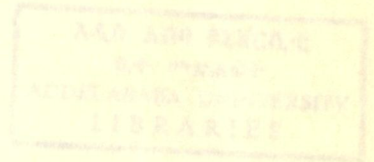
STUDY OF MALARIA ANTIGENS IN BLOOD AND URINE USING SOLID-PHASE RADIOIMMUNOASSAY

A Thesis

Presensited to the

School of Graduate Studies

Addis Ababa University



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By

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School of Graduate Studies

Study of Malaria Antigens in Blood and Urine Using Solid - Phase Radioimmunoassay.

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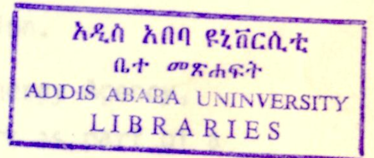

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ABSTRACT

The aim of this study was to investigate the presence of malarial antigens in the body fluids (blood and urine) and consider their application in the immunodiagnosis of malaria. One hundred and twenty-one urine samples and one hundred and nineteen red blood cell (RBC) samples collected from different localities of Ethiopia were assayed by using antibody-binding inhibition solid-phase radioimmunoassay (solid-phase RIA). The samples included acute and convalescent *Plasmodium falciparum* and *P. vivax* cases, and non-malarious cases from endemic and non-endemic areas.

The results obtained demonstrate the presence of malarial antigens in the urine of patients with an active falciparum infection. In the test of urine samples of convalescent cases, the gamma-activity bound was higher than that of acute cases, suggesting a decrease in the amount, or elimination, of malarial antigens. Thus, the application of detection of malarial antigens in urine for diagnosing malarial infection and conducting post-therapeutic follow-ups may be promising.

In the examination of the lysates of RBC, high antibody-binding-inhibition activities were calculated from the results of samples from acute and convalescent cases. The cause of high inhibition values in the convalescent cases was not clear. It has been proposed that this could be attributed to several factors, including the presence of malarial antigens within the RBC two weeks after treatment, or interference by antibodies.

The antigens used for coating the microtitre plate wells were from a Zairian isolate of *P. falciparum* . In the present study, this isolate was sufficiently cross-reactive with Ethiopian strains. This feature of cross-reactivity is important for the development of standardized immunodiagnostic methods based on antigen detection.

CHAPTER 1
I N T R O D U C T I O N

1.1. Malaria: An Ancient Disease Defying Solution

1.1.1. History

Malaria is a febrile disease caused by protozoan parasites belonging to the genus *Plasmodium*. The malarial agent was discovered by Alphonse Laveran in 1880 (cited by Bruce-Chwatt, 1981). The host-parasite relations of the plasmodia involve two hosts, one a vertebrate (mammal, bird, reptile) and the other an invertebrate (a mosquito of the genus *Anopheles)*.

The word malaria has been known in the literature since 1740. The history of malaria, as a disease, however, is as old as man's recorded history. Before the name malaria came in existence it was known by names such as 'marsh-fever','intermittent fever', or 'ague' (Bruce-Chwatt, 1981).

The name malaria is of Italian origin. It refers to bad or spoiled air (mal'aria) because of the general belief, at the time before the discovery of the aetiological agent, that some noxious vapour or miasma was responsible for the disease. Such a belief might have also existed in Ethiopia. For example, in Tigrignia (a language spoken in Northern Ethiopia) the word for malaria 'asso' is derived from the verb 'assewe', which means that an area has become humid, marshy, and smelling in the presence of flourishing fungi. On the other hand, the Amharic (official language in Ethiopia) name is 'weba' though it is also known by the name 'nidad' (burning). As

it can be inferred from a note given by Desta (1970), the word 'weba' was probably adapted from the Arabic word 'webaa'. According to Elias' Modern Dictionary of Arabic, 'webaa' is defined as an infectious epidemic disease (Elias & Elias, 1972). The adaptation of this word in Amharic for malaria possibly reflects the wide occurrence of malaria epidemics in Ethiopia at the time the word was introduced.

1.1.2. The Malaria Problem

1.1.2.1. The Global Malaria Situation

In many tropical countries, malaria endangers the health of both individuals and of communities. If the greatest harm to the greatest number of persons is taken as a standard of importance, one of the four forms of human malaria, falciparum malaria, is one, if not the most (Ifediba and Vanderberg, 1981) important infectious diseases of man. The effect of malaria is not limited to the impairment of health but it also paralyses the normal life of an area and has great impact on the overall socio-economic development.

During the two decades following the end of the Second World War, great successes were achieved by malaria eradication programmes. Malaria was eliminated from many regions (Bruce-Chwatt, 1981), but the risk has been eliminated for only about 1/5 of those living in malarious areas. As at mid-year 1978, taking 1947 as a reference year, out of 143 countries or areas (excluding China) where malaria was originally endemic and for which information was available, 37 countries or areas have been freed from malaria, in 15 others the risk is minimal, and in the remaining 91 the risk ranges from moderate to high (WHO, 1982). Furthermore, malaria control programmes based

on the application of anti-vectorial and plasmodicidal agents are facing great obstacles.

1.1.2.2. The Malaria Situation in Africa

In Africa it is estimated that more than 150 million malarious persons are living (Quinn and Plorde, 1981). In tropical Africa, because the application of malaria control measures has remained rudimentary (Wernsdorfer, 1979) malaria has great impact on the health of the population. Even though there are no precise nor exhaustive statistical data it is estimated that about one million African children die of malaria every year (Anon., 1980). As to malaria's effect on socio-economic development in Africa, some experts think of it as the major impediment to economic development (Maugh, 1977).

1.1.2.3. The Malaria Situation in Ethiopia

In Ethiopia 50% of the population live in malarious areas (Eyassu *et al.*, 1981). Malaria incidence ranges from the very high stability (occurrence throughout the year) along the courses of permanent rivers or settlement areas near marshy regions in the lowlands, to the unstable (occurring seasonally or during epidemics outbreak) malaria which characterizes the incidence in the cooler highlands.

During the 1958 malaria epidemic in Ethiopia, it was estimated that more than 150,000 lives were lost (Fontaine *et al.*, 1961). This led to the establishment of the National Malaria Eradication Service (NMES) and the opening of the Malaria Training Centre at Nazareth in 1959. Because of this programme it was possible to reduce the number of malaria epidemics and parasite rates in some areas, but elimination of the disease from the country has not yet been achieved. Since 1972, the name of the project has been changed

to Malaria Control Program and it is being integrated into the general health services (Ethiopia, Ministry of Health, 1982). The malaria outbreak in Ethiopia in 1981 is a reminder that there should be no room for complacency about the existing malaria situation.

1.1.2.4. Malaria Resergence Stimulates a World-wide Concern

The period 1972-76 was marked by a dramatic increase in the prevalence of malaria in the world. This included not only the tropical countries but also an increased number of malaria cases imported into Europe and the United States (Ellis *et al.*, 1979; Ellis, 1981; Gentilini *et al.*, 1981; Quinn and Plorde, 1981; Holvoet *et al.*, 1982).

The malarial comeback has been of world-wide concern, and many have reached the conclusion that malaria will stay with us for some time to come. The key problems to stress are that (a) in many of the poorest countries of the world, people are under the scourge of malaria, (b) there is a risk of malaria being introduced into malaria-free or malaria-freed areas, (c) there is a danger of non-immune persons being infected when travelling or working in malarious areas, and (d) there are increasingly difficult problems confronting malaria control programmes. To find solutions to these problems or to reduce the incidence of malaria it is reasonable enough that reorganized health plans against malaria be undertaken.

1.2. The Life Cycle of the Human Malarial Parasite

Features of *Plasmodium* species, in common with other parasites, are molecular diversity (genetic heterogeneity) coupled with great structural and life cycle complexity. As shown in FIGURE 1, the malarial parasite

alternates between the female *Anopheles* mosquito and man. The stage that infects the vertebrate host is the sporozoite. When an infected mosquito bites a man, the sporozoites are introduced into the new host.

The sporozoites have never been directly demonstrated in the circulating blood (Meuwissen, 1979). They reach the liver and invade the parenchymal cells by mechanisms that are not yet clearly understood (Meuwissen, 1979; Perrin *et al.*,1982). Inside the liver cells the sporozoites undergo multiplication and changes in morphology. They develop into trophozoites which in their turn become vegetatively dividing forms called schizonts. The products of asexual reproduction, exoerythrocytic merozoites, are released from the liver cells and may invade other liver cells, in *P. vivax* and *P. ovale* but not in *P. falciparum* and *P. malariae*, or blood cells. This is the exo-erythrocytic development.

The merozoites that have succeeded in invading RBC first appear as ring-shaped, later enlarging to become trophozoites, which have an amoeboid form. This is followed by asexual multiplication forming schizonts containing erythrocytic merozoites. The merozoites break out of the infected cells and rapidly invade other RBC. This is the erythrocytic development. During this course of the continuing cycles of asexual reproduction some parasites convert into sexual forms, the male and female gametocytes.

When a female *Anopheles* mosquito feeds on the blood of the gametocyte-carrying man, the gametocytes reach the stomach of the mosquito. The microgametocyte exflagellates to form motile microgametes. Fusion of a microgamete and a macrogamete results in the formation of a zygote. The zygote is transformed into an ookinete which penetrates the gut wall and develops into an oocyst. Sporogony (multiplication division) takes place within the

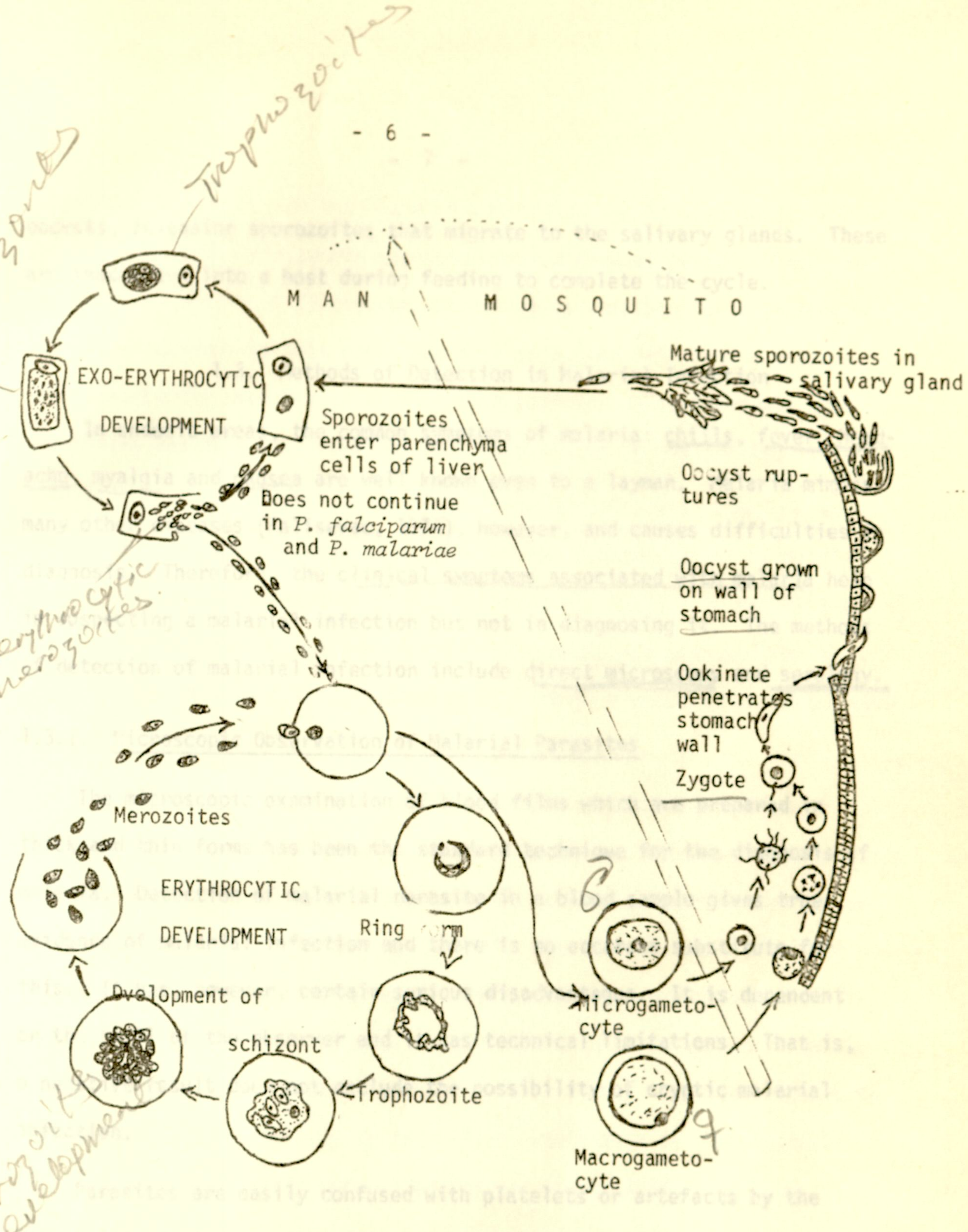


FIGURE 1

The Chain of the Developmental Stages in the Transmission of the Malarial Parasite

oocysts, releasing sporozoites that migrate to the salivary glands. These are inoculated into a host during feeding to complete the cycle.

