortality was demonstrated in the course of the implementation of large-scale trials using insecticide-impregnated mosquito nets (IMN) under various epidemiological settings in tropical Africa.[32–34] The reduction of malaria mortality was not accompanied, however, by a reduction of malaria prevalence, thus indicating the continuation of malaria transmission.[6] Taking into consideration, however, that the IMNs were deployed exclusively among the children of young age, one can assume that malaria transmission among this age group was considerably reduced. In accordance with the proposed hypothesis, partial blockade of the passage of parasites from a non-immune to another non-immune had occurred along with the parallel reduction of the access of mosquitoes to highly virulent parasites of *P. falciparum*. That had led to a reduction of the frequency of severe malaria and number of malaria deaths.

Another example that is not contrary to the hypothesis is the preliminary data on the results of two-year observations on the efficacy of insecticide-impregnated curtains in hyper-endem-ic areas in Burkina Faso.[35] The most important difference between the series of these experiments with that of the use of IMN was that the insecticide-impregnated curtains protected all the inhabitants of the dwellings where they were distributed. As expected, a considerable reduction of malaria transmission had occurred, supported by the results of the entomological observations, like reduction of the density of malaria vector, sporozoite index and inoculation index, although the reduction of parasite index was moderate. Malaria mortality among the children of young ages was about 15% (two-year observations). However, year-wise reduction of mortality indicates that it was only during the first year of ob-

servations, that mortality was considerably reduced (26%), while during the second year it demonstrated a very modest reduction (4% only).

According to the proposed hypothesis, these results indicate that the impact of insecticide-impregnated curtains on malaria mortality was diminishing due to parallel reduction of malaria transmission and the community's immunity as well as the increase in the circulation of highly virulent strains of parasites.

EVALUATION OF HYPOTHESIS

Proposed method of assessment

An attempt was made to develop a mathematical method in order to evaluate the validity of the hypothesis. In doing so, the main assumptions were selected as follows.

It is known that in the foci of hyper-endemic malaria, the proportion of non-immune and immune populations is more or less permanent. Conditionally, four groups of populations can be identified in such foci in relation to their immunity status.

Non-immune population *(l)*: This group (1–4 years old) consists not only of a relatively small group of children of young age (who had not yet encountered malaria infection) but also of a bigger group of children of this age who had already been exposed to some of the circulating strains of *P. falciparum* in this locality.

Semi-immune population *(m)*: Children of older age (> 5 years old), the majority of whom had already encountered most of all the circulating strains of *P. falciparum*, pregnant women as well as some young males (15–25 years old) who are the most mobile in this population group.

Immune population *(k)*: People who have developed immunity to all strains of *P. falciparum*, circulating in the locality, but who are asymptomatic carriers (with gametocytes in the blood).

***l-m-k*-immune population (1):** People who have developed immunity to all circulating strains in the locality, and who are not the carriers of gametocytes of *P. falciparum*.

In the hyper-endemic foci of malaria, practically all non-immune, semi-immune and immune carriers of gametocytes of *P. falciparum* are the reservoirs of malaria infection, which can be expressed as $(l+m+k)$. In accordance with the proposed hypothesis, infection that is received from semi-immunes and immunes with "normal virulence"* by non-immune persons, modifies its virulence and results in the formation of gametocytes with increased virulence. The clinical outcome of such infection is non-complicated malaria and malaria death on rare occasions and in cases of concomitant infection or low nutritional status.

Feeding on the infected and non-immune fraction of the population *(l)*, mosquitoes will be infected with the high-virulence parasite and will distribute this among the total population of the malaria focus. Thus, the proportion of vectors

*An average statistical level of virulence of the strain.

infected with such parasites will be $l^2 \times (k+l+m)$. Conversely, the non-immune fraction of the population will be infected with high-virulence parasites and also be equal to $l^2 \times (k+l+m)$. The outcome of this infection in non-immunes will be cases of severe malaria and malaria-related deaths. Acquisition of highly virulent strains by semi-immunes and immunes will not result in cases of severe malaria or in malaria deaths due to the process of attenuation of parasites' virulence in them. Thus, in hyper-endemic foci, only non-immunes harbour *P. falciparum* parasites with high virulence as suggested by Smith *et al.*[20]

Mathematically, the proportion of non-immunes receiving parasites with high virulence may be expressed as $(m+l) \times l^2 \times (k+l+m)$.

It is not at all unusual in the hyper-endemic malaria foci to encounter malaria superinfection.[20] If that case occurs, i.e. the infection is with a highly virulent strain of *P. falciparum*, death may take place specially in young children with low health status. Therefore, the proportion of lethal cases due to malaria among the population of malaria hyper-endemic foci, in accordance with the proposed hypothesis should not exceed the level of $(m+l) \times l^2 \times (k+l+m)$, or $l^2 \times (k+l+m)$.

Use of the method for evaluation of the hypothesis

As a basis for our calculations, the age structure of Cameroon (% of the total population), which represents many typical demographic features of the countries of tropical Africa was taken which is as follows:

Age < 1 yr = 3.4; 1–4 yr = 11.8; 5–9 yr = 15.1; 10–14 yr = 12.8; 15–19 yr = 12.7; 20–24 yr = 10.0; 25–29 yr = 7.5; and > 30 yr = 26.7.

For purposes of a more precise evaluation, there is a need for identification of the proportion of the total population on the age range between the average age when maternal immunity had already ceased, to the average age when malaria prevalence had reached its highest level. Achidi *et al.*[36] established this range for tropical Africa as 2–3 months and 25–31 years of age accordingly.

With this in mind, parameter l is taken as 15.2% (young children), parameter m as 50.6% and parameter k is taken as the proportion of people above 25 years of age, multiplied by 10% (proportion of gametocyte carriers among the immunes). Thus, parameter k is equal to 3.4%. The results of calculations deploying the formulas $(m+l) \times l^2 \times (l+m+k)$ and $l^2 \times (l+m+k)$ for the establishment of the range of probable mortality due to malaria are 1.05 and 1.60% accordingly, which are in close proximity to widely accepted estimations of 1–2%.[6]

This method was also used by us for evaluation of data obtained in the Garki Project in Nigeria.[29] Parameter l was 13.7%, m was 43.6%, and parameter k was equal to 4.3%. Thus, the range of possible malaria mortality was assessed as 0.66 and 1.08% against factual lethality at 0.86%.[29]

CONCLUSION

The principal points of the proposed hypothesis of the modulation of the virulence of *P. falci-*

parum in the foci of hyper-endemic malaria in tropical Africa are as follows.

(1) Diversity of *P. falciparum* genotypes circulating in the foci of hyper-endemic malaria in the countries of tropical Africa is determined by interaction of various factors.

(2) The level of virulence of different strains of *P. falciparum* is a dynamic process and is closely related to the interaction of the parasite and the immunity status of the host.

(3) The virulence of parasites is modified in the process of passage through the non-immune or immune status of the infected person.

(4) The evolutionary process of adaptation of such a modulation facilitates the parasites to survive in the human community with a considerably high proportion of people with immunity.

For better verification of the hypothesis, it might be of interest to use the data age-wise of parasite/gametocyte index, malaria lethality and various forms of severe/complicated malaria, as well as the annual age structure of the population. It would be useful to utilise data derived from the malaria foci with a different status of the immunity of population. A more reliable verification of the proposed hypothesis could be possible with the deployment of the mathematical modelling of the dynamics of the proportion of the immunes in the community. Since the hypothesis relates only to the foci of endemic malaria, the next step might be the verification of the hypothesis in the territories under the risk of malaria epidemics.

The proposed hypothesis allows a better understanding of certain epidemiological peculiarities of hyper-endemic malaria in the countries of tropical Africa, particularly those related to the incidence of severe malaria and malaria mortality in areas with various levels of malaria transmission.

It demonstrates the necessity to consider the role of each age group of population in the development of the epidemiological process of malaria in every epidemiological setting. It is with such an assessment, particularly with the quantitative one, that the planning and implementation of malaria control activities will have to be done.

Large-scale trials with the insecticide-impregnated mosquito nets in various epidemiological settings in tropical Africa demonstrated that its use among young children contributed to a considerable reduction of malaria-related mortality. In accordance with the proposed hypothesis, this phenomenon can be explained by a considerable reduction of the transmission of malaria strains with high virulence, in parallel with the retention of the transmission at the original level of malaria strains with normal virulence. Thus, the epidemiological justification of the expansion of the use of IMNs to the population groups other than that of young children, in the foci of hyper-endemic malaria, should be assessed very carefully.

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Naturally Occurring Plasmodia-Specific Circulating Immune Complexes in Individuals of Malaria Endemic Areas in India

PADMAWATI TYAGI[a] and SUKLA BISWAS[a]

Blood samples collected from individuals belonging to malaria endemic areas were assayed for antigen-specific circulating immune complexes in polyethylene glycol precipitates of serum by enzyme immunoassay. Sera were tested from patients with acute *P. vivax* and *P. falciparum* infections, from clinically immune individuals and also from healthy normals. Circulating immune complexes (CICs) containing immunoglobulin G and M isotypes were found to be abundant in individuals with ongoing and past infections and also in clinically immune donors. In patients with acute infection but without any past history of malaria, CICs of IgM type were found to be significantly higher. Demonstration of antigen/antibody specific CICs could be a useful indicator of active, ongoing and recent/past infection, also of the status of immune responses of individuals belonging to various endemic areas.

Keywords: Enzyme immuno assay, Immune complexes, Immunoglobulins, Malaria

INTRODUCTION

Plasmodium vivax and *Plasmodium falciparum* are the two main species responsible for nearly 65% and 35% of malaria in India. Protective immunity to malaria is primarily a premunition, i.e. a resistance to super-infection and general immune responses in malaria infection is evoked through both the effector limbs of the immune system.[1] Humoral as well as cellular immune responses are adequately elicited in malaria infection.[2,3]

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However, the evasive strategies of *Plasmodium* spp to evade and modulate the immune responses of the infecting host have contributed immensely to their extended survival and propagation in human hosts. In spite of effective elicitation of immune responses, acquisition of sterile immunity is rare, and relapses and recrudescence do exist in malaria. Mechanisms of slow development of immunity in individuals living in endemic areas is also not clearly understood.[4]

Continual exposure of the host to a variety of antigens and subsequent polyclonal activation of B-cells[5] contribute grossly in the formation of immune complexes (ICs) in the circulation. *In vivo* immune complex formation is a continuous process whenever an antibody meets the antigen, whether specific or non-specific. Several viral, bacterial and parasitic diseases demonstrate immune complex formations; malaria is one of them. Formation of immune complexes in malaria may be one of the outcomes of persistent infection. Low-grade persistent infection and poor antibody response jointly lead to the formation of immune complex and chronicity of the disease. Circulating immune complexes (CICs) have been documented to play an essential role in modulation of the immune response to various antigens.[6] Their presence in murine, rodent, simian and human malaria have been reported.[7,8] However, in human malaria very little is known about CICs and antigens and the antibodies involved in their formation.

In the present study, an attempt has been made to determine the plasmodial CICs of IgG and IgM classes directed against *P. vivax* and *P. falci-parum* antigens.

MATERIALS AND METHODS

Parasite antigen preparation

***P. falciparum* antigen:** The Indian *P. falciparum* isolate from Delhi, FDL-B was used for antigen preparation, as described earlier.[9] Parasites were grown and adapted *in vitro* using O + RBCs and AB + serum by the candle jar technique. The culture was harvested when parasitaemia reached almost 10% and a large number of late trophozoites and schizont stages were observed. Parasites were freed by saponin lysis and a soluble extract was obtained by sonication in MSE Soniprep at 14 μA for 90 sec. The antigen was purified after affinity adsorption with rabbit anti-human erythrocytes antibody, and the protein concentration was determined.

***P. vivax* antigen:** Blood samples were collected from patients who visited the malaria clinic of MRC, Delhi and suffering from *P. vivax* infection after obtaining consent for venipuncture. Nearly 5–10 ml blood was collected aseptically from patients showing 1% or more parasitaemia in heparinised tubes.

Plasma was aspirated and stored frozen; RBC pellet was resuspended in an equal volume of sterile PBS. The suspension was overlayed on 3% dextran sulphate solution, then incubated for 45 min at 37 °C keeping the tube in a slanting position. The supernatant containing WBCs was removed, and the RBC pellet was washed thrice in PBS. The antigen was prepared by saponin lysis, followed by centrifugation, sonication and affinity adsorption.

Collection of blood samples: Blood samples for sera were collected during the transmission season (April to November 1996) from individuals living in various endemic pockets in Delhi, Ghaziabad (PHC-Loni) and belonging to age groups of 15–50 yr. Study subjects were categorised under four groups according to their malaria experience. Blood samples collected from four groups were as : patients suffering from acute *P. vivax* infection and with a history of repeated *P. vivax* and *P. falciparum* infections (n = 29); patients with acute *P. falciparum* infection without any history of past infection (n = 24); clinically immune adults who earlier suffered repeatedly from malaria but did not show any clinical symptoms at the time of blood collection (n = 15); and healthy adults who were residents of endemic zones but without any history of malaria (n = 20). A volume of 1–2 ml blood was collected aseptically from each individual after obtaining informed consent. Patients diagnosed for malaria were treated with the recommended doses of chloroquine by the clinician. Sera were separated and stored in aliquotes at – 20 °C for their use in a number of studies.

Isolation of CICs: Circulating immune complexes were isolated by precipitating at 2.5% (w/v) polyethylene glycol (PEG), mol wt 6000 (SISCO Research Laboratories, India) following the published method.[10] Briefly, 100 µl of serum was diluted five times in PHS-EDTA (0.15 M PBS, pH 7.2 and 0.01 M EDTA, pH 7.6). Equal volumes of freshly prepared 5% PEG in PBS were added to the diluted serum, then incubated overnight at 4 °C. The precipitates were obtained by centrifuging at 1000 x g for 30 min at 20 °C, then washed once with 2.5% PEG. Finally, precipitates were reconstituted to the original volume by dissolving thoroughly in PBS and kept at 37 °C for 2 h. The dissolved precipitates were stored at – 20 °C till use.

Detection of malarial antigen specific CICs: The CICs in PEG precipitates were detected by simple indirect ELISA. Polystyrene round-bottom 96 well ELISA plates were coated separately with 50 µl per well *P. vivax* and *P. falciparum* crude antigens diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6 at a concentration of 10 µg/ml, then they were incubated overnight at 4 °C. After removing excess antigens, the plates were washed twice with PBS-Tween-20 (PBS-T) and blocked with 1% BSA for 2 h at 37 °C. PEG precipitates diluted in PBS at a dilution of 1: 100 (50 µl) were added in quadruplicate wells in both *P. vivax* and *P. falciparum* antigen coated plates and incubated overnight at 4 °C. After washing five times with PBS-T, anti-human IgG and IgM conjugated with HRPO were added in respective wells (50 µl) in duplicate and incubated for 1 h 30 min at 37 °C. Enzyme-specific substrate, orthophenylene di-amine/H_2O_2 (50 µl) was added in wells after washing five times with PBS-T and kept for reaction in the dark for 30 min at room temperature. Enzyme-substrate reaction was terminated by adding 50 µl of 8 N sulphuric acid and the plates were read at 492 nm in an ELISA reader (Titertek Multiskan Plus, Flow Laboratories, UK).

Statistical analysis: Mean ELISA O.D. values of each group were compared using student's *t-test* and probability values for the

CICs in each group were determined.

RESULTS

Sera tested in this study were collected from a group of individuals suffering from both *P. vivax* and *P. falciparum* malaria. Some of them had multiple attacks, while some of them had no history of malaria infection. The IgG and IgM type CIC profiles of these samples detected against *P. vivax* and *P. falciparum* antigens are shown in Fig. 1. In healthy normals (Group A), *P. vivax* and *P. falciparum* specific IgG and IgM type immune complexes were found to be much lower. A few samples demonstrated slightly higher values in both cases. In *P. vivax* patients (Group B), CICs of both the Ig isotypes were found to be elevated when tested against two antigens, indicating their present and recent past infections. The patients with acute infection of *P. falciparum* (Group C) demonstrated significantly higher IgM type CICs with *P. falciparum* antigen since these patients were suffering from a fresh bout of *P. falciparum* infection. In the clinically immune donors (Group D), CICs of IgM type were substantially high as observed by the reactivity of PEG ppt with both *P. vivax* and *P. falciparum* antigens. These may indi-

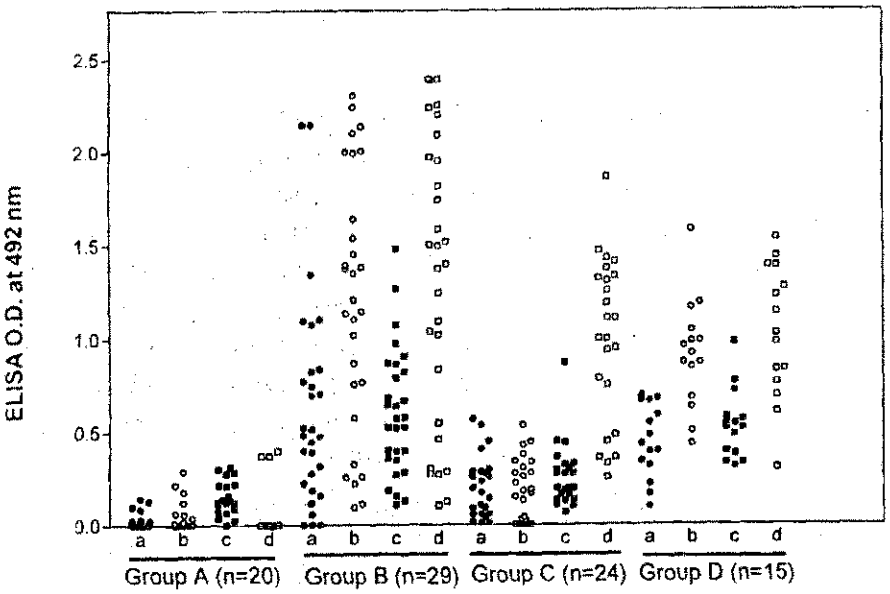

Fig. 1: Scatter diagram of plasmodial-specific CICs of IgG and IgM classes in sera from individuals.
Groups are normal healthy control (A), acute *P. vivax* and *P. falciparum* patients (B and C), clinically immune individuals (D). The graph is plotted with ELISA O.D. values at 492 nm.

Profile of CICs: a = IgG type with *Pv* Ag; b = IgM type with *Pv* Ag; c = IgG type with *Pf* Ag; d = IgM type with *Pf* Ag.

Table 1. Plasmodial (*P. vivax* and *P. falciparum*) CICs (IgG and IgM types) in PEG precipitates of sera from healthy normals, patients and immune individuals

Group	Sample size	ELISA O.D. values with *Pv* Ag.		(Mean ± S.D.) with *Pf* Ag.	
		IgG	IgM	IgG	IgM
A (Healthy)	20	0.03 ± 0.04	0.05 ± 0.08	0.15 ± 0.09	0.06 ± 0.14
B (*Pv* case)	29	0.62 ± 0.55	1.25 ± 0.80	0.60 ± 0.34	1.29 ± 0.75
C (*Pf* case)	24	0.20 ± 0.12	0.23 ± 0.16	0.22 ± 0.09	1.04 ± 0.41
D (Immune)	15	0.45 ± 0.19	0.91 ± 0.28	0.54 ± 0.18	1.03 ± 0.34
Statistical analysis					
A vs B		NS	NS	$p = 0.05$	$p = 0.05$
A vs C		$p < 0.05$	$p < 0.05$	$p < 0.001$	$p < 0.001$
A vs D		$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.001$

NS: Not significant.

cate that clinically immune individuals might be harbouring submicroscopic/subclinical infection in the system, which, in turn, provides immunity to the disease. These results with their statistical significance are shown in Table 1.

DISCUSSION

Observations of elevated levels of plasmodial CICs of IgG and IgM types in endemic areas are understandable as intense exposure to malaria may elicit humoral immune response in individuals residing in endemic areas.[11, 12] Immune complexes are formed during the immune response to an introduced or exposed antigen by binding to the newly formed specific antibodies to that antigen.[13]

The endemic areas where the study subjects belong offered ideal malariogenic conditions. In these areas malaria is seasonal: early, prolonged monsoons are responsible for intensive transmission of both *P. vivax* and *P. falciparum*. Coexistence of both *P. vivax* and *P. falciparum* CICs in individuals may indicate the high prevalence and infestation rate of these species in the community. Malariometric surveys conducted in northern India have shown the presence of both *P. vivax* and *P. falciparum* in many patients.[14] There are reports in which cross-reactivities among different strains of *Plasmodium* have been demonstrated[15,16]. These studies suggest that certain antigens — or at least some of their epitopes — might be common to different species of plasmodia. High levels of IgM-class CICs directed to *P. falciparum* antigens in patients with clinically acute *P. falciparum* infection may indicate their first encounter to this infection, as IgM is the predominant immunoglobulin produced during the primary immune res-

ponse[17]. Immune individuals have shown high IgM-type CICs which may be due to subclinical infection consisting of a new clonal subpopulation of parasites with a very low level of parasitaemia. This group of individuals was also having CICs of IgG type, but their level was found to be lower than the IgM-type complexes. The continuous boosting of the immune system with low-grade parasitaemia might be helping in acquisition of immunity. High levels of IgM in areas of fresh malaria outbreak have also been reported.[18] The polyclonal B-cell activation by plasmodial antigens produces arrays of antibodies which may not be protective to the host. The high load of CICs detected in sera may be both protective and non-protective antibody-bound complexes. It is important to identify antigen-antibody complexes relevant to the development of host protective immunity in people living in malaria endemic areas of India. In various species including human, ICs are formed during the immune response. A small proportion of the ICs is trapped in B-cell follicles on the cellular processes of follicular dendritic cells and can be retained there for a long period of time[19] via binding to Fc receptor[20] and complement C3 receptor.[21] Antigen preserved in this way is believed to play a crucial role in the generation of memory B-cell and the maintenance of long-term humoral immune responses. *In vitro* performed ICs have been shown to be much more efficient in inducing humoral immune responses *in vivo* than the native antigen.[22] This specific potential of ICs may suggest their use as vaccine antigens. Presence of CICs in clinically immune population living in endemic areas may explain the low infection rate in these individuals.