1.3. Methods of Detection in Malarial Infection

In endemic areas, the common symptoms of malaria: chills, fever, headache, myalgia and nausea are well known even to a layman. Malaria mimics many other diseases (Falisevac, 1974), however, and causes difficulties in diagnosis. Therefore, the clinical symptoms associated with malaria help in suspecting a malarial infection but not in diagnosing it. The methods of detection of malarial infection include direct microscopy and serology.

1.3.1. Microscopic Observation of Malarial Parasites

The microscopic examination of blood films which are prepared in thick and thin forms has been the standard technique for the diagnosis of malaria. Detection of malarial parasite in a blood sample gives true evidence of malarial infection and there is no accurate substitute for this. It has, however, certain serious disadvantages. It is dependent on the skill of the observer and it has technical limitations. That is, a negative result does not exclude the possibility of cryptic malarial infection.

Parasites are easily confused with platelets or artefacts by the inexperienced microscopist. Microscopic diagnosis of malaria may be a problem not only to the unskilled technician but also to an experienced microscopist. For example, in falciparum malaria, the parasite is not common in the peripheral blood. This is because schizogony (asexual multiplication) in *P. falciparum* occurs, almost exclusively, in the blood vessels of the viscera. Furthermore, the time at which the blood film is

prepared with respect to the stage of multiplication is important. A 20-fold increase of parasitaemia can occur over a 48-hour period. Thus, because of the nature of falciparum malaria it is possible for a microscopist of long experience to miss parasites even in a good film from a patient who subsequently dies two days later from cerebral malaria and who even terminally has only one red blood cell parasitized in 100 (Ellis, 1981).

In asymptomatic carriers with low-grade parasitaemia, even the most painstaking prolonged examination, part of the recommendation by Belding (1965), may not disclose the infection (Heineman, 1972). In the USSR the blood of a suspected blood donor (among 23 others) was examined for 30 minutes more than 22 times without result, though eventually parasites were found (see Bruce-Chwatt, 1974).

The Giemsa method is certainly the most widely used technique of staining blood films. In many American laboratories another stain, Wright's is the preferred stain, but this does not keep well under tropical or subtropical conditions (Russel *et al.*, 1963). The Giemsa stain works best within a narrow range of neutral pH. Variation in pH may prevent manifestation of parasites. Moreover, heavy losses in parasites occur during the dehaemoglobinization and staining of thick films. This contributes to the shortcomings of the routine thick-film examination in the diagnosis of scanty malaria parasitaemias (Dowling and Shute, 1966).

Density-gradient centrifugation, sedimentation, free-flow electrophoresis (WHO, 1980) and high magnetic-field gradient (Paul *et al.*, 1981) techniques can be used for the separation of infected from uninfected erythrocytes and, to some extent, the stage-specific separation of infected erythrocytes (Heidrich *et al.*, 1982). But these techniques have not been

widely used in the diagnosis of malarial infections.

The above shortcomings must be considered when an observer encounters a patient who has typical symptoms of malaria but shown negative results on blood film examination. Provided the necessary precautions are taken, it can be concluded that direct microscopy is entirely satisfactory in acute infections and in individual cases. A problem arises when direct microscopy is used in (1) diagnosis of many patients (e.g. in areas where seasonal malaria occurs or when there is an epidemic outbreak) and (2) large-scale epidemiological investigations.

In many regions, microscopists in anti-malaria programmes and other health institutions examine millions of blood slides each year. The data obtained from such examinations have been used to measure and characterize epidemiological situations. The data include malaria cases reported by anti-malaria programme laboratories, hospitals, clinics, and results obtained from annual examination of blood films prepared from the population living in malarious areas. In such instances, however, direct microscopy is time-consuming, requiring highly skilled personnel and is very tedious - especially when the vast majority of smears are negative, and not sensitive when the concentration of parasites is low in the blood or parasites are absent at the time of testing (Lopez-Antunano, 1979).

Parasitaemia is influenced by the immune status and by use of anti-malaria drugs, and often occurs only intermittently during malaria infection (Meuwissen, 1974). Thus, in areas with low-grade infections (a phenomenon common where the level of immunity is stable) parasites are often missed (Field *et al.*, 1963) and consequently estimated prevalence and transmission rates in endemic areas will be lower than the rates actually existing. Those undetected in these surveys may form an important reservoir, leading

to the re-establishment of malaria in those areas where eradication had been thought to be successful (Thomas *et al.*, 1980).

One of the consequences of freedom from malaria is that the absence of cases may result in loss of skills relating to the clinical and microscopical diagnosis of malaria. Also, there is no opportunity for training based on actual cases (WHO, 1982). In areas that are vulnerable to malaria introduction, such loss of skills would favour the re-establishment of malaria.

1.3.2. Serodiagnostic Techniques in Malaria

The drawbacks of microscopic observation of blood films stimulated work on serodiagnosis. In the opening years of this century the work of many investigators made it clear that antibody formation was triggered by bacterial diseases. In the immunology of malaria, following Eaton's (1937) work (reviewed by Kuvin *et al.*, 1962a) on passive immunization of birds against malaria, through antiserum transfer, several reports suggested that malarial infection stimulates the production of immunoglobulin by inducing the formation of antibodies. Holmes *et al.* (1955) conducted a study on samples from Uganda to investigate the serum protein pattern in relation to diet and malaria. Based on their observation, they suggested that the gammaglobulins represented the humoral element of malarial immunity or products of hypersensitization of the lymphoid-macrophage system. Kuvin *et al.* (1962a, b) showed that antibody production correlated with the initial appearance of malarial parasites in the blood. The study of serology in malaria had then reached an important point and it offered a new hope for the development of improved methods of detection of malarial infection.

Serological tests have been useful tools for research and for indi-

vidual and community diagnosis. Serological methods can be of great help in situations where there is a low-grade malarial infection/transmission and especially where the transmission has been disturbed by the use of insecticides or drugs (Meuwissen, 1974). The clinician who is concerned with individual patients, and public health officials who are interested in the prevalence of malaria in the population, would appreciate the benefits from such improved tests.

The aims of serodiagnosis (WHO, 1974b; Lobel and Kagan, 1978; Wilson, 1979) are:

i. Diagnosis of individual patients
- arriving at the correct diagnosis and identification, if possible, of the *Plasmodium* species responsible for the infection.
- for the exclusion of the probability of malaria in patients with fever of unknown origin, hepatosplenomegaly, anaemia, and nephrotic syndrome.
- for the screening of blood donors.
- for post-treatment follow-up of patients.

ii. Epidemiological investigation
- for the establishment of the geographic distribution of malaria, the degree of stability and species prevalence in an area.
- to assess the effect of an eradication programme or to indicate the establishment of malaria in malaria-free or malaria-freed areas.
- to identify areas or communities which require priority action with regard to malaria.

These objectives may be achieved by performing tests that can detect: a. antibodies in serum
b. immune complexes
c. parasite antigens.

1.3.2.1. The Demonstration of Antibodies in Serum

As mentioned above, the malarial infection generates a vigorous humoral immune response, about a week after the completion of the prepatent period (interval of time between infection and appearance of initial detectable parasitaemia). This is marked by an increase in the total levels of the immunoglobulins IgG, IgA, and IgM, in parallel with serum antimalarial antibodies (Neva *et al.*, 1970).

Serologic examination for antibodies gathers period prevalence data because of the persistence of antimalarial antibodies for long after an infection has been eliminated. It thus provides information about the total infection of groups with malaria. Since the individual's immune response is affected by several factors such as age, immunologic competence, cumulative exposure to malaria antigens, and the kind and amount of specicific therapy, the interpretation of serologic data requires detailed knowledge of the local epidemiology of malaria (Meuwissen, 1974).

In the early 1960's, it was shown that the antibodies produced in response to malarial infection could be reliably detected (Draper, 1979). Now there are several serological methods for the detection of malaria antibodies (reviewed in Voller and Houba, 1980; Voller and Draper, 1982). Some of the most extensively used antibody-detecting tests include gel precipitation tests, indirect haemagglutination test and indirect fluorescent antibody test. IgG, IgM and to a lesser extent IgA are involved in these interactions.

1.3.2.1.1. Gel-precipitation tests

Among the techniques of precipitation tests in gel are the double-diffusion-in-gel (DD) technique and crossed immunoelectrophoresis (CIE).

Precipitin tests were used for studying the changing patterns of antibodies in relation to age in infants (Molineaux *et al.*, 1978); in the study of antibodies formed against soluble antigens (McGregor *et al.*, 1968; Wilson *et al.*, 1976); and for quantitative studies of antimalarial antibodies (Jepsen and Axelsen, 1980).

Gel-precipitation tests have the following basic advantages (WHO, 1974b; Wilson, 1979):

- they are technically simple and inexpensive to perform,
- suitable for comparative purposes owing to the visual appearance of precipitation lines which are easy to evaluate,
- when dried and stained they can be kept for future reference, and
- make possible the study of a large number of sera daily.

Limitations to the wide application of gel-precipitation techniques include:

- consumption of a large amount of antigen,
- time-consuming process of antibody titre determination,
- relative degree of insensitivity, and
- occurrence of false negatives in young children (WHO, 1974b).

1.3.2.1.2. Indirect haemagglutination (IHA) test

Stein and Desowitz (1964) used the indirect haemagglutination test for measuring antibody titres from *P. vivax* patients against homologous and heterologous antigen preparations. Based on their findings, these authors suggested the application of the test for following the immunological phenomena during the course of a malarial infection and for determining the interspecies antigenic relationship in *Plasmodium* species. Since then it has been evaluated by many authors (Rogers *et al.*, 1968; Kagan *et al.*,

1969; Kagan, 1972; Meuwissen, 1974; Mathews *et al.*, 1975) under different working conditions and the results were in favour of its use for work in epidemiological studies of malaria.

The IHA technique has been widely used in a large number of application areas. It has been used for establishing the degree of malaria endemicity and the state of immunity in an area (Desowitz and Saave, 1965; Mathews *et al.*, 1970b; Meuwissen, 1974; Mamtani *et al.*, 1979); to study the changing patterns of antibodies in relation to age (Bagchi *et al.*, 1978; Molineaux *et al.*, 1978) and in relation to malaria control measures (Brogger *et al.*, 1978), for studying the level and persistence of antibodies in relation to curative treatment (Wilson *et al.*, 1971), and to detect re-introduction of malaria in an area where eradication had been successful (Mathews *et al.*, 1970a).

The IHA test is simple to carry out and it can be used easily for large numbers of samples. It has medium to high sensitivity, and it is suitable for field use where action can be taken in accordance with results obtained the day the sera are collected (WHO, 1974b). It can show, however, false-negatives in parasite carriers. Examples are children, individuals tested during the initial attack, and those patients readily treated (Meuwissen, 1974; Meuwissen *et al.*, 1974; Collins *et al.*, 1975). Children are epidemiologically often the most important age-group (Draper, 1979). False-positivity also occurs in IHA tests due to nonspecific agglutination factors (Meuwissen, 1974). A further problem with IHA test also related to technical aspects is that small variations in the sample collection and test procedures can drastically affect the results (Voller and Houba, 1980). The availability of antigens suitable for the test from only a few centres (WHO, 1974b) is a serious disadvantage, especially for underdeveloped countries.

1.3.2.1.3. Indirect fluorescent antibody (IFA) test

Indirect fluorescent antibody test was first described for the detection of malarial infection by Kuvin *et al.* (1962a). Initial problems related to the antigens have been overcome by using a thick smear and antigen prepared from washed, parasitized erythrocytes (Sulzer *et al.*, 1969). The availability of *P. falciparum* from *in vitro* culture (Lopez-Antunano, 1974; Thomas and Ponnampalam, 1975; Hall *et al.*, 1978) has also facilitated the preparation of antigens.

Until very recently, IFA test offered the highest degree of sensitivity, specificity, and reproducibility (Lopez-Antunano, 1979). In children and in individuals with primary infection, antibodies are detected earlier and more efficiently by using IFA than IHA (Wilson *et al.*, 1971; Meuwissen, 1974; Collins *et al.*, 1975). A further advantage is that antigen preparation is relatively simple and sometimes this test can be used to indicate species prevalence (WHO, 1974b). Another great advantage of IFA test is that air-dried finger prick blood is used. Papers can be easily stored, then air-mailed to recognised test centres.