It may be concluded that determination of CICs in endemic areas could provide valuable data for evaluation of active transmission, current infection rate as well as the immune response profile of the host.

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Population Dynamics of Mosquito Immatures and the Succession in Abundance of Aquatic Insects in Rice Fields in Madurai, South India

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Studies on the breeding pattern of mosquito immatures and the successional changes in the abundance of aquatic insects were conducted in rice fields near Madurai, south India. The population of late (III/IV) larval instars of culicines peaked on Day 28 and pupae (*Culex tritaeniorhynchus, Cx. pseudovishnui* and *Cx. vishnui*) peaked on Day 9 after transplantation, whereas the population of late (III/IV) larval instars of anophelines peaked on Day 19 and pupae of *Anopheles subpictus* and *An. vagus* on Days 7 and 9 respectively, after transplantation of paddy. A total of 14 families (consisting of 17 subfamilies) of aquatic insects belonging to six different orders, Coleoptera, Diptera, Ephemeroptera, Hemiptera, Anisoptera and Zygoptera were collected during the study period and the abundance of each group of insects is discussed in detail. The Shannon-Weaver Diversity Index, applied to study the diversity of different groups of aquatic insects, showed a clear pattern in diversity of surface predators, bottom predators and non-predators of mosquito immatures. The multiple linear regression model reveals that notonectid adults, coenagrionids, libellulids and veliids act as important predators of mosquito immatures in rice fields.

Keywords: Abundance and diversity of insects, Breeding pattern, Mosquito immatures, Predator-prey relationships, Rice fields

INTRODUCTION

The changes in aquatic insect fauna in rice fields during each stage of plant growth may either increase or decrease. As a result, the community structure of aquatic insect fauna is progressively

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altered.

The succession of mosquito species in relation to different stages of plant growth in rice fields has been studied in India,[1–4] Sarawak,[5] Louisiana[6] and in the Philippines.[7] Although several studies have been reported to show the changes in the abundance of aquatic insects in rice fields in California,[8–11] Japan[12] and in the Philippines,[7] the successional change in the abundance of aquatic insect predators in rice fields in south India has not been studied thoroughly so far, where the *Culex vishnui* subgroup species, viz. *Cx. tritaeniorhynchus, Cx. pseudovishnui* and *Cx. vishnui*, the most important vectors of Japanese encephalitis, mainly breed in rice fields. The present study was carried out in rice fields near Madurai, south India to determine the population dynamics of mosquito immatures, cycles of abundance of aquatic insects and their diversity in rice fields and predator-prey relationships in rice fields.

MATERIALS AND METHODS

Study site

Studies were conducted in a farmer's rice fields in Melur near Madurai (9.58°N, 78.10° E) Tamil Nadu, during *Navarai* crop season in 1992, to study the breeding pattern of mosquitoes and abundance of aquatic insects. In Madurai district, rice is a major crop and two crops, *Kuruvai* (June–October) and *Samba* (July–January) are normally raised with water from the Periyar canal irrigation system. The third crop, *Navarai* (January–May) is very restricted, and fields are irrigated mainly from wells during this crop season. Average annual rainfall ranges from 3.6 to 213.2 mm and the mean monthly temperature recorded ranges from 21.8 to 36.6 °C. Rainfall is influenced by southwest monsoon (June–August) and northeast monsoon (October–November) and the *Kuruvai* and *Samba* crop seasons synchronise with them, respectively.

Sampling of mosquito immatures and other aquatic insects

Samples were taken by using a 0.1 sq m (0.3 x 0.3 m) metal quadrat, 15 cm high, open at both ends, since the earlier studies conducted in rice fields near Madurai in Tamil Nadu have shown that using 0.1 sq m quadrat is superior to conventional dipper samples for the collection of mosquito immatures and other aquatic insects present in different ecological niches.[13] Ree[14] also found that a static quadrat was more effective than dipper sampling for the estimation of aquatic invertebrates in rice fields in South Korea. The quadrat was pushed well into the soil in rice fields at random sites. The mosquito immatures (III and IV instar larvae and pupae) and other aquatic insects present inside the quadrat were collected by draining the water using an enamel bowl and then counted. When the water level within the quadrat went down, a pipette was used to collect the fauna from the bottom. After counting the insects separately according to their taxonomic classification, they were released in the same field, and then the density of each group was converted to numbers/sq m. Representatives of each group of aquatic insects were collected from rice fields in the study area, stored in 70% ethyl alcohol, and

identified in the laboratory up to species level and then listed. The mosquito pupae collected were brought to the laboratory for species identification after emergence.

In the present study, three rice fields (F1, F2 and F3), each 400 sq m were selected. Rice variety ADT 36 was cultivated in F1, and IR 50 in F2 and F3. Samples were taken three times in a week after two days of transplantation, and on each sampling occasion five quadrat samples were taken in each field. Sampling was continued up to six weeks, since earlier studies conducted in rice fields in Madurai district have shown that maximum breeding of mosquitoes occurs only during the first few weeks after transplantation and there is a natural decline in the population of mosquito immatures from six weeks onwards.[15, 16] Factomphos (12 kg/acre) was applied basally on the day of transplantation. In the second week after transplanting, urea (N = 46%) at 30 kg/acre and potassium at 12 kg/acre were also applied. Phosphomidon (125 ml/acre) was sprayed during the fifth week after transplantation.

Water temperature, pH, depth and plant height were also noted on each sampling occasion. Water temperature and pH were measured by using a digital water analyser (Hanna Instruments, India) and water depth and plant height were measured by using a 30-cm scale randomly at 5 different sites in the rice fields and the average values were noted.

Data analysis

The Shannon-Weaver Diversity Index (H')[17] was used to describe the diversity of various groups of aquatic insects in relation to plant growth in rice fields.

$$H' = -\sum_{i=1}^{S} \frac{n_i}{N} \frac{\log\left(\frac{n_i}{N}\right)}{\log 2}$$

Where, S = number of species in a sample; n_i = number of individuals of each species; and N = total number of individuals in the sample.

Data on the population of mosquito immatures (III/IV larval instars and pupae of both anophelines and culicines) and aquatic insect predators were analysed after transformation to log (n+1). Pearson's correlation analysis was used to relate the abundance of mosquito immatures with that of individual groups of predators.[18] Multiple regression analysis by the backward elimination method was used to find out the relationship between each group of predators and mosquito immatures. Since the predators have different trophic niches, bottom predators and surface predators were analysed separately. The SPSS/PC$^+$ statistical package version 4.0.1 (SPSS Inc., Chicago, IL., USA, 1984–1990) was used for these analyses.

RESULTS

The average height of the paddy seedlings at the time of transplantation was 19.8 cm, and it reached 48.3 cm when the last sample was taken after six weeks. The average water temperature, measured at around 1000 hrs, in the study fields ranged from 29.2 to 35.8 °C, while the

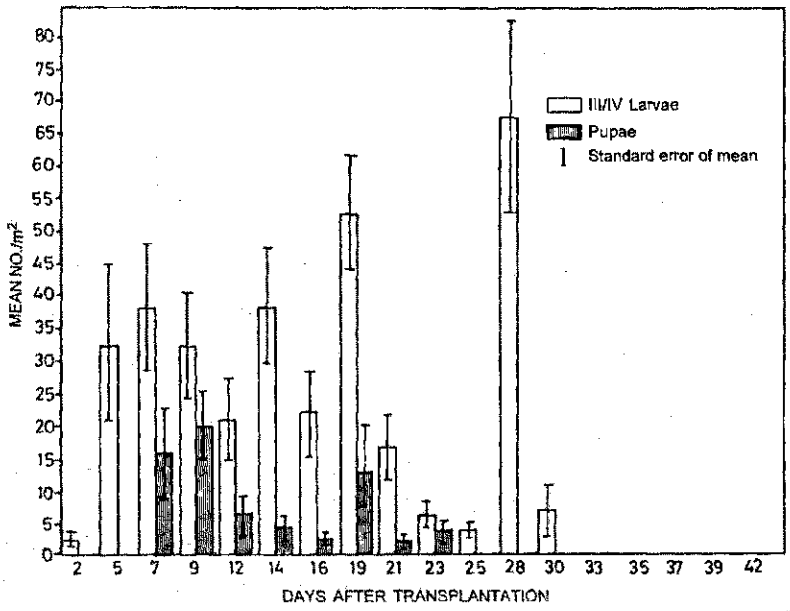

Fig. 1: Population dynamics of culicine immatures in rice fields

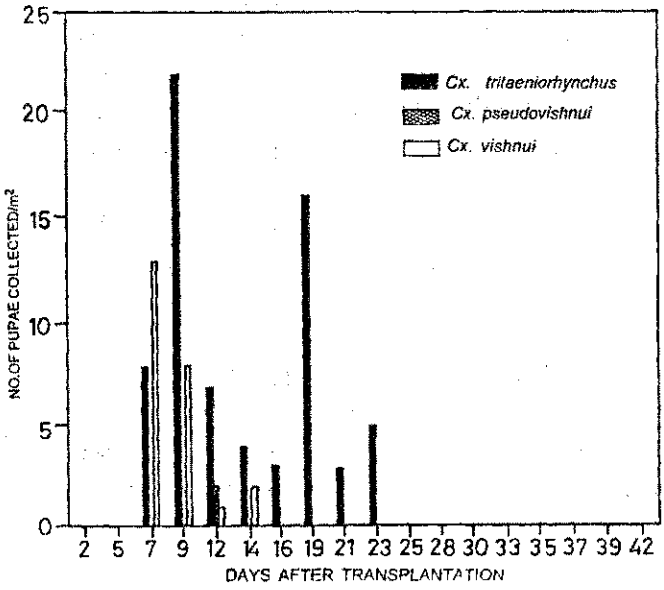

Fig. 2: Abundance of species of the *Culex vishnui* subgroup in rice fields

pH of the water ranged from 7.1 to 8.2. The greatest water depth recorded was 7.3 cm during the first week after transplantation and from the fourth week after transplantation, it remained at only about 3.0 cm.

Biotic factors

Dynamics of populations of mosquito immatures: The late (III/IV) larval instars and pupae of both anophelines and culicines were collected up to 30 days (fifth week) after transplantation of paddy, after which no larvae or pupae were found. The population of late instar larvae of culicines peaked on Day 28 (fourth week) and that of the pupae peaked on Day 9 (second week) after transplantation (Fig. 1). No pupae were found after 23 days (fourth week) post-transplantation. Among the total culicine mosquito species identified in rice fields during the study period, *Cx. tritaeniorhynchus* was predominant, consisting of 72.3% of the total culicines, followed by *Cx. vishnui* (25.5%) and *Cx. pseudovishnui* (2.2%). The highest density of pupae of *Cx. vishnui* (13) was present on Day 7 after transplantation and thereafter, it declined (Fig. 2). The population of *Cx. tritaeniorhynchus* pupae was found to be highest (22) on Day 9 (second week) after transplantation and the pupal population remained up to fourth week after transplantation, whereas *Cx. pseudovishnui* pupae (2) were found to be present during the second week only (Fig. 2).

The population of anopheline late (III/IV) larval instar peaked on Day 19 (third week) after transplantation, whereas the population of pupae peaked on Day 9 (Fig. 3). The pupae remained present up to 21 days after transplantation. *An. subpictus* (92.5%) and *An. vagus* (7.5%) were the two anopheline species present in the rice fields during the study period. The pupal population of *An. subpictus* was found to be highest

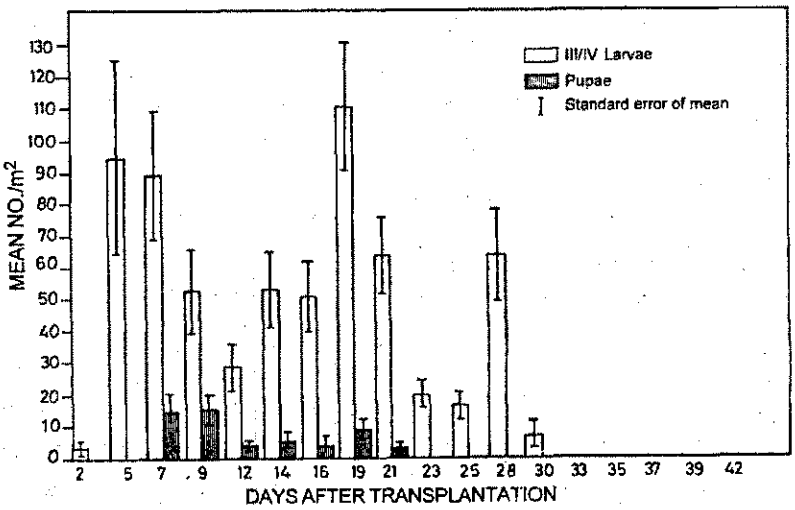

Fig. 3: Population dynamics of anopheline immatures in rice fields

from on Days 7–9 after transplantation and then declined gradually. The pupae of *An. vagus* were collected in small numbers during the second and third week after transplantation.

Cycles of abundance of other aquatic insects: Different groups and stages of aquatic insects were collected in rice fields during the study period (Table 1). Totally, 14 families (consisting of 17 subfamilies) of aquatic insects belonging to six different orders, Coleoptera, Diptera, Ephemeroptera, Hemiptera, Anisoptera and Zygoptera were collected.

The aquatic beetles collected during the study period were dytiscids, hydrophilids and noterids. Four genera of the family Dytiscidae, belonging to two subfamilies (Dytiscinae and Laccophilidae) were identified. The population of adult Dytiscidae, known predators of mosquito immatures, peaked on Day 19 (during the third week) after transplantation and thereafter decreased as the paddy height increased. Hydrophilinae, the deep water beetles, comprised two subfamilies, Berosinae and Hydrophilidae. *Berosus indicus*, a vegetarian beetle, was collected in large numbers during the third week after transplantation, and the highest number collected was 160 per sq m on Day 21 after transplantation. The number of the subfamily Hydrophilinae, bottom dwelling and predatory beetles, was found to be high during the first three weeks after transplantation, peaking during the third week. Thereafter, the population declined and disappeared totally by Day 33 after transplantation. Noterids, which are not known to be predators of mosquito larvae, peaked on Day 28 after transplantation and subsequently gradually decreased in abundance.

Dipterans other than mosquito immatures included larvae of Chironomidae and Stratiomyiidae. The family Chironomidae was represented by a single genus, *Chironomus* sp (subfamily Chironominae) and the abundance was high during the first week after transplantation, declining thereafter. Only one larva of soliderfly (Stratiomyiidae) was collected. The population of ephemeropteran nymphs (*Baetis* and *Cloeon*) increased to a peak on Day 21 after transplantation and then decreased.

Hemipterans belonging to five different families, viz., Corixidae, Gerridae, Hydrometridae, Notonectidae and Veliidae, were collected during the study period. Two species, *Anisops barbata* and *Anisops bouvieri*, of the subfamily Anisopinae (Notonectinae family) were collected in rice fields. Both nymphs and adults are known predators of mosquito immatures. The nymphs were collected from the second to the fourth weeks, showing a peak in abundance on Day 12 after transplantation, with the highest estimate of 55 per sq m. The adults appeared from Day 19 onwards and were not encountered after Day 25, showing a peak in abundance on Day 21 after transplantation. The highest estimate of adults was 26 per sq m.

Two species of Anisopterans, *Brachythemis contaminata* (Sympetrinae subfamily) and *Pantala flavescens* (Trameinae subfamily) of the family Libellulidae and one species of Zygopteran, *Ceriagrion aurantiacum* (Pseudogrioninae subfamily) of the Coenagrionidae family were collected during the study and the

Table 1. Aquatic insects collected in rice fields during *Navarai* crop season (1992)

Order	Family	Subfamily	Species	Stages collected*
Coleoptera	Dytiscidae	Dytiscinae	*Eretes sticticus*[c]	A
			Cybister limbatus[a]	L,A
			Dytiscus sp[a]	A
		Laccophilinae	*Laccophilus flexuosus*[a]	A
	Hydrophilidae	Berosinae	*Berosus indicus*[c]	A
		Hydrophilinae	*Hydrophilus* sp[a]	L,A
	Noteridae	Not identified	Not identified	A
Diptera	Chironomidae	Chironominae	*Chironomus* sp[c]	L
	Culicidae	Anophelinae	*Anopheles* spp	L,P
		Culicinae	*Culex* spp	L,P
	Stratiomyiidae**	Not identified	Not identified	L
Ephemeroptera	Baetidae	Baetinae	*Baetis geminatus*[c]	N
			Cloeon bimaculatus[c]	N
			Cloeon bengalensis[c]	N
Hemiptera	Corixidae	Corixinae	*Corixa graveleyi*[c]	N,A
		Micronectinae	*Micronecta thyseta*[c]	A
	Gerridae	Gerrinae	*Limnogonus* sp[b]	N,A
	Hydrometridae	Hydrometrinae	*Hydrometra greeni*[b]	A
	Notonectidae	Anisopinae	*Anisops barbata*[a]	N,A
			Anisops bouvieri[a]	N,A
	Veliidae	Rhagoveliinae	*Rhagovelia nigricans*[b]	A
Anisoptera	Libellulidae	Sympetrinae	*Brachythemis contaminata*[a]	N
		Trameinae	*Pantala flavescens*[a]	N
Zygoptera	Coenagrionidae	Pseudogrioninae	*Ceriagrion aurantiacum*[a]	N

*A: Adult; L: Larva; N: Nymph; P: Pupa; **Collected on only one occasion; [a]Bottom predators; [b]Surface; [c]Non-predators.

populations of these insects appeared from Day 19 onwards and peaked on Day 28 after transplantation.

Diversity of aquatic insects: Aquatic insects other than mosquitoes were grouped into three different categories based on trophic niches and their feeding behaviour, particularly in relation to mosquito immatures, following Mogi *et al.*[19] These categories were surface predators (Gerridae, Hydrometridae and Veliidae), bottom predators (Notonectidae, Coenagrionidae, Libellulidae, Hydrophilidae and Dytiscidae) and non-predators of mosquito immatures (corixids, chironomids and ephemeropteran nymphs). The Shannon-Weaver Diversity Index was applied to study the diversity of these different groups of aquatic insects. A clear

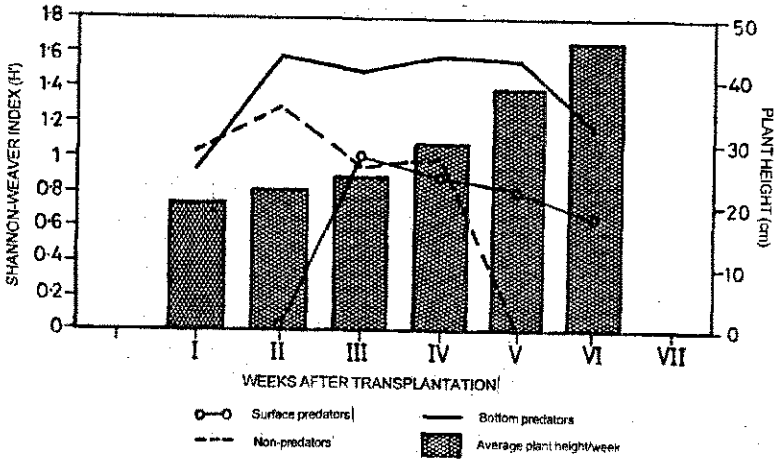

Fig. 4: Shannon-Weaver Diversity Index for aquatic insects in rice fields

pattern was noticed in the diversity of these three groups of aquatic insects in relation to paddy growth in rice fields during the study period. The surface predators appeared in the third week after transplantation when the diversity of the species was highest and the plant height was 24.7 cm (Fig. 4). Thereafter, as the plant height increased the diversity index decreased. The diversity of the bottom predators was high between the second and the fifth weeks, when the plants' heights ranged from 22.5 to 38.6 cm, and later declined. The diversity of non-predators was high during the first two weeks (average plant height 22.5 cm), and then decreased as the plant canopy became thick.