IFA has been the most widely used technique for epidemiological work (Draper, 1979). In non-malarious areas it is useful for the serodiagnosis, post-therapeutic follow-up and study of certain immunopathological aspects of malaria. In endemic areas its application is in epidemiology (Ambroise-Thomas, 1974, 1976). It has been applied to the investigation of:

- malarial experience of the population, the local levels of endemicity and transmission, and effect of control measures (Collins *et al.*, 1968b; Voller and Bruce-Chwatt, 1968; Jeffrey *et al.*, 1975; Brogger *et al.*, 1978; Thomas *et al.*, 1980),

- the pattern of malaria antibodies with respect to age (Collins *et al.*, 1967; Molineaux *et al.*, 1978),
- the pattern and persistence of antibodies after chemotherapy (Collins *et al.*, 1968a; Wilson *et al.*, 1970),
- the return of malaria to an area from which it had disappeared (Ambroise-Thomas, 1976), and
- the disappearance of malaria from an area (Bruce-Chwatt and Draper, 1973).

In individual cases it has been used for detecting previous infection (Bruce-Chwatt *et al.*, 1972), and for detecting antibodies in kidney tissue (Bhamarapravati *et al.*, 1973). It has also been used for determining serologic relationships between isolates of *P. inui* (Collins *et al.*, 1970).

The use of IFA is not limited to diagnostic or field work. It has been a very important tool in laboratory-based research work, for example in selection of monoclonal antibodies. It has, however, certain disadvantages. The necessity for expensive microscope equipment is one disadvantage. Another limitation is that the reading of results has to be done critically by a skilled person with experience of the test system (Wilson, 1979). Furthermore, the evaluation procedure is time-consuming in that a relatively small number of samples can be examined by one worker in a day (Collins *et al.*, 1975). Malaria parasite carriers can occasionally give seronegative reactions (Wilson *et al*, 1971).

1.3.2.1.4. Concluding points on demonstration of antibodies in serum

The various techniques developed and investigations undertaken in the past have been of scientific and practical importance. Their contribution to the understanding of immunological phenomena in malaria and their use as diagnostic and epidemiological tools have been very encouraging.

The demonstration of serum antibodies, however, has certain inherent limitations from a diagnostic point of view, since the mere presence of antibodies indicates experience of malaria but does not distinguish between current and recent, or remotely past but burnt-out infections. Due to the kinetics of the immune response, antibodies may persist long after the disease has been cured. After repeated attacks, malaria antibodies my be detectable for even up to 30 years after the last episode of malaria (Draper and Sirr, 1980). Even after a single infection they may persist for 13 years (Kagan, 1972).

Moreover, from the practical point of view, the presently available tests for detecting malaria antibodies are inadequate for many reasons. According to Voller *et al.* (1980) the exact role of serology /based on detection of antibodies/ is debatable.

Thus, the need is evident for a battery of simple tests to show (1) the existence of an active infection, (2) a past host-parasite contact, and (3) the degree of protective immunity. The present study was planned with the hope that it would shed some light towards a solution to the first two needs.

1.3.2.2. The Demonstration of Immune Complexes

Immune complexes are formed during malaria infection and they may be found both free in the serum and bound in tissues. Sensitive methods have recently been developed for the detection and purification of immune complexes (Lambert *et al.*, 1979). This aspect of serology may make possible the development of new immunodiagnostic tests.

1.3.2.3. The Demonstration of Antigens

Malarial antigens are a very complex group. They represent structural

elements of the parasite in its various stages, host elements expressed as antigens because of the presence of parasites or metabolites, or secretions of parasitic origin which include soluble antigens.

Structural Components: Each of the developmental stages shown in FIGURE 1 is associated with specific antigens. Moreover, there is antigenic variation in a chronic infection and amongst different strains of a *Plasmodium* population in an area. However, there is also an extensive antigenic cross-reactivity between sporozoites, exoerythrocytic forms, blood stage forms, variants and strains. At the species level, some antigens are species-specific while others cross-react between species. There are about 100 species of *Plasmodium* affecting a variety of vertebrates. Some of the publications indicating the diversity of *Plasmodium* antigens as well as their cross-reactivity are given in TABLES 1 and 2.

Soluble Malarial Antigens: McGregor *et al.* (1968) detected soluble antigens of *P. falciparum* in the sera of Gambian children suffering from acute falciparum malaria. The antigens are a heterogenous group. At least 29 different serum antigens have been demonstrated by matching very large series of patients' sera and immune sera (McGregor and Wilson, 1971). The antigens could be placed into three groups on the basis of their sensitivity/resistance to heat treatment (Wilson *et al.*, 1969). The groups are; the Labile (L) antigens - destroyed or precipitated by heating at 56°C for 30 minutes, the Resistant (R) antigens - stable at 56°C but are destroyed at 100°C, and the Stable (S) antigens - not destroyed by boiling at 100°C for 5 minutes. These major groups are further divided into subdivisions, example, into La, Lb etc. Most studies have been carried out on the S antigens (Wilson *et al.*, 1975a, 1975b; Wilson, 1980). Wilson and Ling (1979)

and Wilson (1980) suggested that the S antigens might serve as markers for serotyping isolates of *P. falciparum*. On the basis of aminoacid incorporation studies, Wilson suggested that antigens of the S group might represent altered components of host tissues (McGregor, 1974).

TABLE 1

Antigenic Diversity in *Plasmodium* parasites

Claimed Description of Antigen	Findings Supporting the Description	Source
Variant-associated	Five antigenic variants isolated from successive relapses of a *P. knowlesi* infected monkey	Voller & Rossan (1969)
	Antigenic variation demonstrated in six stabilates isolated from relapses following a parental infection of *P. knowlesi*	Brown & Brown (1965)
	Failure of protective effect of antiserum of known protectivity against the homologous antigenic variants of *P. knowlesi* in *M. mulatta*	Brown *et al.* (1968)
Stage-specific	Antisporozoite antibodies tested against sporozoites and infected red blood cells of *P. berghei*, *P. knowlesi* and *P. falciparum* detected sporozoite-specific antigens	Nardin & Nussenzweig (1978)
	Progressive degree of antigenic maturation was observed in salivary gland sporozoites	Nussenzweig & Chen (1974)
	Study of 11 *P. knowlesi* antigens obtained from ring-, trophozoite-, and schizont-infected red blood cells showed trophozoite-, schizont and merozoite-dependent antigens	Deans & Cohen (1979)
	Monoclonal antibodies exclusive to antigens on *P. yoelii* merozoite were raised	Freeman *et al.* (1980)
	Three, five, and five polypeptides believed to be either predominant or specific for stages in ring forms, trophozoites and schizonts of *P. falciparum*, respectively were isolated	Perrin *et al* (1981a)
Strain-specific	Agglutination mediated by antigens on surface coat of *P. knowlesi* merozoites was greatest against homologous strains of *P. knowlesi*: Malaysian and Philippines	Miller *et al.* (1975)
Species-specific	Out of 11 *P. knowlesi* antigens studied against *P. cynomolgi bastianellii* one was *P. knowlesi* specific	Deans (1979)

TABLE 2

Antigenic Cross-reactions in *Plasmodium* parasites

Description of Antigen	Findings Supporting the Description	Source
Cross-reacting between stages	Most of the 40 labelled polypeptides identified were common to the different erythrocytic stages of *P. falciparum*	Perrin *et al.* (1981a)
	Antigens common to sporozoites, exoerythrocytic and blood stage schizonts	Deans & Cohen (1979)
	Antigens common to sporozoites, blood stages and gametes in *P. berghei*, *P. knowlesi* and *P. falciparum* were detected	Nardin & Nussenzweig (1978)
Cross-reacting between strains	The same antibody titres were obtained when sera from two volunteers infected with Chesson strain *P. vivax* and two others with Venezuelan strain *P. vivax* were allowed to react with the homologous and heterologous strains of *P. vivax*	Tobie *et al.* (1962)
	Reaction between antigens of an antiserum against sporozoites of *P. falciparum* strains of Burma and Mark and of other species showed cross-reactivity	Nussenzweig & Chen (1974)
	Antigens expressed on surface knobs of human and monkey erythrocytes parasitized with Southeast Asian and two African strains of *P. falciparum* were cross-reactive	Langreth & Reese (1979)
Cross-reacting between species	Antigen prepared from mature schizonts of *P. knowlesi* reacted with antibodies produced against 10 species of primate plasmodia	Rogers *et al.* (1968)
	More than four proteinaceous antigens on the surface of erythrocytes with schizonts of *P. falciparum* and *P. knowlesi*	Schmidt-Ulrich *et al* (1982)
	Reaction between B strain of *P. cynomolgi* and the Chesson and Venezuelan strains of *P. vivax* with sera from five normal volunteers infected with the B strain of *P. cynomolgi* was cross-reactive	Tobie *et al* (1962)

The soluble malarial antigens might be excretory-secretory products of the parasite or host-cell components. Excretory-secretory antigens have been isolated from *in vitro* cultures of *P. falciparum* (Thelu *et al.*, 1982) and *P. berghei* (Weissberger *et al.*, 1979).

As to the chemical nature of the antigenic determinants they may involve proteins or glycoproteins (Bannister, 1977; Kilejian, 1980; WHO, 1980), glycolipids or other complex macromolecules (WHO, 1980).

The diversified nature of malarial antigens poses a problem to the development of an improved diagnostic test. It entails much labour and time for identification and characterization. Nevertheless, at present, detection of antigens is considered to be preferable to detection of circulating antibodies.

One reason why antigen detection is prefered to circulating antibody detection is (Houba, 1980) that the detection of antigens is associated with a present or recent infection, since the time for elimination of malarial antigens could be expected to be shorter than that of antibodies in circulation. Besides, McGregor *et al.* (1968) have suggested the presence of soluble antigens which are possibly of weak immunogenicity. Some of the soluble antigens may then be freely circulating, not forming antigen-antibody complexes, and would then be easier to detect. Urine is another body fluid in which antigens may be detected. This would be a further advantage obviating the need for blood samples.

Malarial Antigens Excreted in the Urine: The occurrence of malarial antigens in the urine of infected individuals has not been investigated.

Some work has been done to demonstrate antigens in several parasitic infections, for example, malaria, schistosomiasis, onchocerciasis, and

toxoplasmosis. Malaria antigens have been detected with a relatively high degree of sensitivity in a preparation of lysed RBC from malaria patients (Mackey *et al.*, 1980a). Circulating antigens have been demonstrated by Capron and co-workers in the serum and in the urine, and also in the milk of mothers infected with *Schistosoma mansoni* (Deelder, 1982) and in the body fluids of animals infected with *S. mansoni*, *S. japonicum* and *S. haematobium* (Santoro *et al.*, 1978; Deelder, 1982). In the sera of onchocerciasis patients, circulating *Onchocerca volvulus* antigens have been demonstrated by Ouaissi *et al.* (1981). *Toxoplasma gondii* antigens were detected in *Toxoplasma* lysate, in peritoneal fluid of mice, and in sera from humans acutely infected with *T. gondii* (Araujo *et al.*, 1980).

If fully developed, a test based on antigen detection could provide a method for detecting active, and possibly, recent infections. It could also be used to monitor chemotherapy, and if applicable on a large scale, such as in antibody-surveys, could be a useful sero-epidemiological tool. One of the main problems in detecting circulating antigens is that the concentration of these substances in body fluids (serum, milk, urine) is usually very low, and highly sensitive techniques are needed for their measurement (Houba, 1980).

Enzyme-linked immunosorbent assay and radioimmunoassay tests are the present candidates for a new and improved immunodiagnostic technique to be used in the detection of antigens.

1.3.2.3.1. Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay was studied and developed as an alternative to radioimmunoassay (*vide infra*) for the detection of soluble antigens and antibodies (Engvall and Perlman, 1971, 1972; Engvall *et al.*,

1974) because it has the advantages of more stable reagents and less requirements of specialized equipment. Though ELISA has been found to be of low sensitivity for malarial antibody detection (Voller *et al.*, 1975, 1976, 1980; Bidwell and Voller, 1981), recently, Mackey *et al.* (1982) have used ELISA for the detection of *P. falciparum* antigens from infected red blood cells and found promising results. Parasites were detected at a level of 8 parasites/10^6 RBC. These authors feel that greater precision can be expected when purified malaria antigen and antibody reagents become available.

1.3.2.3.2. Solid-phase radioimmunoassay (RIA)

Solid-phase radioimmunoassay employs isotope-labelled reagents for the measurement of antigen-antibody reactions. In this test, the antigen or antibody is immobilized most often to a plastic surface (solid-phase RIA). The radiolabelled reactant is applied either directly or indirectly. The antigen-antibody reactions which have occurred on the surface of the support can be detected by measuring the amount of radioactivity fixed.