Predator-prey relationship: Pearson's correlation analysis on log (n+1) transformed data was used to relate the abundance of mosquito immatures with the abundance of individual groups of predators. The late (III/IV instar) culicine larvae showed statistically significant positive correlations with hydrophilids ($r = 0.88$, $p < 0.001$) and dytiscids ($r = 0.87$, $p < 0.001$). Similarly, culicine pupae also showed significant positive correlations with hydrophilids ($r = 0.64$, $p < 0.01$) and dytiscids ($r = 0.72$, $p < 0.01$). But culicine pupae showed significant negative correlations with coenagrionids ($r = -0.65$, $p < 0.01$), hydrometrids ($r = -0.67$, $p < 0.01$) and veliids ($r = -0.74$, $p < 0.001$). The late (III/IV) larval instars of anophelines showed significant positive correlations with hydrophilids ($r = 0.94$, $p < 0.001$) and dytiscids ($r = 0.88$, $p < 0.001$ and anopheline pupae with dytiscids ($r = 0.62$, $p < 0.01$) only. The population of anopheline pupae had significant negative correlation with coenagrionids ($r = -0.67$, $p < 0.01$), veliids ($r = -0.80$, $p < 0.001$) and hydrometrids ($r = -0.76$, $p < 0.001$).

Since predator-prey relations in the rice field ecosystem involve a whole complex of predators, it was felt more appropriate to use multiple

regression analysis. Log (n+1) transformed data were analysed using the multiple linear regression model with the backward elimination method. The bottom predators and surface predators were analysed separately since they had different trophic niches. The bottom predators included in the multiple regression equation were notonectid nymphs and adults, libellulids, coenagrionids, dytiscids and hydrophilids. The model eliminated notonectid nymphs from the equation for larvae and pupae of both anophelines and culicines. Other insect groups eliminated from the equation were notonectid adults for culicine pupae and anopheline (III/IV) instar larvae; hydrophilids for anopheline and culicine pupae; coenagrionids for late instar larvae of culicines and anophelines and culicine pupae; and libellulids for late (III/IV) instar culicine and anopheline larvae and anopheline pupae. Notonectid adults showed negative coefficients which are statistically significant in the equation for culicine late (III/IV) instar larvae (adjusted $R^2 = 0.89$, $p < 0.001$) and anopheline pupae (adjusted $R^2 = 0.79$, $p < 0.001$) (Table 2). Similarly, coenagrionids interacted negatively with anopheline pupae and libellulids interacted negatively with culicine pupae (adjusted $R^2 = 0.84$, $p < 0.001$). For culicine and anopheline larvae, the regression equation accounted for 89 and 91% respectively of the total variance ($p < 0.001$), and for culicine and anopheline pupae, the regression equation accounted for 84 and 78% of total variance respectively. Results of the multiple regression analysis show that notonectid adults act as important predators of culicine larvae and anopheline pupae, coenagrionids for anopheline pupae, and libellulids for culicine pupae.

Table 2. Multiple linear regression model relating mosquito immatures and bottom predators (n = 6)

Stages of mosquitoes	Intercept	β-coefficients						Adjusted R^2	Significance of 'F'
		Notonectids (Nymphs)	Notonectids (Adults)	Hydrophilids	Dytiscids	Coenagrionids	Libellulids		
Culex (III/IV instar) larvae	−0.23	NS	−0.34	0.54	0.61	NS	NS	0.89	$p<0.001$
Culex pupae	−0.05	NS	NS	NS	0.95	NS	−0.63	0.84	$p<0.001$
Anopheles (III/IV instar) larvae	−0.16	NS	NS	0.67	0.32	NS	NS	0.91	$p<0.001$
Anopheles pupae	0.14	NS	−0.38	NS	0.75	−0.57	NS	0.78	$p<0.001$

n = No. of observations included in the regression equation; NS = Not significant; R = Multiple regression coefficient.

Table 3. Multiple linear regression model relating mosquito immatures and surface predators (n = 16)

Stages of mosquitoes	Intercept	β-coefficients			Adjusted R^2	Significance of 'F'
		Veliids	Hydrometrids	Gerrids		
Culex (III/IV instar) larvae	1.58	−0.68	NS	0.62	0.36	$p<0.05$
Culex pupae	1.10	−0.74	NS	NS	0.51	$p<0.05$
Anopheles (III/IV instar) larvae	1.79	0.65	NS	0.63	0.35	$p<0.05$
Anopheles pupae	0.98	−0.80	NS	NS	0.61	$p<0.05$

n = No. of observations included in the regression equation; NS = Not significant; R = Multiple regression coefficient.

Hydrophilids showed significant positive coefficients for late (III/IV) instars of culicines and anophelines, but for pupae they were not significant. Similarly, dytiscids showed significant positive coefficients for late (III/IV) instar larvae and pupae of anophelines and culicines.

Data for surface predators were analysed separately. Groups included in the equation were veliids, hydrometrids and gerrids. The regression model eliminated hydrometrids from the equation for larvae and pupae of both anophelines and culicines. Veliids showed statistically significant negative coefficients for larvae of culicines and anophelines (Table 3). For anopheline and culicine pupae, the equation accounted for 61 and 51% respectively of the total variance ($p < 0.001$ and $p < 0.05$ respectively). For larvae of culicines and anophelines, the regression model was not as good a fit, as shown by R^2 values (0.36 and 0.35 respectively). Among the surface predators, veliids acted as important predators of larvae and pupae of both anophelines and culicines. However, it is clearly known that surface predators are less important than bottom predators, as shown by smaller R^2 values for the former.

DISCUSSION

Agricultural practices in rice fields can influence mosquito production and the potential for mosquito-borne disease. The use of fertilisers, pesticides, the type of tillage, irrigation and cropping systems determine the dynamics and abundance of the biotic community in rice fields.[20] In the present study, the density of culicine immatures was highest during the first few weeks after transplantation, as has been reported in Pondicherry[4] and in Madurai district.[15,16] There was a clear pattern in the succession of mosquito species in relation to paddy growth. *Culex*

vishnui was found to breed only up to 14 days after transplantation and then disappeared, whereas *Cx. tritaeniorhynchus* continued to breed up to 23 days by replacing *Cx. vishnui*. Similar results were obtained in rice fields in North Arcot district.[3] On the other hand in newly transplanted fields, the population of *Cx. tritaeniorhynchus* peaked in Malaysia[5] and in fallow fields in the Philippines.[7] The anopheline species *An. subpictus* and *An. vagus* were most abundant during the first few weeks after transplantation. Sen,[2] Chandrahas and Rajagopalan,[4] and Mogi and Miyagi[7] also reported that these species were most abundant during the early stages of paddy growth.

Other groups of aquatic insects also showed distinctive patterns of abundance in relation to growth of rice plants. The populations of dytiscids and hydrophilids, known predatory beetles, were high during the third week after transplantation and declined thereafter. However, the peaks in their abundance coincided with those of mosquito larval populations. Mogi and Miyagi[7] found that dytiscids were abundant in fallow fields, but without any clear trends. *Berosus indicus*, a vegetarian beetle, was collected in large numbers in the present study. In Japan, Mogi[21] also found its predominance in rice fields in summer.

Chironomids were most abundant during the first week after transplantation. Miura *et al.*[9] also found that the population of chironomids was highest during the early stage of paddy growth and then decreased as paddy height increased. The decrease in the population density may be because of the appearance of potential predators two weeks after transplantation. Ephemeropteran nymphs showed an increase–peak–decrease trend in their abundance. Miura *et al.*[9] found that populations of mayfly nymphs were very low in rice fields with dense vegetation in California. However, Kramer *et al.*[11] and Mogi and Miyagi[7] found no clear pattern in the abundance of ephemeropteran nymphs in rice fields. Corixids (non-predatory bugs) were abundant during the first week only. These results were in conformity with the results of the studies conducted in Japan[7] and California.[9, 11]

The population of notonectids decreased as the plant height increased. This pattern was consistent with those obtained in rice fields in California[8–11] and the Philippines.[7] This could be due to the elimination of 'open water' habitat as suggested by Miura *et al.* [9] Gerrids were collected in small numbers only, since they usually prefer habitats with constant flowing water.[22] The populations of veliids and hydrometrids increased as the paddy height increased, showing a peak during the fifth week after transplantation. The shade provided by the dense plant canopy may be the favourable microclimate for these surface inhabiting insects. However, it has been found that the abundance of veliids is independent of plant growth in rice fields in the Philippines.[7] Hemipterans such as nepids and belostomatids, known predators of mosquito immatures, were not collected in rice fields during the present study. However, they were collected in rice fields in Thailand[19] and in the Philippines.[23] Dragonfly nymphs appeared from the third week after transplantation. However, in Japan, Ban and Kiritani[12] found that the population of dragonfly nymphs disappeared when

the rice plants became thick and the aerial oviposition was disturbed by the plant canopy. Damselfly nymphs also appeared from the third week after transplantation. However, Kramer *et al.*[11] found that the abundance of damselfly nymphs was high during the early stage of paddy growth and then declined, while Miura *et al.*[9] found that the population of damselfly nymphs increased as the paddy height increased.

Surface predators appeared in the third week after transplantation and the diversity of species was highest during the third week. The diversity of bottom predators was high between the second and fifth weeks, and that of non-predators was high during the first two weeks after transplantation. Mogi and Miyagi[7] have observed that the total abundance of bottom predators and surface predators decreased when the fields were ploughed and slowly recovered as the plants matured, but non-predators showed no clear trend. Palchick,[24] applying the Shannon-Weaver Diversity Index, found no clear difference in the diversity of predators collected at different water depths in rice fields in California. These patterns in the diversity of insect groups in rice fields show that each group of insects prefers a particular phase of plant growth for its colonisation, which provides a favourable environment for its survival and development.

Notonectids, dragonfly and damselfly nymphs, hydrophilids and dytiscids have been reported potential predators in rice fields.[9, 23, 25–27] As suggested by Mogi *et al.*,[26] the predation of mosquito immatures in a rice field ecosystem is not by a single species, but a complex of predators are involved. In the present study, multiple linear regression analysis shows that bottom predators such as notonectid adults, nymphs of coenagrionids and libellulids and veliids, which are surface predators, are the effective predators of mosquito immatures in rice fields. The appearance of these insect groups in the third week after transplantation coincides with the decrease of mosquito immatures. However, hydrophilids and dytiscids, known predators, show positive relationships with mosquito immature populations suggesting that some common environmental factors influence the high abundance of both these predators and mosquito immatures during the same period.

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Patterns of Parasitaemia, Antibodies, Complement and Circulating Immune Complexes in Drug-suppressed Simian *Plasmodium knowlesi* Malaria

SUKLA BISWAS[a]

Rhesus monkeys were inoculated intravenously with 1×10^4 *P. knowlesi* infected erythrocytes. After about three days prepatency, peripheral smears were found positive and the animals were cured with chloroquine phosphate when parasitaemia reached about 15–25 per cent. The monkeys were repeatedly exposed with three bouts of infection. The first and second bouts were cured but after the third challenge, all 10 monkeys showed a longer prepatent period, lower parasitaemia and then self-recovery. Sera were collected in different phases of infection to assess immune responses. Antimalarial IgG and IgM responses were measured by ELISA. The presence of IgM antibody was associated with every bout of infection. With repeated infections until self-recovery, a substantial amount of IgG was found in circulation. A significant level of schizont-infected cell agglutination antibody was also detected in the animals after survival from challenge infection. Antigen-specific circulating immune complexes, both of IgG and IgM types, appeared in various phases of infection, but their appearance did not coincide with the acquired immune responses of the animals. During the self-recovery phase, almost all monkeys had an elevated level of serum C3 and C4.

Keywords: Antibodies, Circulating immune complexes, Complement, Immunity, *P. knowlesi*, Rhesus monkey

INTRODUCTION

Plasmodium knowlesi of macaques is remarkable for its unique quotidian periodicity in the blood and almost 100% lethality in its unnatural host, *Macaca mulatta*.. If the disease is arrested by the administration of quinine, the parasitaemia declines followed by severe haemolytic

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anaemia. This condition seems to be similar to the fatal haemolytic anaemia in children in *P. falciparum* infections.[1] The nature of responses of *P. knowlesi* infection in its natural host, *Macaca fascicularis*, is different from the unnatural, highly susceptible host, *M. mulatta*. The natural host synthesises the protective antibody of broad serological specificity within two weeks of infection and develops mild chronic parasitaemia, while the unnatural host shows no protective antibody and suffers rapidly from fatal malaria.[2]

In malarial infection, the host is exposed to a series of antigens of great complexity. The combined effect of antigens helps the hosts to develop immune responses which are partly nonspecific and partly specific. During malarial infections, there is a marked increase in serum immunoglobulin levels, part of which is specific to malaria. However, there is a poor correlation between immune status and the levels of antimalarial antibodies.[3]

The immunity acquired by blood stage parasites is species and stage-specific, and mediated by a specific effector mechanism. This effector mechanism may include several factors, such as: specific antibody and its action in collaboration with complement or macrophages, direct T-cell cytotoxicity and the production of cytotoxic mediators by the interaction of T-cells with malaria antigen.[4] A good helper T-cell response may be necessary to ensure production of antiplasmodial antibody in sufficient quantities to check the parasites.[5] The protective antimalarial antibody is of both IgG and IgM types, and the former is predominant in established infections. Cell mediated, as well as antibody-dependent mechanisms, cooperate in the immune response against malaria.[6] The merozoite invasion inhibitory antibody shows correlation with clinical immunity in human and monkey malaria. A prolonged prepatent period and a modified course of parasitaemia developed in rhesus monkeys after repeated infection with *P. knowlesi* and cure. The sera of this group of monkeys demonstrated schizont infected cell agglutination (SICA) antibody and merozoite invasion inhibitory antibody. However, there was no correlation between functional immunity and *in vitro* tests.[5,7]

The appearance of antimalarial antibodies and malarial antigens in the serum are closely associated with a depression of serum C3 and C4 levels and the presence of circulating immune complexes (CICs). The pathogenic role of immune complexes in malarial nephritis and development of other tissue lesions are well established. Glomerular deposits of IgM preceded the appearance of CICs, whereas deposits of IgG and C3 were concomitant with the appearance of CICs.[8,9] Immune complexes (ICs) have some role in immune response in addition to pathogenesis. The cells, like polymorphs, lymphocytes and monocytes, can be activated in presence of circulating immune complexes.[10] The ICs were shown to consist of IgG, IgM, complement and malarial antigens, and their presence in the serum of acutely infected animals may protect the plasmodia from the activities of macrophages.[11,12]

The study on acquired immune responses to malaria in simian *P. knowlesi* is very important

in the light of human malaria. In human malaria clinical immunity develops after repeated exposure in individuals living in endemic areas. It has been shown that by immunisation or by drug-limited infection, rhesus monkeys may develop immunity on challenge with *P. knowlesi* instead of lethal infection.[5]

In the present study, rhesus monkeys were repeatedly infected with *P. knowlesi* and then cured until self-recovery. Blood was collected from these animals in various phases of infection. Sera were tested for determining schizont agglutination antibody, antimalarial IgG and IgM isotype levels, appearance of CICs and serum C3 and C4 levels.

MATERIALS AND METHODS

Animal: Male rhesus monkeys (*Macaca mulatta*) weighing 3–4 kg were kept under quarantine and were screened for tuberculosis. A normal diet of fresh fruits, vegetables, nuts and soaked grams was given, water was provided *ad libitum*.

Strain of parasite: *P. knowlesi* strain W1 variant, chloroquine sensitive, was obtained from Guy's Hospital Medical School, London, UK. This strain is uniformly lethal to rhesus monkeys.

Infection and collection of sera: Fifteen rhesus monkeys (5 control and 10 experimental) were infected intravenously with 1×10^4 parasitised RBC. The course of parasitaemia was monitored daily by examining peripheral blood smears. All animals were cured at 15–25% parasitaemia with chloroquine phosphate, with a first dose of 29 mg/kg and a second dose of 15 mg/kg body weight. Ten monkeys of the experimental group were infected thrice on Days 1, 30 and 60 with the same strain and cured until self-recovery. Blood was collected at different phases of infection.

Detection of anti-*P. knowlesi* antibodies IgG and IgM types: Anti-*P. knowlesi* antibodies in sera were estimated by indirect ELISA using *P. knowlesi* antigen as described.[13, 14] Anti- monkey IgG and IgM antisera were raised in rabbits and purified immunoglobulin fractions were conjugated with horse radish peroxidase by the standardised method.[15] These conjugates were used as a second antibody for the assay.

Detection of SICA antibody: *P. knowlesi* schizont stages were isolated from whole blood and a final suspension of 4×10^7 cells per ml was prepared in physiological saline. In 96 well 'U' bottom plate, two fold dilutions of individual serum (25 µl) was prepared in saline containing 1% normal monkey serum. An equal volume of schizont suspension was added in each well. The cells were allowed to settle for 3 h at room temperature and agglutination reactions were then assessed microscopically as described elsewhere.[16]

Sandwich ELISA for detection of CICs—IgG and IgM types: Polystyrene microtitre plates were coated with rabbit anti-*P. knowlesi* antibody (50 µg/ml). Test sera at a dilution of $^1/_4$ in borate buffered saline were added in duplicates and incubated overnight at 4 °C. Spe-

cies-specific anti-IgG and IgM conjugates were used to determine the class of immunoglobulins complexed with antigen.

Determination of serum C3 and C4 levels: Serum C3 and C4 concentrations were estimated by single radial immunodiffusion.[17] Commercially prepared anti-human C3 and C4 (raised in goat) were used for the test. Anti-C3 and C4 incorporated 0.8% agarose was poured on micro-slides. After solidification in a moist chamber at 4 °C, 2-mm-size wells were punched and 5 µl of serum samples were added in each well. The slides were incubated for 42 h at room temperature. The slides were then washed in normal saline, dried and stained in Coomassie blue. The diameter of the precipitin ring was measured, and protein concentration was determined by comparison with the standard curve.

Statistical analysis: The significance of the difference in parasitaemia between the control and experimental groups was determined by an unpaired t-test and the correlation coefficient (r-value) for the immunological profile was determined by the Spearman rank test.

RESULTS

Control monkeys (n = 5) were monitored for parasite load and all immunological parameters. They were cured with chloroquine phosphate. Experimental monkeys (n = 10) were subjected to three bouts of infection on Days 1, 30 and 60. These animals were cured with chloroquine phosphate after the first and second infections as parasitaemia crossed 15%. After the third challenge, all of them showed self-recovery. The parasitological profiles of the third self-recovery bout of 10 monkeys and 5 control monkeys are shown in Table 1, which depicts the prepatent period, cumulative, maximum and duration of parasitaemia. The average prepatent period for the control group remained 3.2 days after the first bout, and in the experimental group it remained 6.2 days after the third bout of infection. The average maximum parasitaemia per µl blood was 2,960 in the control group, whereas it was 656 in the experimental group. Differences in prepatency were found to be significant ($p < 0.001$).

The humoral immunity of these animals for specific antimalarial antibodies was assessed by ELISA on different days after challenge and rechallenge. A substantial amount of IgG response at the immune state was observed, and also a marked IgM response was noticed after fresh infection (Table 2).

The sera of all 10 monkeys showed significant SICA-antibody titre at immune state (Table 3). An increase in SICA-antibody titre has been observed from Day 30 onwards. After repeated infection with homologous *P. knowlesi* strain, the antibody level remained high. A positive correlation exists between antimalarial IgG and agglutinin ($r = 0.86$; $p < 0.01$).

Antigen-specific CICs in sera were detected by sandwich ELISA using rabbit anti-*P. knowlesi* antiserum as the first antibody and antimonkey IgG or IgM as the second antibody. The appearance of CICs of IgG and IgM types on different days is shown in Table 2. After the first

Table1. Parasitological profile of control and experimental monkeys

Monkeys (nos.)	Prepatent period (days)	Parasitaemia duration (days)	Cumulative parasitaemia	Parasitaemia index	Maximum parasitaemia
C1	3	6	3120	520	2500
C2	3	7	3755	536	2300
C3	3	7	5890	841	4000
C4	3	4	4560	1140	3000
C5	4	4	4100	1025	3000
E1	6	20	1400	70	900
E2	6	18	910	50.5	700
E3	6	16	1540	96	800
E4	7	19	1450	76.3	830
E5	6	16	118	7.4	30
E6	6	20	180	9.0	100
E7	6	20	1590	79.5	700
E8	7	24	1300	54.2	800
E9	6	20	1090	54.2	900
E10	6	19	1800	94.7	800

C1 — C5: Control animals; E1— E10 : Experimental animals; Parasitaemia index = Cumulative parasitaemia/ Parasitaemia duration.

bout of infection, both IgM class antibody and CICs were detectable on Day 5, whereas the IgG level was built up after repeated infection. The IgG class CICs were also found to be a little elevated along with the availability of free antibody ($r = 0.68$, $p < 0.01$). The appearance of IgG class CICs was more initially, but later there was an overlapping: no marked changes were observed at the recovery phase.

The concentration of serum C3 and C4 levels increased after every fresh infection. After the third challenge, the level of C3 was maintained at higher than normal. In the case of C4, a fall was recorded initially, but later the C4 level also increased (Table 2).