There are various test procedures for carrying out solid-phase RIA. These include competitive binding RIA technique, noncompetitive (direct) RIA binding technique, noncompetitive (indirect) RIA binding technique, or antibody-binding-inhibition RIA technique.

Antibody-binding-inhibition RIA technique was used in the present study. This test is based on the ability of malarial antigens in the test samples to combine with radiolabelled antibodies and thus prevent the subsequent interaction of the later with antigens fixed on the surface of microtitre plates.

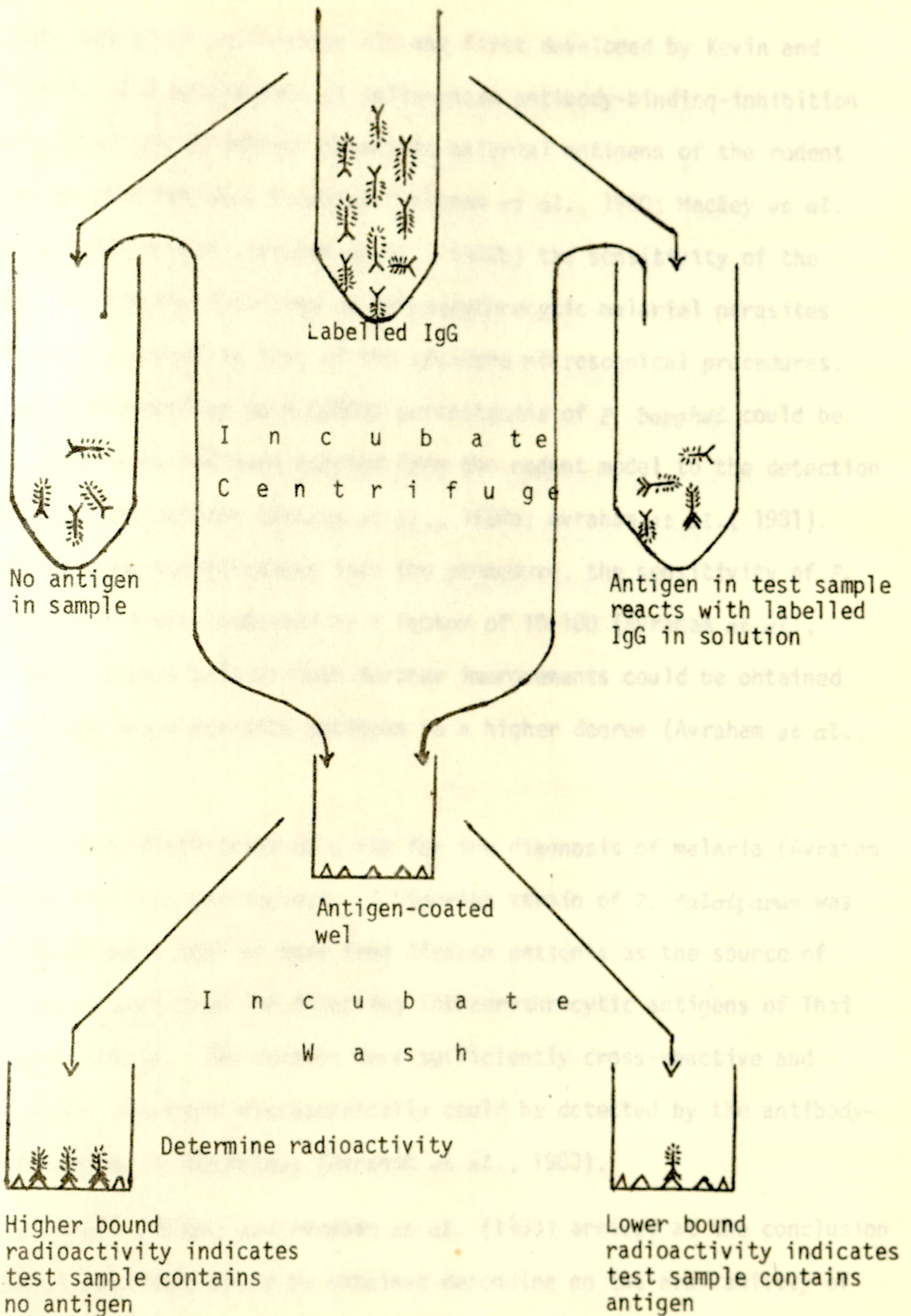


FIGURE 2

The Inhibition Method of RIA
for Assay of Antigen

The tube method of solid-phase RIA was first developed by Kevin and Tregear (1967). The application of solid-phase antibody-binding-inhibition RIA for the detection of intraerythrocytic malarial antigens of the rodent parasite *P. berghei* has been reported (Avraham *et al.*, 1980; Mackey *et al.*, 1980b). In a later report (Avraham *et al.*, 1982b) the sensitivity of the inhibition test for the detection of intraerythrocytic malarial parasites was found to be superior to that of the standard microscopical procedures. An infection corresponding to 0.00005% parasitaemia of *P. berghei* could be detected. This assay has been adapted from the rodent model to the detection of *P. falciparum* infections (Mackey *et al.*, 1980a; Avraham *et al.*, 1981). By introducing some modifications into the procedure, the sensitivity of *P. falciparum* detection was increased by a factor of 10-100 (Avraham *et al.*, 1982a). These authors believe that further improvements could be obtained by purifying the major parasite antigens to a higher degree (Avraham *et al.*, 1982a).

A preliminary field trial of a RIA for the diagnosis of malaria (Avraham *et al.*, 1983) has been carried out. A Nigerian strain of *P. falciparum* was used as antigen and a pool of sera from African patients as the source of antibody. These were used for detecting intraerythrocytic antigens of Thai *P. falciparum* strains. The strains were sufficiently cross-reactive and almost all cases confirmed microscopically could be detected by the antibody-binding-inhibition RIA technique (Avraham *et al.*, 1983).

Mackey *et al.* (1980a) and Avraham *et al.* (1983) arrived at the conclusion that maximum sensitivity would be obtained depending on the availability of defined reagents. In this respect, a judiciously selected single-specificity antibody, produced using the hybridoma technique of Kohler and Milstein

(1975) may have great value (SWG, 1981).

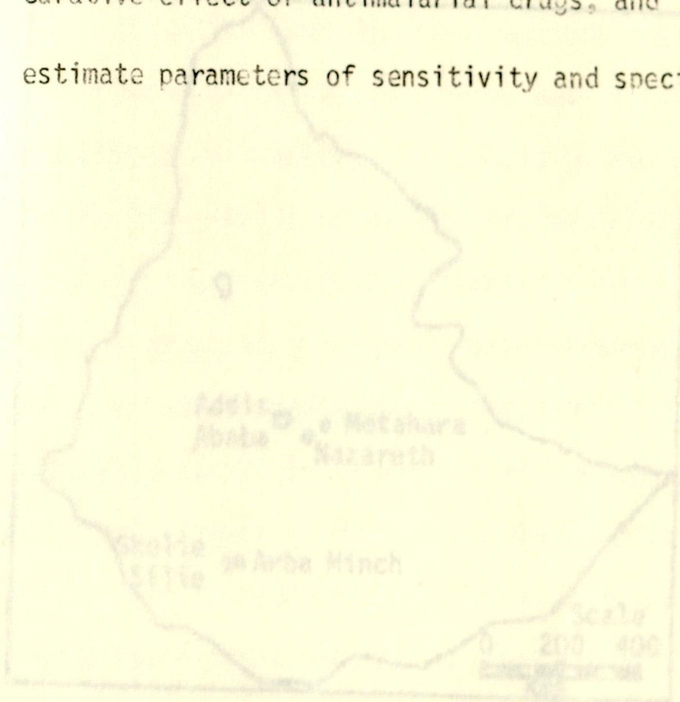
An emphasis of the research of the development of monoclonal antibodies against malaria parasites has been on the identification and characterization of the protective antigens (Nussenzweig, 1982). A number of monoclonal antibodies against various stages of different *Plasmodium* species have been produced (Freeman *et al.*, 1980; Rener *et al.*, 1980; Yoshida *et al.*, 1980; Cox, 1981; Epstein *et al.*, 1981; Holder and Freeman, 1981; Perrin *et al.*, 1981b; Er-Hsiang, 1982; McBride *et al.*, 1982). Characterization of appropriate antigens for immunodiagnostic requirements will add to the efforts in developing specific, sensitive serologic tests in malarial infections.

CHAPTER 2

OBJECTIVES OF THE PRESENT STUDY

The present study was undertaken to establish:

- the applicability of solid-phase RIA to the detection of intraerythrocytic parasite antigens of the Ethiopian strains of *P. falciparum*, circulating malarial antigens, or parasite antigens present in urine,
- the possible application of the test for evaluating the curative effect of antimalarial drugs, and
- estimate parameters of sensitivity and specificity.



CHAPTER 3

MATERIALS AND METHODS

3.1. Collection of Test Samples

3.1.1. Sampling Areas

The names and locations of the sites where samples were collected are indicated in FIGURE 3.

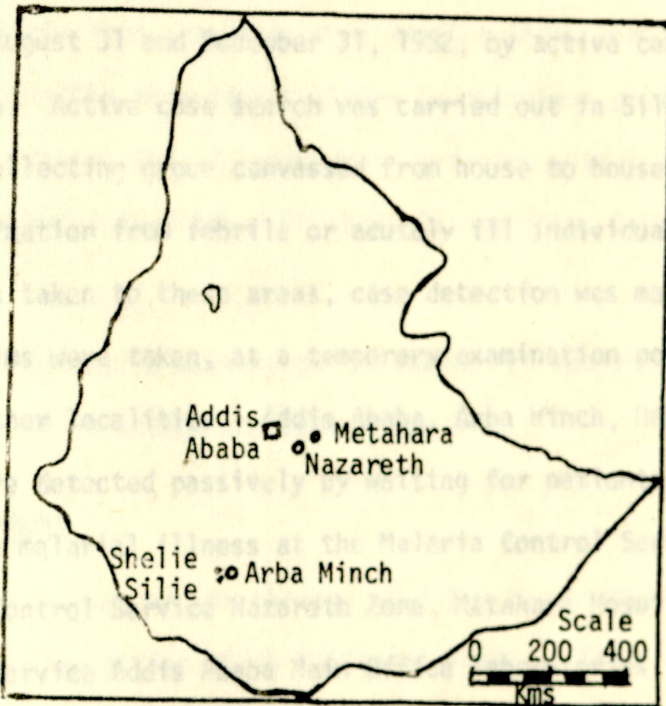


FIGURE 3

Map of Ethiopia with Location of Sampling Areas

Arba Minch lies in the lower region of the Great Rift Valley, which bisects

the Ethiopian highlands, at an altitude of about 1200 m (505 Kms south of Addis Ababa). Nazareth is located in the central region of the Rift Valley at an average altitude of about 1600 m (99 Kms south-east of Addis Ababa). Silie and Shelie (of about the same altitude as Arba Minch) are cotton estate farms located at about 18 and 26 Kms, respectively, south-west of Arba Minch. Metahara (about 950 m altitude) is one of the three sugar factories (Wenji, Shewa and Metahara) located along the Awash River. Metahara is about 95 Kms east of Nazareth.

3.1.2. Case Detection and Subjects

Test samples from patients with acute falciparum malaria were collected between August 31 and December 31, 1982, by active case search and passive detection. Active case search was carried out in Silie and Shelie. The sample collecting group canvassed from house to house, taking blood films for examination from febrile or acutely ill individuals. Since a microscope was taken to these areas, case detection was made on the same day blood films were taken, at a temporary examination post under a big tree. In the other localities - Addis Ababa, Arba Minch, Nazareth, and Metahara - cases were detected passively by waiting for patients seeking treatment for acute malarial illness at the Malaria Control Service Arba Minch Zone, Malaria Control Service Nazareth Zone, Metahara Hospital, and Malaria Control Service Addis Ababa Main Office laboratories.

Thick blood films stained by the Giemsa technique for identification of malarial parasites were examined on the days of collection before any treatment was given. Out of 1320 patients screened for falciparum malaria, 95 were found to be positive. Samples were not collected from some patients because of the serious health condition which required immediate treatment

(WHO, 1974a) or problems of sampling.

The following samples were taken from each of 43 falciparum malaria patients (38 males and 5 females; age ranging from 9 to 60 with a mean of 23 (±11): thick-thin blood films, venous blood, capillary blood, and urine. The patients were asked to come back 12-16 days after the completion of treatment, for follow-up sampling.

Information recorded for each subject included name, age, sex, type of treatment, date when symptoms started, probable geographical area where malaria was acquired, and locality of residence.

Control samples were collected from 10 individuals without known exposure to malaria from a malaria-free area of Addis Ababa (5 males and 5 females), 10 healthy individuals living in malarious areas of Arba Minch and Nazareth (males) and 12 individuals infected with *P. vivax* from Nazareth post (9 males and 3 females). Of these 12 patients, 6 came back for follow-up sampling.

TABLE 3 presents a summary of the number of subjects in relation to probable site of infection and collection area.

3.1.3. <u>Treatment</u>

The formulations of the therapy used for the treatment of acute falciparum malaria were different in different places. Some patients were treated with only chloroquine, others with chloroquine and primaquine, and a third group with amodiaquine and primaquine. The vivax patients were given a treatment of amodiaquine and primaquine.

Chloroquine Treatment: This involved administration of 1000 mg of chloroquine (4 tablets) immediately after sample collection, to be followed

by 500 mg (2 tablets) 6 hours later and 500 mg daily for 2 days following the first day of treatment.

TABLE 3

Number of Falciparum Cases Included in the Study with Respect to Collection Area and Probable Site of Infection

Sample Collection Site	Probable Site of Infection	No. of Patients Sampled
Shelie	Shelie	8
Silie	Silie	9
Arba Minch	Arba Minch	4
	Shelie	2
	Elgo	1
Metahara	Metahara Sugar Factory	3
Nazareth	Wolenchity	3
	Dengore	2
	Nazareth?*	1
	Wonji	1
	Melka Sedi	1
	Koka Dam	1
	Kucurfa	1
	Feto	1
	Borcheta	1
	Buta	1
	Chercher	1
	Kobo Lito	1
Addis Ababa	Kutaber	1
TOTAL		43

* may have been infected out of Nazareth.

Chloroquine and Primaquine Treatment: This involved administration of

chloroquine as outlined above plus 75 mg (10 tablets) base of primaquine taken in divided doses of 15 mg (2 tablets) base per day over a period of 5 days.

Amodiaquine and Primaquine Treatment: Immediately after sample collection the patient took 600 mg (4 tablets) of amodiaquine to be followed by 300 mg (2 tablets) 6 hours later and 300 mg daily for 2 days following the first day of treatment. Together with this, 75 mg (10 tablets) of primaquine base was taken in divided doses of 15 mg (2 tablets) each over a period of 5 days.

3.1.4. Collection of Serum

Five mls of venous blood were collected by withdrawing into disposable plastic syringes. Immediately, the blood sample was transferred into sterile test tubes and allowed to clot at room temperature for about 6-8 hours. The tube was transferred to $4^{0}C$ and left overnight. The next day the blood sample was centrifuged at setting 2 (Phywe A.G., Cottingen) and the serum removed with a sterile Pasteur pipette, into individually marked Nunc(R) or polycarbonate tubes. Sodium azide (NaN_3) was added to the serum samples to make a 0.1% NaN_3 solution. The tubes containing serum samples were stored at $-20^{0}C$.

3.1.5. Collection of Blood into Capillary Tubes

The finger was cleaned with an alcohol-moistened swab and dried with a piece of dry cotton. The finger was then punctured with a disposable lancet and the first drop of blood wiped off. Blood was collected into heparinized capillary tubes.

The capillary tubes were sealed at one end by plugging with plasticine.

These capillary tubes were allowed to stand at room temperature (RT) to let cells sediment. After this, the open ends were sealed. These were put into labelled Falcon(R) tubes and stored at -20°C.

3.1.6. Preparation of Blood Films

The finger was prepared for puncture as described above for collection of capillary blood. A thick blood film and a thin blood film were made on the same slide. The blood films were air-dried in a horizontal position, after which the thin films were fixed in 100% methanol. The slides were dried, marked and kept in a slide box.

The blood films were prepared for future reference of species identification checking and to find out the extent of agreement between the parasitaemia and amount of malaria antigen detected by RIA.

3.1.7. Collection of Urine Samples

About 25 mls of urine were collected in a clean beaker. The urine was centrifuged at setting 2 (Phywe A.G., Cottingen) to remove any obvious debris. To this sample sodium azide (NaN_3) was added to make 0.1% sodium azide solution. About 10 mls of this urine was transferred into marked plastic bottles. The bottles were screwed tightly and stored at -20°C

3.1.8. Storage and Transport

During the sample collection process the serum, capillary blood and urine samples were stored at -20°C at Arba Minch, Nazareth (Haile-Mariam Mammo), and Metahara Hospitals, after which they were transported in a portable ice-box to the Armauer Hansen Research Institute in Addis Ababa, where they were stored at -20°C or -70°C until the immunoassay tests were carried out.

3.2. Laboratory Methods

3.2.1. Microscopic Determination of Parasite Density

Blood films for parasite density determination were stained with Diff Quick Stain Set (Dade Diagnostic, Inc. Aguada PR00602 - American Scientific Products).

Diff-Quick Stain Set is a modification of the Wright Stain technique, which provides speed. The Stain Set consists of Diff-Quick Fixative (methanolic fixative), Diff-Quick Solution I (a buffered solution of eosin Y - an anionic dye), and Diff-Quick Solution II (a buffered solution of thiazine dyes - cationic dyes). Since the thin blood films were fixed in 100% methanol during the sample collection process, there was no need of using the Diff-Quick Fixative. The blood films were dipped in Solution I for 25 sec and in Solution II for another 25 sec. The slides were rinsed in tap water in between the two steps and after staining in Solution II. The stained slides were air-dried.

Parasite density was determined from the thick films and thin films. When using the thick films, the parasites and white blood cells seen in a field were counted and then the field changed until the number of white blood cells counted reached 200. This was recorded as the number of parasites per 200 WBC. The second method of determination of parasitaemia involved counting the number of parasitized red blood cells per 15,000-25,000 erythrocytes of the thin blood films, and expressed as percentage parasitaemia. A x100 oil-immersion objective and x10 eye piece were used.

3.2.2. RIA Test for the Detection of Malaria Antigens

Test preparations of RBC or urine were mixed with ^{125}I-labelled IgG (IgG^*) and incubated for one hour at 37°C followed by 30 minutes at 4°C. The mixtures were centrifuged at 1600g for 15 minutes and the supernate transferred into microtitre plate wells coated with malarial antigen. After incubation for 12 hours at 4°C the wells were washed three times with borate buffer solution (BBS, 0.2M, pH 8.6), cut out and put into gamma-vials for counting of gamma-activity in a 1270 Rackgamma II gamma counter LKB-Wallac. The antibody-binding-inhibition caused by antigens in test samples was calculated by comparing with that of antibody alone suspended in BBT (BBS + 0.05% Tween 80 + 2%BSA). Statistical analysis of the results obtained for acute and convalescent cases was done by using a t test for paired samples.

3.2.2.1. Preparation of Lysate of RBC

The volume of the capillary tubes used for collecting blood samples was 0.1 ml in the total length of 7.5 cm. The length of the capillary tube with sedimented RBC was measured and the volume calculated. The RBC from each capillary were lysed with BBS + 0.3% NP40 (a non-ionic detergent) to obtain a 1:10 dilution of RBC.

3.2.2.2. Preparation of Urine Samples

The pH of the urine was adjusted by adding 10 µl of 10x concentrated BBS to 90 µl of each urine sample to be tested.

3.2.2.3. Preparation of Antigen-coated Plate Wells

A preparation of *P. falciparum* (M25, an isolate from Zaire - kindly

provided by Dr. Renu Dayal) was sonicated three times for 15 to 30 sec at 50W in an ice-bath using a B12 sonifier (Branson sonic power company - Danbury, Connecticut). The sonicate was diluted with BBS to a concentration of 50 µg protein/ml BBS. 50 µl of the solution was used to coat each well of flexible polyvinyl chloride microtitre plates (Dynatech 1-220-29).

Microtitre plates containing the coating antigen were incubated at 37°C for 3 hours. These plates were used immediately or left at 4°C. Microtitre plates containing the coating antigen could be stored for at least 2 weeks (Mackey *et al.*, 1980a, b).

3.2.2.4. Reconstitution of IgG*

An aliquot of IgG* prepared by Dr. Renu Dayal from pooled sera of immune individuals was diluted 30 times in BBS + 0.05% Tween 80 + 2% bovine serum albumin (BSA). The specific activity of the diluted IgG* used was 2.4×10^7 cpm/ml for the RBC assay and 1.16×10^7 cpm/ml for the urine assay. The different specific activities were a result of the radioactive decay of the labelled IgG.

3.2.2.5. Assay Procedure

The assay procedure is illustrated in the flow chart, FIGURE 4.

3.2.2.6. Control Reagents

3.2.2.6.1. Negative control antigen

The negative control antigen was prepared from washed normal RBC lysed in BBS + 0.3% NP40. This preparation was used to determine the non-specific inhibition of binding and also as a diluent for parasite antigens. In addition to this, urine from a healthy person was used to

determine the non-specific inhibition of binding and also as a diluent for parasite antigens in the assay of the urine samples.

3.2.2.6.2. Positive control antigen

Infected RBC from *in vitro* cultures of *P. falciparum* of known parasitaemia were lysed in 9 volumes of BBS + 0.3% NP40. The lysate was used for preparing eight five-fold dilutions. The dilutions were made in the NRBC lysate or normal urine (negative control antigens).

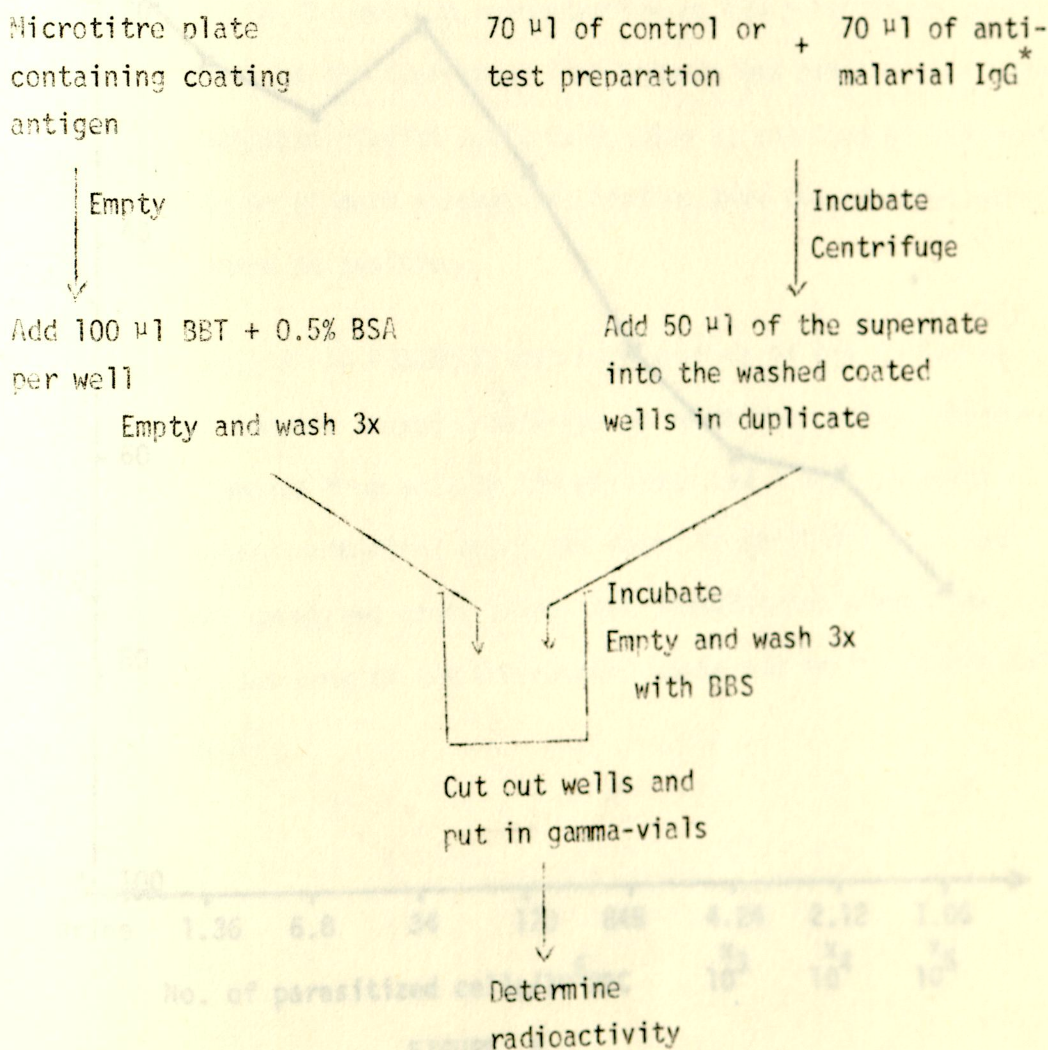


FIGURE 4

Assay Procedure

CHAPTER 4

R E S U L T S

4.1. RIA of Urine Samples

RIA test of the control antigen serially diluted in urine gave the results shown in FIGURE 5. The level of parasite detection corresponded approximately to 170 parasitized cells/10^6 RBC (500 cpm).