DISCUSSION

Many attempts have been made to determine a positive correlation between development of the clinical immunity and the presence of a specific antimalarial antibody. From the data of several studies, it is quite clear that the immunoglobulin

Table 2. Profile of antibodies, CICs and serum C3 and C4 levels in various phases of infection

Status	Days	IgG	IgM	CICs IgG	CICs IgM	C3 (μg/ml)	C4 (μg/ml)
Infection	1	0.09±0.03	0.07±0.02	0.03±0.01	0.04±0.01	1496±12	356±24
Treatment	5	0.07±0.02	0.14±0.03	0.07±0.02	0.36±0.08	1366±56	263±26
	10	0.10±0.04	0.20±0.04	0.18±0.04	0.44±0.06	1283±29	344±84
	15	0.21±0.09	0.34±0.17	0.15±0.09	0.39±0.03	1373±62	398±28
	20	0.23±0.09	0.15±0.09	0.17±0.07	0.34±0.06	1399±78	365±56
Infection	30	0.27±0.07	0.10±0.06	0.30±0.12	0.19±0.09	1347±56	273±33
Treatment	40	0.88±0.37	0.22±0.05	0.27±0.10	0.21±0.06	1152±101	313±41
	45	1.09±0.43	0.20±0.06	0.22±0.12	0.13±0.03	1175±13	401±73
Infection	60	0.68±0.34	0.06±0.02	0.38±0.05	0.33±0.10	1302±278	315±22
	75	1.36±0.57	0.41±0.25	0.30±0.10	0.27±0.04	1368±89	453±174
	90	1.37±0.56	0.20±0.09	0.33±0.10	0.25±0.08	1802±162	415±132

Table 3. SICA antibody in various phases of infection

Monkeys (no.)	SICA antibody titre ($-\log_2$)					
	15d	30d (second bout)	45d	60d (third bout)	75d	90d (Self-recovery)
1	3	6	6	7	8	10
2	2	4	5	7	7	9
3	2	3	5	8	8	10
4	2	3	4	5	6	8
5	2	4	4	6	4	7
6	0	2	2	5	5	8
7	0	2	4	6	6	9
8	0	0	5	7	8	10
9	0	0	4	5	7	9
10	0	2	4	5	7	9

fraction, particularly IgG of the sera of the immune or semi-immune human or primate has protective action against homologous malarial parasites. Antimalarial protective antibody has been found in both IgG and IgM classes, with IgG antibody being predominant in established infections.[2] The appearance of malaria-specific IgG and IgM antibodies has been demonstrated by ELISA. With every bout of infection, the presence of IgM antibody has been observed in the present study. Though it has been mentioned that B lymphocytes producing IgM become IgG producers under continuing antigenic stimulation;[18] in case of infection, the live parasites may recall the IgM producing B-cell memory which in turn evokes a synthesis of IgM to the renewal infection, or it can be the result of antigenic variations in *P. knowlesi* parasites which confuses the immune system and leads to production of a new set of IgM class antibody. After first exposure to parasites, there was not much IgG antibody in circulation, though its appearance has been observed in later phases. With repeated infection until immunity a substantial amount of IgG was found in circulation, which may indicate that the presence of malaria-specific IgG may help the animals to build up protective immunity.[19]

Many stage-specific proteins are expressed in the life cycle of plasmodia, some of which are said to be capable of stimulating protective immunity, a proportion of these proteins also exhibit antigenic diversity within the species, which may be the phenotypic variation as observed in *P. knowlesi* as SICA antigen. Acute infection of *P. knowlesi* in rhesus monkeys when controlled by drug treatment produces a chronic infection characterised by the sequential appearance of antigenically distinct populations. The sera of these chronic monkeys contain agglutinin at high levels, and these sera can agglutinate all variants of a given strain but not those of other strains of *P. knowlesi*.[16] In the present study significant SICA-antibody titre was detected in the animals, who overcome the challenge infection and showed correlation with protective immunity.

By a heterologous sandwich ELISA malarial antigen-specific CICs have been detected following drug-suppressed *P. knowlesi* infection. In murine *P. berghei* infection, the presence of tissue immune complexes in kidney and brain correlated with the appearance of CICs at different time intervals after infection. The depositions of IgM, IgG and C3 were found in tissue ICs by immunofluoroscence assay. The presence of IgM deposits was observed from Day 3, whereas those of IgG and C3 were found from the second week.[8] The appearance of CICs of IgG and IgM types in circulation did not show any correlation with acquired immunity in the present study. The peak of CICs usually coincides with decreased levels of C3 and C4 components of complement as observed in murine *P. berghei* and human *P. vivax* and *P. falciparum* infections.[8, 20] In the present study, decreased levels of C3 and C4 have been observed after every fresh infection which coincided with the appearance of immune complexes in circulation. Elevated levels of C3 and C4 were seen in the animals which survived the challenge infection, and the raised levels were demonstrated in the sera even after a month.

Based on the findings of the present study, it may be assumed that a similar phenomenon may

arise in malaria endemic areas where individuals experience repeated infections. If there is not much variability in the parasite strains of both *P. vivax* and *P. falciparum*, acquired immunity may build up as a cumulative effect of repeated infections and cure.

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A Controlled Study on Haemograms of Malaria Patients in Calcutta

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A study was carried out at the Urban Health Centre, Chetla, Calcutta to evaluate the efficacy of quantitative buffy coat (QBC) analysis of haemograms in malaria patients suffering from fever with bodyache and chill and/or rigour attending the Fever Treatment Depot during a three months period (March–June 1996) who had undergone both malaria parasite study and haematological investigation by the QBC method, from blood samples collected by finger prick. To avoid bias, malaria parasite studies and haemograms were done separately, and investigators were kept 'blind' about the results of other investigations. The haematological findings obtained of 180 slide-positive malaria cases were compared with a sample of 177 age- and sex-matched slide-negative controls selected by random sampling. The results revealed that haemoglobin levels (g %), haematocrit values (%), WBC and platelet counts of malaria cases were significantly lower than in the matched controls. Thus, QBC estimation correlates well with existing knowledge about malarial haematology. This relatively easier, quicker and reliable method of taking haemograms may be recommended for field testing for assessing haematological parameters of malaria cases under field conditions, before its introduction for large-scale use.

Keywords: Malaria, Quantitative buffy coat analysis

INTRODUCTION

The quantitative buffy coat (QBC) method of haemogram is known to give a strong correlation with the conventional methods. Moreover, this method is comparatively time-saving and is advocated by many in field conditions[1, 2].

Thus, the present study was conducted in a highly endemic area of malaria to elucidate the

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haemogram findings in malaria by the QBC analysis method in a case-control design to examine the reliability of this relatively quicker method of investigation, to characterise the haematological parameters usually associated with the disease.

MATERIALS AND METHODS

This cross sectional, clinic-based study was undertaken in the Urban Health Centre, Chetla, the field practice area of the All India Institute of Hygiene and Public Health, Calcutta, for a period of three months during the months of March to June 1996. The reference population of this study belonged to a "high-risk" urban area for malaria where annual parasite incidence (API) ranged from 45.1 to 61.3 per thousand population in the last five years, with high annual blood examination rate (ABER) ranging from 17.8 to 22.6%, and high slide positivity rate (SPR) ranging from 24.1 to 27.2% (as per criteria of the Accelerated Urban Malaria Scheme).[3]

Adult patients aged 15 yr and above with history of fever with chill and/or rigour were examined clinically, and blood from finger prick was taken both for malaria parasite study[4] as well as in a precoated capillary tube for haemogram by QBC analysis. The haemogram was done by receiving blood from the finger prick in a precision-bored glass capillary tube. The capillary tubes were centrifuged within 30 min, and the results were seen in a QBC computerised analyser.[2] The capillary tubes were centrifuged in a standard micro-haematocrit centrifuge at 10,500 rpm for 5 min. The RBC settled at the bottom, followed by granulocytes, agranulocytes, platelets and plasma, in that order. The bands were observed and quantified in a computerised spectrophotometric unit as devised by Clay Adams of Becton Dickenson of New Jersey. The blood smears positive for malaria parasite were defined as "case". The age- and sex-matched "controls" were selected by sampling from the fever cases, whose blood smears were negative for malaria. In order to validate the suitability of the QBC method for assessing haemograms during malaria infection, instead of "normal" controls from non-endemic areas, malaria "cases" were compared with controls selected from this highly endemic area only, who might have had past experience of malaria with its effect on the haematological system. Out of 180 malaria parasite positive blood smears (cases), only 9 (5.0%) were *P. falciparum* and the remaining 171 (95.0%) were *P. vivax*. The 177 slide-negative for malaria fever cases (controls) were subsequently diagnosed as: respiratory tract infections 62 (35.0%); viral fever or pyrexia of unknown origin 95 (57.73%); urinary tract infection 11 (6.2%); kala-azar cases 6 (3.4%); and cases of gastroenteritis 3 (1.7%). Since malaria is highly endemic in the study area, slide-negative persons with a past history of slide-positivity for malaria in the recent past, i.e. the last six months of the recall period were excluded from the control group (three cases). Moreover, persons with history of bleeding disorders or haematemesis and melaena were excluded from the sample population. In order to avoid bias, malaria parasite studies and QBC analyses

were carried out separately by different investigators and they were kept 'blind' about the results of other investigations, as well as the clinical status of the study samples.

RESULTS

Table 1 shows that the haematocrit value was below 40% among 146 (81.1%) cases and 124 (70.1%) of the controls. This difference was significant statistically ($\chi^2 = 5.95$, $p < 0.001$). Moreover, the mean haematocrit value of 'cases' was lower (34.9 ± 7.6) compared to that of the control group (36.8 ± 6.7). This difference was also significant statistically ($Z = 2.48$, $p < 0.01$).

Table 2 shows that the haemoglobin was less than 14 g% in 170 (94.4%) cases and 141 (79.7%) of controls, with mean haemoglobin

Table 1. Distribution of patients according to haematocrit values (%)

Haematocrit value	Malaria parasite* in blood smear		Total (n = 357)	Test of significance
	Positive (n = 180)	Negative (n = 177)		
< 40%	146 (81.1)	124 (70.1)	270 (75.6)	$\chi^2 = 5.95$ $p < 0.001$
40% and above	34 (18.9)	53 (29.9)	87 (24.4)	
Mean haematocrit	34.9 ± 7.6	36.8 ± 6.8		$Z = 2.48$ $p < 0.01$

*Out of 180 malaria parasite positive blood smears, 9 (5.0%) were *P. falciparum* and the remaining 171 (95.0%) were *P. vivax*; Values within parentheses indicate percentages.

Table 2. Distribution of patients according to haemoglobin percentages

Haemoglobin (g%)	Malaria parasite* in blood smear		Total (n = 357)	Test of significance
	Positive (n = 180)	Negative (n = 177)		
< 14%	170 (94.4)	141 (79.7)	311 (87.1)	$Z = 4.25$ $p < 0.001$
14 to 18	10 (5.6)	36 (20.3)	46 (12.9)	
Mean haemoglobin	11.5 ± 2.1	12.2 ± 2.08		$Z = 3.16$ $p < 0.001$

*Out of 180 malaria parasite positive blood smears, 9 (5.0%) were *P. falciparum* and the remaining 171 (95.0%) were *P. vivax*; Values within parentheses indicate percentages; The cut-off point for anaemia by the QBC method is 14 g%.