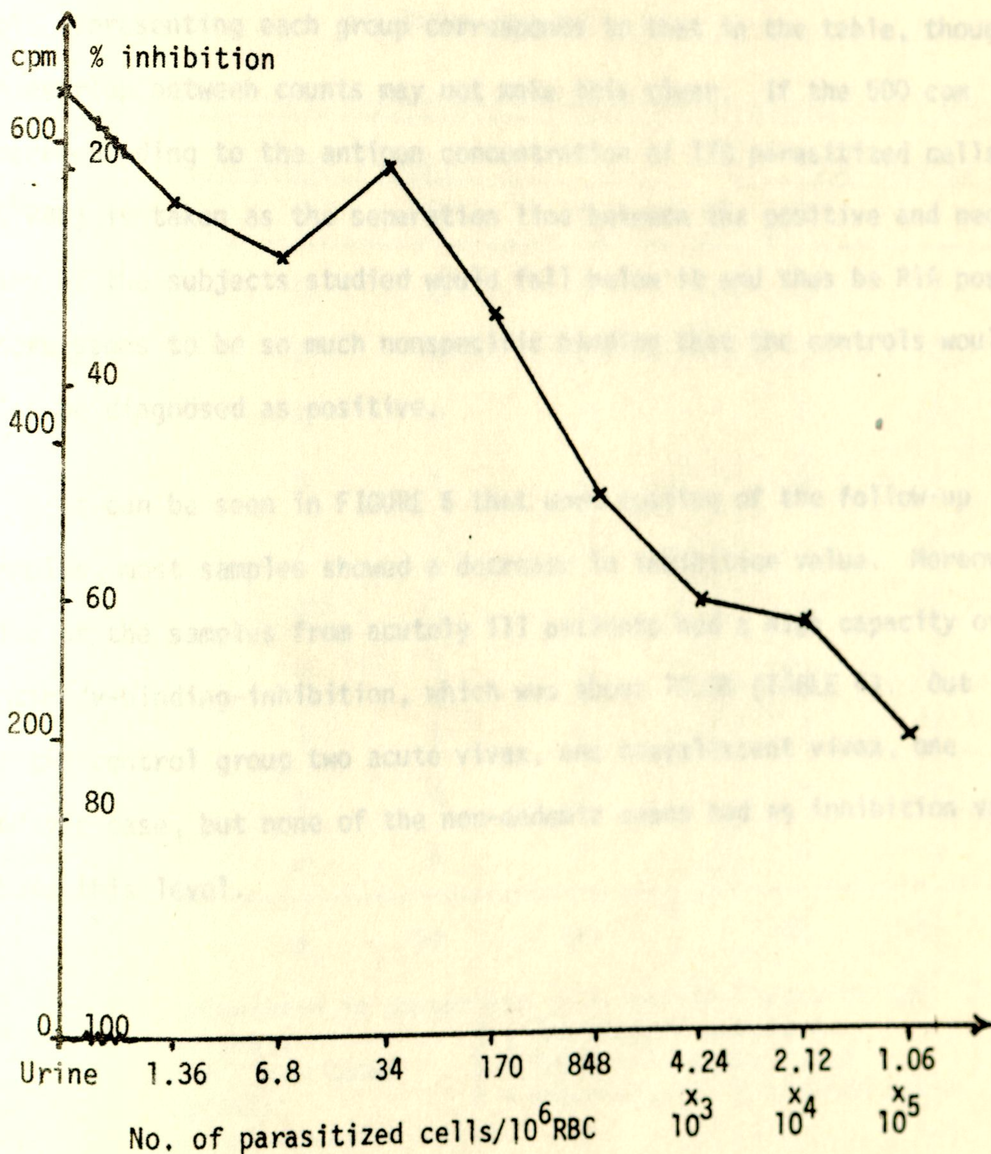


FIGURE 5

RIA Carried Out on Control Antigen Diluted in Urine

After this dilution, there was inconsistency in the pattern of bound gamma-activity suggesting a decrease in the sensitivity of the RIA test.

The results of the RIA of the test samples are shown in TABLE 4 and FIGURE 6. It can be seen that the number of crossing lines in the experimental group (FIGURE 6) is 40 and not 41 (TABLE 4). This discrepancy is due to a missing sample of one acute case. But the number of dots representing each group corresponds to that in the table, though an overlap between counts may not make this clear. If the 500 cpm (corresponding to the antigen concentration of 170 parasitized cells/ 10^6RBC) is taken as the separation line between the positive and negative, many of the subjects studied would fall below it and thus be RIA positive. There seems to be so much nonspecific binding that the controls would also be diagnosed as positive.

It can be seen in FIGURE 6 that upon testing of the follow-up samples, most samples showed a decrease in inhibition value. Moreover, nine of the samples from acutely ill patients had a high capacity of antibody-binding-inhibition, which was above 70.0% (TABLE 4). Out of the control group two acute vivax, one convalescent vivax, one endemic case, but none of the non-endemic cases had an inhibition value above this level.

TABLE 4

Antibody-binding-inhibition
by
121 Urine Samples

Range of % Inhibition*	Control Group				Experimental Group	
	1[a]	2	3	4	5	6
≤ 9.9	0	0	0	0	0	1
10.0 - 19.9	0	0	0	0	0	0
20.0 - 29.9	0	0	0	0	0	3
30.0 - 39.9	1	0	1	0	0	2
40.0 - 49.9	1	1	1	0	6	4
50.0 - 59.9	4	3	4	4	8	20
60.0 - 69.9	3	1	3	6	20	10
70.0 - 79.9	2	1	1	0	8	1
80.0 - 89.9	0	0	0	0	1	0
≥ 90.0	0	0	0	0	0	0
TOTAL	11	6	10	10	43	41

* % inhibition calculated in comparison with cpm obtained with IgG* in BBT

a 1 = Acute vivax cases
2 = Convalescent vivax cases
3 = Endemic subjects
4 = Non-endemic subjects
5 = Acute falciparum cases
6 = Convalescent falciparum cases

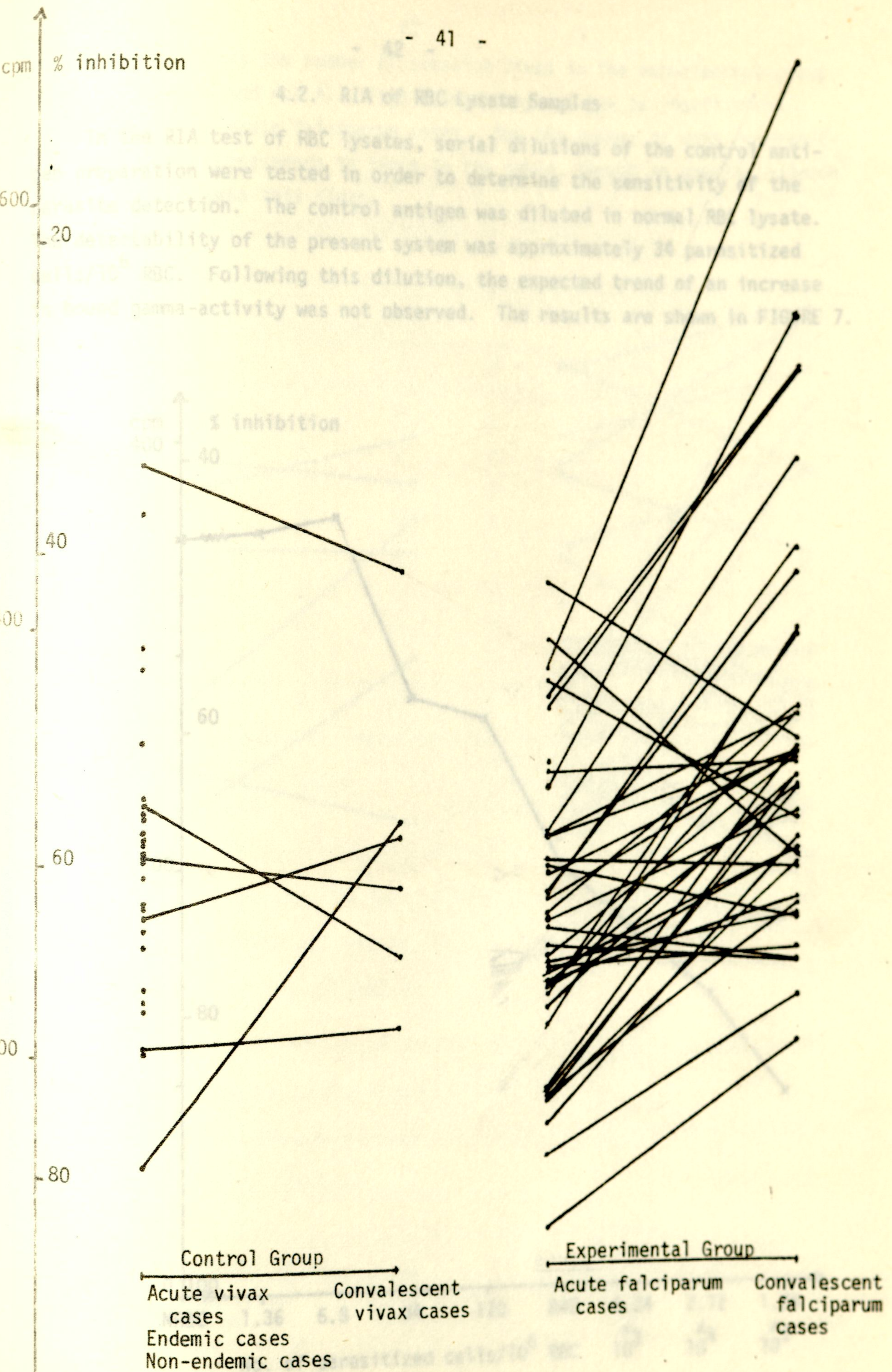


FIGURE 6

4.2. RIA of RBC Lysate Samples

In the RIA test of RBC lysates, serial dilutions of the control antigen preparation were tested in order to determine the sensitivity of the parasite detection. The control antigen was diluted in normal RBC lysate. The detectability of the present system was approximately 34 parasitized cells/10^6 RBC. Following this dilution, the expected trend of an increase in bound gamma-activity was not observed. The results are shown in FIGURE 7.

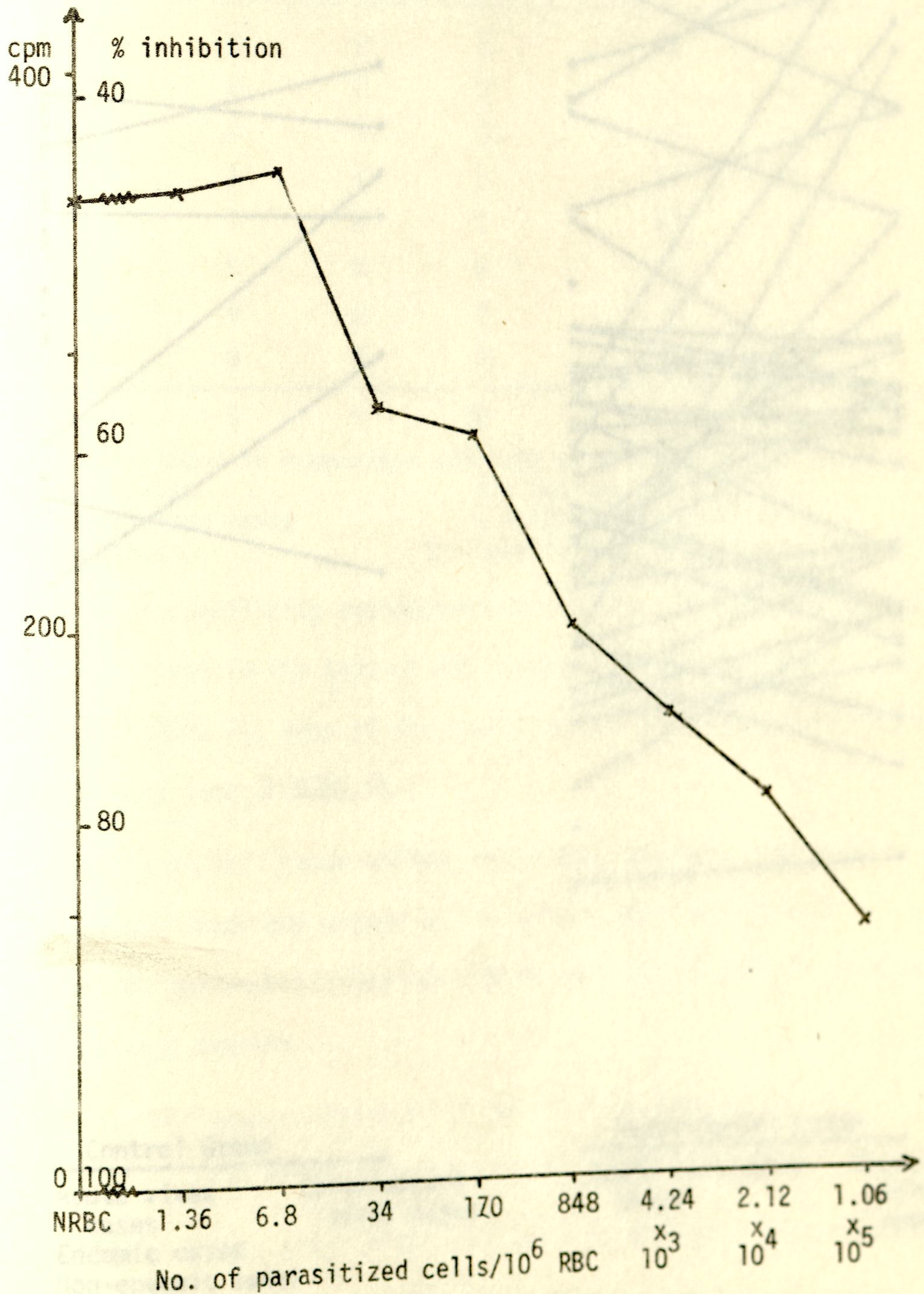


FIGURE 7

It can be seen that the number of crossing lines in the experimental group (FIGURE 8) is 38 and not 41. This discrepancy was due to insufficient samples of three acute falciparum cases. But the number of dots representing each group corresponds to those in the table, though an overlap between counts may not make this clear.

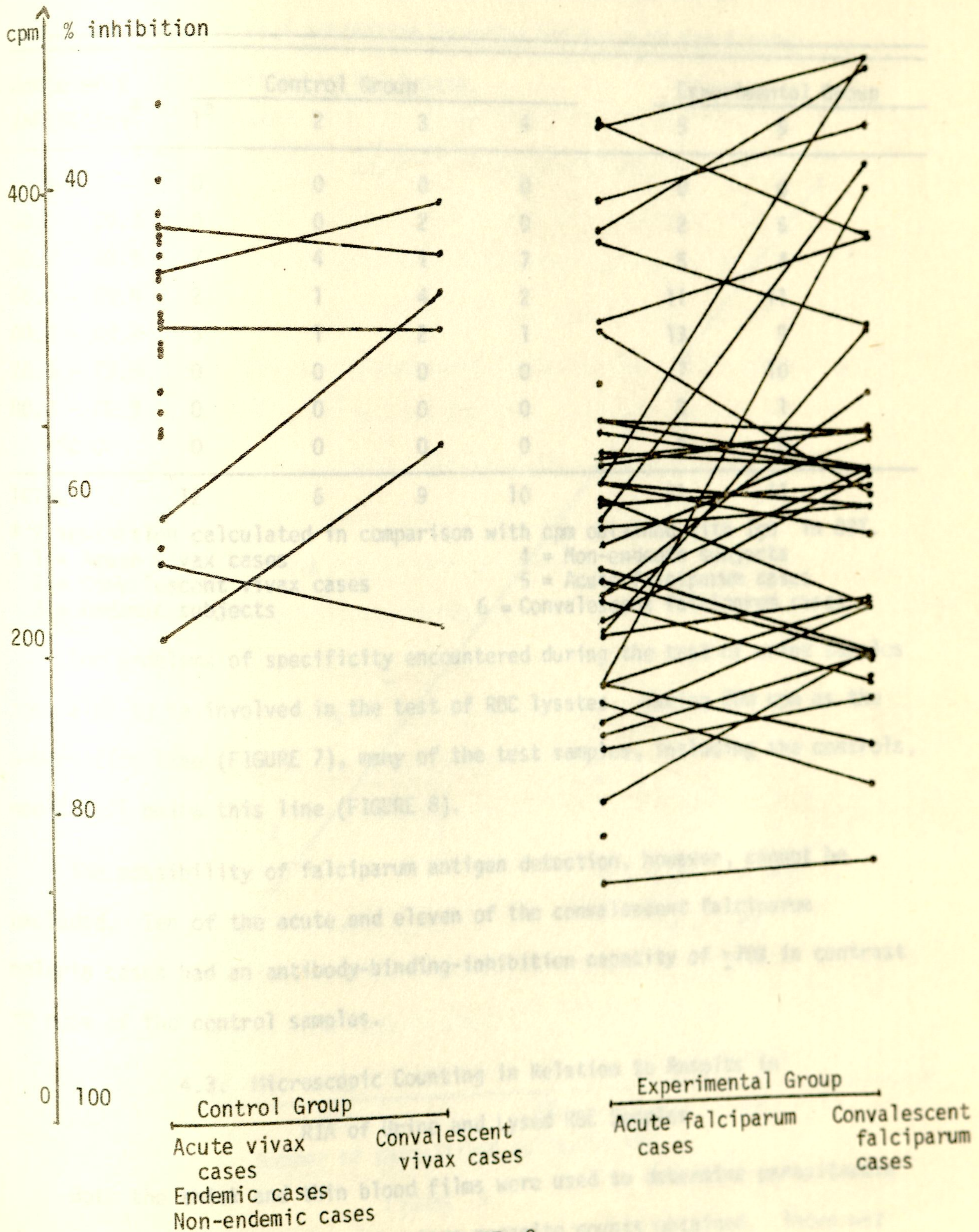


FIGURE 8

Results of RIA Carried Out on RBC Lysates

TABLE 5

Antibody-binding-inhibition by 119 RBC Lysates

Range of % Inhibition*	Control Group 1[a]	2	3	4	Experimental Group 5	6
≤29.9	0	0	0	0	0	0
30.0 - 39.9	0	0	2	0	2	6
40.0 - 49.9	7	4	1	7	5	4
50.0 - 59.9	2	1	4	2	11	11
60.0 - 69.9	3	1	2	1	13	9
70.0 - 79.9	0	0	0	0	7	10
80.0 - 89.9	0	0	0	0	3	1
≥90.0	0	0	0	0	0	0
TOTAL	12	6	9	10	41	41

* % inhibition calculated in comparison with cpm obtained with IgG* in BBT.

a 1 = Acute vivax cases
2 = Convalescent vivax cases
3 = Endemic subjects
4 = Non-endemic subjects
5 = Acute falciparum cases
6 = Convalescent falciparum cases

The problems of specificity encountered during the test of urine samples seem also to be involved in the test of RBC lysates. Taking 280 cpm as the demarcation line (FIGURE 7), many of the test samples, including the controls, would fall below this line (FIGURE 8).

The possibility of falciparum antigen detection, however, cannot be excluded. Ten of the acute and eleven of the convalescent falciparum malaria cases had an antibody-binding-inhibition capacity of ≥70% in contrast to none of the control samples.

4.3. Microscopic Counting in Relation to Results in RIA of Urine and Lysed RBC Samples

Both the thick and thin blood films were used to determine parasitaemia in order to cross-check the percentage parasite counts obtained. There was

a highly significant degree of correlation between the results (FIGURE 9; Spearman's rank correlation coefficient, R = 0.89; P<0.01).

When the values obtained by microscopy were related with those by RIA test of RBC and urine the degree of correlation was low (R = 0.12 for % parasitaemia vs % inhibition by urine and R = 0.08 for % parasitaemia vs % inhibition by RBC lysate.

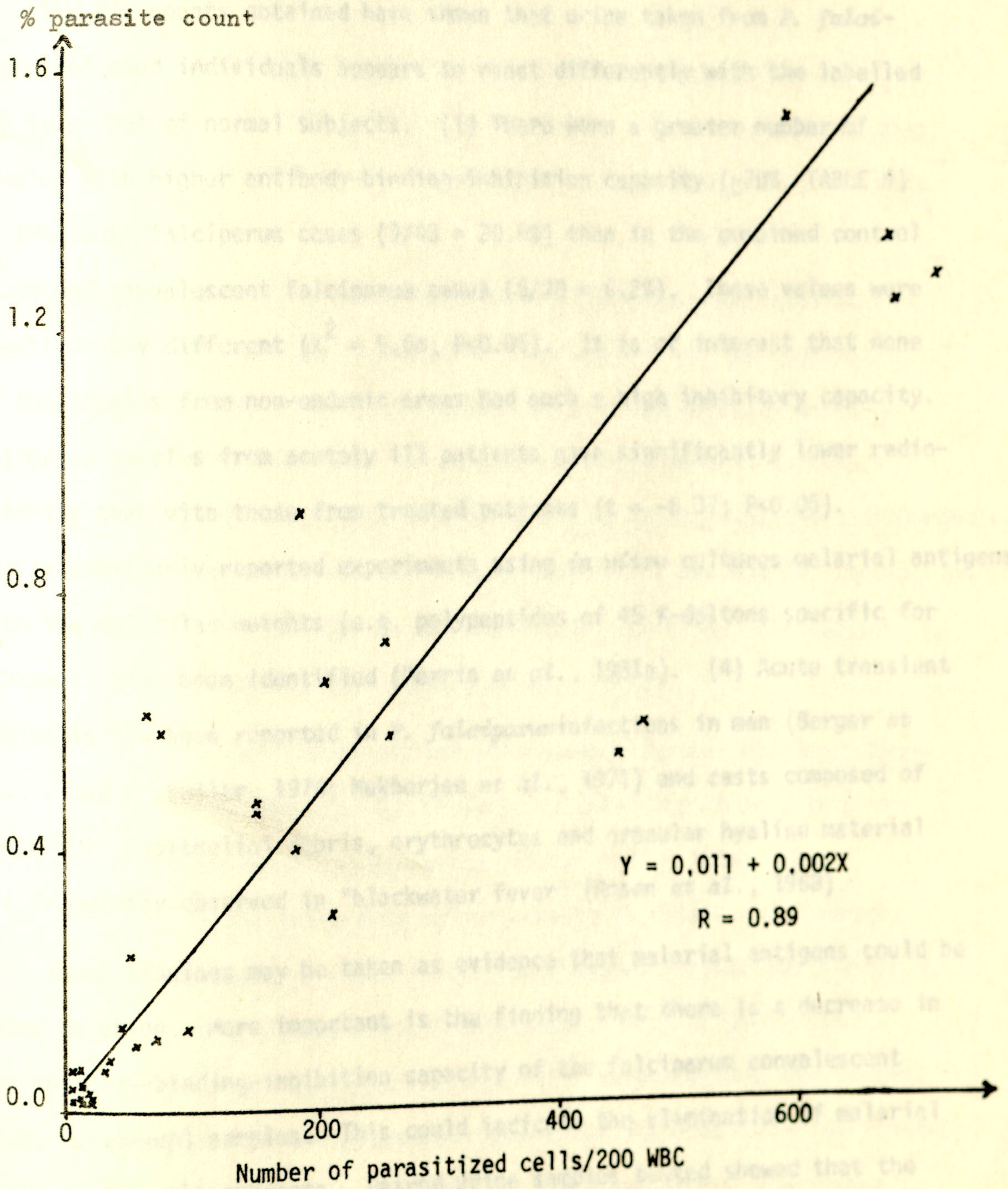


FIGURE 9

Correlation of Percentage Parasite Counts and

CHAPTER 5

D I S C U S S I O N

5.1. RIA in the Detection of Malarial Antigen in Urine

The RIA counts obtained have shown that urine taken from *P. falciparum* infected individuals appears to react differently with the labelled IgG from that of normal subjects. (1) There were a greater number of samples with higher antibody-binding-inhibition capacity ($\geq$70%, TABLE 4) in the acute falciparum cases (9/43 = 20.9%) than in the combined control group and convalescent falciparum cases (5/78 = 6.2%). These values were significantly different ($X^2 = 5.68$; $P<0.05$). It is of interest that none of the samples from non-endemic areas had such a high inhibitory capacity. (2) Urine samples from acutely ill patients gave significantly lower radioactivity than with those from treated patients ($t = -6.37$; $P<0.05$). (3) In previously reported experiments using *in vitro* cultures malarial antigens with low molecular weights (e.g. polypeptides of 45 K-daltons specific for schizont) have been identified (Perrin *et al.*, 1981a). (4) Acute transient nephritis has been reported in *P. falciparum* infections in man (Berger *et al.*, cited by Voller, 1974; Mukherjee *et al.*, 1971) and casts composed of hemoglobin, epithelial debris, erythrocytes and granular hyaline material are frequently observed in "blackwater fever" (Rosen *et al.*, 1968).

These findings may be taken as evidence that malarial antigens could be found in urine. More important is the finding that there is a decrease in the antibody-binding-inhibition capacity of the falciparum convalescent (i.e. follow-up) samples. This could indicate the elimination of malarial antigens from the subjects. Paired urine samples tested showed that the inhibition was significantly different ($P<0.05$) in the acute as compared

to the convalescent samples. According to Sitprija *et al.* (1967), 78% of the patients with malaria have normal renal function, and if this is the case, the detection of malarial antigens may not be attributed to leakage due to renal abnormalities.