Table 3. Distribution of patients according to WBC count

Lympho/Mono WBC count (billion/l)	Malaria parasite* in blood smear		Total (n = 357)	Test of significance
	Positive (n = 180)	Negative (n = 177)		
<4.3%	12 (6.7)	5 (2.8)	17 (20.7)	Z = 1.72 p > 0.05
4.3 to 10	146 (81.1)	120 (67.8)	266 (74.5)	
> 10	22 (12.2)	52 (24.4)	74 (20.7)	
Mean WBC count	7.5 ± 2.5	8.8 ± 3.5		Z = 4.22 p < 0.001

*Out of 180 malaria parasite positive blood smears, 9 (5.0%) were *P. falciparum* and the remaining 171 (95.0%) were *P. vivax*; Values within parentheses indicate percentages: The cut-off point for leucopenia by the QBC method is 4.3 billion/l.

Table 4. Distribution of fever patients according to granulocyte count

Granulocyte count (billion/l)	Malaria parasite* in blood smear		Total (n = 357)	Test of significance
	Positive (n = 180)	Negative (n = 177)		
< 2	26 (14.4)	12 (6.8)	38 (10.6)	Z = 2.37 p < 0.01
2 to 8.8	153 (85.0)	153 (86.4)	306 (85.7)	
> 8.8	1 (0.6)	12 (6.8)	13 (3.6)	
Mean granulocyte	3.7 ± 1.7	4.3 ± 2.8		Z = 2.37 p < 0.01

*Out of 180 malaria parasite positive blood smears, 9 (5.0%) were *P. falciparum* and the remaining 171 (95.0%) were *P. vivax*; Values within parentheses indicate percentages; The cut-off point for granulocytopenia is 2 billion/l.

being 11.5 ± 2.1 in cases and 12.2 ± 2.08 in the controls. Statistical testing revealed that the proportion of anaemic cases among malaria patients was significantly higher ($Z = 4.25$, $p < 0.001$) than the controls. Moreover, the mean Hb% levels of cases was also significantly lower than that of controls ($Z = 3.16$, $p < 0.001$).

Table 3 shows that the mean WBC count (in billion/1) among malaria cases was 7.5 ± 2.5 and among controls 8.8 ± 3.5, and this difference was significant statistically ($Z = 4.22$, $p < 0.001$). But leucopenia, i.e. WBC count below 4.3 billion, was observed in 12 (6.7%) malaria cases and 5 (2.8%) among the controls.

This difference was, however, not significant statistically [Z (test of proportions) = 1.72, $p > 0.05$].

Table 4 shows that the mean granulocyte count was 3.7 ± 1.7 among malaria cases, and 4.3 ± 2.8 among controls, and this difference was significant statistically ($Z = 2.37$, $p < 0.01$). Moreover, granulocytopenia (i.e. count below 2 billion/l) was more among malaria cases (14.4%) than controls (6.8%). This difference was also statistically significant ($Z = 2.37$, $p < 0.01$).

Table 5 shows that the lymphocyte/monocyte count was lower than normal only in two malaria cases and in one control, and the mean

Table 5. Distribution of fever patients according to lymphocyte/monocyte count

Lympho/Mono count (billion/l)	Malaria parasite* in blood smear		Total (n = 357)	Test of significance
	Positive (n = 180)	Negative (n = 177)		
< 1.2	2 (1.1)	1 (0.56)	3 (0.84)	Z = 1.90 p > 0.05
1.2 to 5.3	163 (90.5)	150 (84.75)	313 (87.68)	
> 5.3	15 (8.33)	26 (14.69)	41 (11.48)	
Mean WBC count	3.76 ± 1.70	3.84 ± 1.65		Z = 0.45 p > 0.05

*Out of 180 malaria parasite positive blood smears, 9 (5.0%) were *P. falciparum* and rest 171 (95.0%) were *P. vivax*; values within parentheses indicate percentages.

Table 6. Distribution of fever patients according to platelet count

Platelet count (billion/l)	Malaria parasite* in blood smear		Total (n = 357)	Test of significance
	Positive (n = 180)	Negative (n = 177)		
< 140	115 (63.89)	72 (40.56)	187 (52.38)	$\chi^2 = 19.2$ p < 0.001
140 and above	65 (36.11)	105 (59.32)	170 (47.62)	Z = 5.22 p < 0.001
Mean platelet count	132.79 ± 55.97	165.56 ± 62.05		Z = 4.51 p < 0.001

*Out of 180 malaria parasite positive blood smears, 9 (5.0%) were *P. falciparum* and rest 171 (95.0%) were *P. vivax*; values within parentheses indicate percentages; the cut-off point for throbocytopenia by QBC method is 140 billion/l.

agranulocyte count was 3.76 ± 1.70 among cases and 3.84 ± 1.65 among controls. This difference was not significant statistically ($Z = 0.45$, $p < 0.05$).

Table 6 shows that the thrombocytopenia was significantly higher ($\chi^2 = 19.2$, $p < 0.001$) among malaria cases (63.9%) compared to that in the controls (40.6%). Further, the mean platelet count was also lower among malaria patients (132.8 ± 55.9) than in the controls (165.6 ± 62.0). This difference was also statistically significant ($Z = 4.5$, $p < 0.001$).

DISCUSSION

Parasitisation of RBC and their subsequent breakdown largely explains the lowering of haematocrit level in anaemia.[5–7] There was a general trend of anaemia in the study population living in a highly endemic area of malaria. Nonetheless, the proportion of anaemia (with cut-off point for haemoglobin being 14 g% in QBC analysis) in malaria cases was far more than in the controls. Interiorisation of *Plasmodium* species inside RBC, resulting in their sequestration and subsequent destruction in the spleen lead to such low haemoglobin and anaemia, which is more marked in chronic vivax and acute falciparum cases. Moreover, *P. vivax* has been known to have an affinity for reticulocytes, leading to diminished reticulocyte count.[7]

The leucopenia observed in this study (6.7 and 2.8%) can be ascribed to general infection, and the absence of a significant difference of leucopenia between malaria cases and others corroborates earlier findings.[4] Nevertheless, granulocytopenia seems to be more significant among malaria patients than others. Thus, a relative agranulocytosis can well be acknowledged among them.[7]

Thrombocytopenia among the malaria cases is significantly higher than in the control group of patients, and various studies have shown merozoites within circulating platelets in acute *P. vivax* infection.[7] However, the platelet counts did not diminish to such an extent so as to cause bleeding disorders. But from the findings of this study, it may be concluded that acute vivax infection could well lead to bleeding manifestations, particularly with other concomitant predisposing factors.[7]

Thus, QBC estimation of haemograms correlates well with the available knowledge about malaria haematology. This relatively easier, quicker and reliable method of taking haemograms may be recommended for field testing for assessment of haematological parameters of malaria cases, before its introduction for large-scale use.

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SHORT NOTE

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Comparative Study on Microscopic Detection of Malarial Parasites under Conventional Thick Film and Concentration by Saponin Haemolysis

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Diagnosis of malaria is confirmed by detection of parasites under the microscope. In the national programme, microscopic detection of malaria parasites is done by examining standard thick and thin blood films taken on microslides.[1] In practical work, in malariology only a portion (1 to 2% area) of thick film is examined; the area of a film, one cm in diameter, is about 300 sq mm.[1] Many non-immune individuals take antimalarial drugs prophylactically and this reduces the chances of detecting malaria parasites in the blood. Therefore, a thorough search in one or more thick blood films is required to demonstrate parasites in such cases.[2] Dehaemoglobination of thick film may also cause loss of parasites.[3]

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The XIV WHO Expert Committee on Malaria recommended that WHO should give encouragement and full support to the development, issue and evaluation of a field kit based on the existing model of hand-operated centrifuges.[4] The present study deals with the comparison of microscopic diagnosis of malaria by conventional thick film, and concentration by saponin haemolysis. This evaluative study was conducted at the Urban Malaria Scheme area at Vellore of Tamil Nadu during 1996.

Among those reporting with fever in the malaria clinic of the Urban Malaria Scheme, Vellore and the Headquarters Government Pentland Hospital, Vellore, 285 patients were blood-screened for the study. In these units, microscopic diagnosis of malaria is done by thick film examination.

Routine blood screening for malaria by thick film examination[1] was done for all the 285 patients. Blood samples of 2–3 µl were collected by finger prick utilising sterile Hagedorn's needles. The blood samples were made into blood films measuring one cm in diameter on clean glass slides. Thin smears were also made as a routine practice. Dehaemoglobinised thick blood films were stained in JSB-I and II[1] solutions and examined under oil immersion of compound microscope. In each thick film, 100 fields were examined and the results recorded.

Two ml of venous blood was drawn by venipuncture from each patient after obtaining the consent. Each blood sample with anticoagulant was haemolysed with 1% saponin solution and subjected to centrifugation at 3,500 rpm for three min. The deposits were taken as smears on standard micro-slides and stained with Leishman stain.[5] For all the 285 patients, blood smears were made by the concentration technique. As in the case of thick films, 100 microscopic fields were examined at 100 x and the results recorded.

Under the conventional thick film technique, a total of 22 malaria-positive cases were detected of which 21 were *P. vivax* and one *P. falciparum*, whereas with the concentration technique 29 malaria-positive cases were detected of which 28 were *P. vivax* and one mixed infection. All the 21 cases of *P. vivax* detected by the conventional thick film technique were also detected with the concentration technique. The only single *P. falciparum* case detected under the conventional thick film technique was actually a case of mixed infection (*Pf* + *Pv*) as detected by the concentration technique, obviously with scanty *P. vivax* parasites.

Apart from this, 7 more malaria cases of *P. vivax* were detected by the concentration technique. The morphology of the parasite in the blood films by concentration technique was preserved to species identification level as in the case of conventional thick films. The advantage of selective haemolysis of erythrocytes by 1% saponin solution in the concentration technique gives room for a clear microscopic field, in which malaria-positive film exhibits aggregates of only leucocytes and parasites over a fairly clear, white background.

Though impracticable in the field of active surveillance for malaria, concentration technique

is greatly advocated in hospitals and dispensaries, where the quality of medical care is impaired due to inadequate diagnosis of malaria[6] among patients reporting with varied degrees of parasitaemia. The feasibility of adoption of the concentration technique at hospitals and dispensaries is high, as all the equipment and laboratory articles required for this technique are available in the clinical pathology departments of such institutions. The only additional requirement would be purified saponin powder, and an orientation to the already existing laboratory technicians.

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