High antibody-binding-inhibition results were obtained with urine samples taken from patients with low parasitaemia. This may indicate that malarial antigens present in low concentration may be detected by RIA with high sensitivity.

There was, however, a great range of overlap between the antibody-binding-inhibition capacity of the acute test samples and the control samples. For the occurrence of false-negatives or low antibody-binding-inhibition, the explanation may lie in one or more of several factors: antigenic differences among the Ethiopian isolates of *P. falciparum*, differences between cultured parasites used for the preparation of antigen for the coating of the wells and the parasites in the test blood, the stage of infection, or the nature of the reagents used (Mackey *et al.*, 1980a).

The occurrence of false-positives represents a further problem. Anti-*Mycobacterium leprae* antibody-like activity in urine interferred in a mycobacterial antigen-detecting RIA assay (Olcen *et al.*, 1983). The occurrence of Ig in the urine from normal persons (Berggard and Peterson, 1967) and patients with non-renal infectious diseases (Jensen and Henriksen, 1974) are well known. In the present experiment, three of the control groups which gave an antibody-binding-inhibition above 70% are from endemic areas. This may lead to the assumption that these subjects could have been infected with *P. falciparum* in the past and developed anti-*P. falciparum* antibodies which could be excreted in urine, though interference by falciparum antigens associated with a current inapparent or recent infection is another

possibility.

In *P. falciparum* infections, a mild to severe proteinuria develops between one and two weeks after infection (Voller, 1974) and glomerular Ig (IgG, IgM and IgA) deposits can be shown in renal biopsies (Bhamarapravati *et al.*, 1973). The latter investigators demonstrated that an eluate of immunoglobulin from renal tissue of malaria patients showed a strong antimalarial property. In the present case, however, interference due to antimalarial antibodies did not appear to be a major problem. Had it been so, the number of convalescent samples having a higher capacity of antibody-binding-inhibition would have been greater than the results indicate (TABLE 4). If there were interfering antibodies they could compete with the IgG* for the coated antigens and give lower counts of bound radioactivity. The presence of nonspecifically binding urine components might have been a crucial problem. A malarial infection is well known for its polyclonal B-cell activation (Wigzell, 1982). The presence of such non-specifically binding urine components and non-specific antibodies, including auto-antibodies in the IgG* used, could decrease the efficiency of the present RIA system.

It is hoped that production of monoclonal antibodies will disclose the complexity of such a study system. The present findings seem to demonstrate that the principle of antigen detection in urine for the immunodiagnosis of malarial infection is practicable. Thus, the detection of malarial antigens in urine for diagnosing an active infection and for determining the effect of therapeutic drugs is very promising. If the immunoassay methods for the detection of malarial antigens could be improved to achieve a higher degree of sensitivity and specificity, they would be

extremely advantageous for large malarial field surveys in epidemiological studies and malaria control programmes.

5.2. RIA in the Detection of Intraerythrocytic Antigens

This study supports the findings of other investigators (Mackey *et al.*, 1980a, Avraham *et al.*, 1983) in that RIA may be used to detecte falciparum malaria antigens in infected human blood. Samples of a high antibody-binding-inhibition capacity were found amongst those from falciparum malaria patients. The bound gamma-activity was significantly higher in the convalescent group than in the acute falciparum group ($t = -1.97$; $P<0.05$). In the vivax cases, no significant difference was found. This may indicate a decrease in the amount of antigen present in convalescent falciparum RBC.

When all the results of the RIA are considered, however, in addition to the low level of detectability in differentiating the malarious from the non-malarious subjects, the number of samples falling in the range of antibody-binding-inhibition above that of the control group is the same in the acute samples and convalescent samples. Besides this, in contrast to what was expected, many samples showed a decrease in the amount of radioactivity bound, for which there are a number of possible explanations.

One explanation to this problem may be related to the immune status of the subject. Antibodies present in plasma may form complexes in the acute samples and thus decrease the inhibition value or compete with the IgG* and increase the relative inhibition value in the convalescent samples. Mackey *et al.* (1980b) reported that in their murine malaria model, whole RBC incubated with hyperimmune serum had a good capacity of antibody-binding, though it was not as efficient as those samples not treated with hyperimmune serum. This needs further study if it is applicable to the

human malaria situation. When adapting the method for the detection of *P. falciparum* infections, Mackey *et al.* (1980a) used washed RBC. In the present study, unwashed RBC were used and therefore the presence of antibodies could interfere with the RIA results. Another clue which favours this assumption of an interference due to antibodies, was given by patient records. Many of those samples with high antibody-binding-inhibition capacity were collected from Silie and Shelie. Many of the patients were new labourers in the agricultural development project, and many were highlanders coming from non-malarious areas. It could have been their first exposure to malarial infection and thus antibodies might have not developed when the first sampling took place.

Another explanation relates this problem to the effect of treatment. One mechanism, (reviewed by Wyler, 1983),by which chloroquine causes the death of the parasite, is that chloroquine-ferriprotoporphyrin IX complex (FP IX is a product of the degradation of haemoglobin by the parasites) appears to be toxic to the parasite. To the knowledge of the present investigator nothing is known about the longevity of the RBC with dead parasite material in the cells. If these RBC remain in circulation over a long period, lysed RBC examined after two weeks for the presence of antigens would have a high capacity of antibody-binding-inhibition. In this case, antigen detection in blood may be used for diagnosing a current or recent past infection, but not for following up the effect of treatment after just two weeks.

Alternatively, if the RBC haemolyse immediately after treatment or excrete the parasite antigens, it is possible that residual antigen present in the plasma may attach to the membranes of non-infected RBC and that these react in the RIA system. The rate at which antigen ceases to be

detectable in plasma was found to vary (McGregor *et al.*, 1968). Circulating immune complexes occur in the blood of malarious patients and can be detected up to two weeks after treatment (Perrin *et al.*, 1979).

Another important and critical factor is the purity of the reagents used. It has been mentioned above that the malarial antigens associated with the erythrocytic stages include integral components of the various stages of the parasite itself, parasite antigens expressed on the membrane of infected RBC, and host antigens expressed because of an invasion by the malarial parasite. The antibody preparation used in the test must be capable of reacting with all the blood forms, to be of a high degree of sensitivity. Yet, to be of high specificity it must be free of nonspecifically binding antibodies. This would not be easy to achieve if the antibody preparation is made from the sera of immune persons, when it is known that malaria is characterized by hypergammaglobulinaemia which includes auto-antibodies like anti-RBC antibodies. In the presently used RIA system, the NRBC used caused 46.8% of nonspecific inhibition. To make matters more complicated, the RBC of the patients and controls may react differently and have more non-specifically binding antigens than the negative control antigens used.

The possibility of cross-reactivity with other infections or antigens present in the blood cannot be completely excluded. Mackey *et al.* (1980a) reported that no false positive results were found by RIA among the samples from Geneva blood donors. When testing blood samples collected from Gambia, however, 234 (58.8%) were RIA positive out of 398 samples considered to be negative by microscopy. Again, Avraham *et al.* (1983) reported that no false-positive results occurred in a study involving more than 90 healthy

persons in Israel. Yet, they are of the opinion that the question of false-positives remains to be proved. The possible existence of any other antigens cross-reacting with malaria requires investigation.

5.3. RIA in the Detection of Serum Antigens

Previous investigators have studied soluble malarial antigens with an analytical approach and found that they are of a wide diversity (McGregor *et al.*, 1968; Wilson *et al.*, 1969; McGregor and Wilson, 1971). One of the aims of this study was to examine malarial antigens in serum with the hope of probing their application in immunodiagnosis of malaria. The problem was that antibodies are present in the serum of most people living in malarious areas. In non-immune patients, specific antimalarial antibodies could be detected 5-7 days after starting treatment in the case of a first infection (Perrin *et al.*, 1979). The antibodies would compete with the IgG* for the antigens. This would mean antigen-binding-inhibition instead of antibody-binding-inhibition giving false-positives.

Preliminary tests carried out during this study appeared to support this. The RIA method used for urine and lysed RBC samples was thus not found to be suitable for measurement of malarial antigens in serum.

TABLE 6

Preliminary RIA Assay of Serum Samples

Type of Serum Sample	Average cpm		
	1:10	1:100	1:1000
Normal human serum	486	570	680
Endemic control (64)	466	556	654
Non-endemic " (22)	500	568	650
Acute falciparum -			
Case 1 (54)	340	460	651
Case 2 (74)	198	414	566
Convalescent " -		326	574

5.4. Cross-reactivity of the Ethiopian *P. falciparum* strains with the Zairian Strain

Although *P. falciparum* appears to be a single species, considerable geographic antigenic variations occur (Collins *et al.*, 1970; Hommel *et al.*; 1982; McBride *et al.*, 1982). The distribution of different antigenic types, however, did not appear to be related to geographic origin (McBride *et al.*, 1982). Raffaele and Lega (1937) had suggested establishing an *aethiopicum* variety of *P. falciparum*, based on their observations of certain differential characteristics of the parasite. It is important that the present findings appear to suggest sufficient cross-reactivity between the Ethiopian strains and the Zairian strain. In a study made by McGregor *et al.* (1963) to assess the serological identity of the *P. falciparum* parasite in two different parts of Africa, he used the therapeutic effect of West African gammaglobulin in East African patients, and demonstrated the existence of antigenic similarity between the plasmodia of the two regions.

The occurrence of a wide range of cross-reactivity between the plasmodia in different localities is encouraging for the development of standardized immunodiagnostic methods based on antigenic detection.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1. Theoretical Considerations

6.1.1. Research Activities on Plasmodia

This study was designed to investigate the immunodiagnosis of *P. falciparum* infection, by means of antigen detection.

The fatality of *P. falciparum* infection prompts investigators to direct their greatest attention to this parasite. It would also be desirable to investigate the other species; *P. vivax*, *P. malariae* and *P. ovale*. If it were possible to isolate cross-reacting antigens, these would facilitate the work of setting up improved immunodiagnostic assays with the use of, for example, monoclonal antibodies.

6.1.2. Studies on Urine from Malaria Patients

The results obtained suggest that detection of malarial antigens in urine appears to be of promise for diagnosing a falciparum infection and for follow up of the therapeutic effect of drugs. Some studies which would make the present understanding more complete can be proposed:

- Antigenic analysis of malaria antigens in urine
- Detection of malaria antigens in relation to the stage of infection
- Identification of urine components which may be responsible for nonspecific binding.

6.1.3. Studies on Malarial Intraerythrocytic Antigens

The present findings have shown that intraerythrocytic malarial antigens

may be detected before treatment and for two weeks following treatment. The origin of the antigens after treatment is not known. Further investigations that are needed include:

. The fate and longevity of RBC with dead parasites and intact antigens
. The affinity of binding between soluble malarial antigens and the surface membrane of RBC

6.1.4. Studies on Malarial Serum Antigens

Previous investigators have shown that soluble malarial antigens are of a wide diversity. Further understanding in this field is required and includes:

Identification of soluble antigens which are of low immunogenicity and are common to plasmodia in various malarious regions.

6.2. Technical Aspects

6.2.1. Application of RIA in the Diagnosis of Malarial Infections

Development and use of antimalarial monoclonal antibodies would solve the problems of sensitivity and specificity experienced in this study. If defined reagents are available, the technique is simple, rapid, reproducible and free of subjectivity.

. Parasite-host interactions in malaria are so dynamic and complex that they are characterized by an antigenic variation. If application of monoclonal antibodies is tried, monoclonal antibodies to antigens of all differentiation stages of the parasites will be necessary for immuno-

diagnosis. By use of balanced mixtures of different monoclonal antibodies, the inherent danger of loss of sensitivity, when using only one antigen, might be overcome.

. If RIA is to be developed as an immunodiagnostic technique, it is with the view that samples collected will be examined in well-equipped central laboratories. It is not feasible under field conditions. Other disadvantages to the wide application of RIA include the hazard of using radioactive reagents which also are costly and of short life. For these reasons, it has been proposed that ELISA is preferrable to RIA in developing countries.

6.2.2. Preparation of RBC

In this study, RBC from whole capillary blood were used unwashed. Moreover, though the cellular components were sedimented, the layer of serum was not cut off before storage.

The effect of washing the infected RBC in human malaria before the antibody-binding-inhibition test requires investigation.

6.2.3. Preparation of Sera

The assay used presently detects both antigens and antibodies. For antigen detection only material that does not contain antibodies can be used. If the presence of antibodies cannot be avoided, they must be in low concentration so that their effect in the assay is minimal.

Methods that are suitable for measuring soluble malarial

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DECLARATION

I, the undersigned, declare that this thesis is my work and that all sources of material used for the thesis have been duly acknowledged.

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