ACOMPARATIVE STUDY BETWEEN MICROSCOPY AND AN IMMUNOCHROMATOGRAPHIC TEST (ICT) FOR THE DIAGNOSIS OF MALARIA IN KHARTOUM

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Dedication

To my parents ...

For their continuous care and encouragement, hoping that I have succeeded to achieve some of their great wishes for me.

To my Brothers, sister & husband ..

For their tender love and assistance.

To my sons...

&

to all which I love
ABSTRACT

This study was conducted in Elkalakla area (south Khartoum) from February to July, 2001, to assess the performance and the comparative sensitivity and specificity of the ICT in the laboratory diagnosis of malaria.

There were ninety-two patients, suspected of having malaria, enrolled in this study. Finger blood samples were tested with ICT dipstick and with microscopical examination of blood films, the comparative sensitivity and specificity of the ICT were calculated using the results of blood film examination as the gold standard, the intensity of parasitaemia was estimated by using W.H.O. crosses system.

Microscopical examination of Giemsa stained blood films revealed that 54 (58.7%) patients had the malaria parasite: Plasmodium falciparum in their peripheral blood while, the ICT revealed that there were 65(70.7%) who showed malaria-parasite antigen in their peripheral blood. Eleven patients who were negative by blood films examination were positive by the ICT; they were considered as false positives for the ICT and the persistent antigenaemia maybe a more probable cause of those false positive samples. There was no evidence of any positive-blood film that reacted negative in the ICT.

Comparative results in this study showed that the ICT was 100% sensitive and 77.6 % specific when compared with microscopic examination of Giemsa-stained blood films for the diagnosis of malaria. So, it is a valuable diagnostic tool for malaria specially in emergency and field situations which require rapid diagnosis and treatment.
The present study also compared specific data of the patients such as sex, age, fever symptom and treatment with the rate of positive of malaria infection by both techniques: microscopic examination of Giemsa-stained blood films and ICT. Results showed that there was no significant differences in the sex ratio in all samples. While the effect of age as a factor for acquisition of immunity against infection was confirmed by the results that revealed that the parasite positivity rate and density of infection decreased significantly with increase age.

Results of fever revealed that there was a significant correlation between fever and the malaria infection rate, particularly with high level of parasitaemia. While there was strong association between pre-treated patients and the malaria infection rate in both positive blood films and positive ICT.
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CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction:-

Malaria, the most important parasitic disease of man, remains a major cause of morbidity, and economic burden in the world specially in the tropics. Also, it causes a lot of suffering and premature death in children. It is a disease that continues to be one of the most significant health problems, it is endemic in 91 countries with about 40% of the world's population being at risk (Sandra, 2001). Each year there are 300-500 million clinical cases of malaria (90% of them in Africa), resulting in 1.5-2.7 million deaths, most of them are children under 5 years of age (WHO, 1998).

Malaria affects young and old but children and pregnant women are particularly at high risk and it is one of their major killers in tropical Africa (Bruce-Chwatt, 1996). The mortality of children is very high: about 1 in 20 children die from malaria and 1 in 5 die from malaria related diseases i.e. anemia (Tiemoko, et al., 2000). It is considered as one of the most wide-spread transmissible diseases in the world, but it is spread only when a combination of several factors is available including: source of infection, vectors of disease, susceptible population, and favorable natural-climatic conditions.

It is important to recognize that countries that are at present free from malaria may not be able to maintain their malaria free status, since imported malaria may occur with relative speed of modern travel and migration (Rathor, 1996).
At present, in malaria risk areas Sudan is classified as low to moderate, West Africa as moderate to high and New Guinea, Kenya, Nigeria and India as high risk of malaria (Snounou, 2001).

In Sudan, malaria is endemic all over the country with various levels of endemicity from hypo- to holo-level. It accounts for 40% of all infectious diseases, with *Plasmodium falciparum* responsible for 90% of all cases in most parts of the Sudan. In Khartoum, malaria accounts for 14.3% of all out-patients attending health facilities and it is the first cause of hospital admission representing about 30.9% and the constitutes the commonest cause of death estimated to be about 20.3% (National malaria administration, 1998).

The world malaria situation is aggravated due to many factors including the following: climatic changes, population movements, the declining efficiency of affordable drugs such as chloroquine because of the resistance of the parasite to it and the failure of vector control measures (Sharma, et al., 1999).

However many countries have tried to develop realistic malaria control programs adapted to their local needs and their available resources. Inspite of that, malaria still remains a major public health problem and is becoming increasingly difficult to control in these countries. Therefore, the World Health Organization (WHO) established, 1992, a global malaria control strategy with guidelines for its implementation during 1993-2000, its basic elements include: providing early diagnosis and prompt treatment, strengthening local capacities in basic and applied research to permit and prompt the regular assessment of the country's malaria situation, particularly the ecological, social and economic determinants of the disease.
As the WHO recommended, it is necessary to emphasize that the district laboratory investigations will have an important role in both malaria treatment and control aspects. Therefore, accurate and timely laboratory information has become the foundation upon which current disease treatment prevention and control are based. It is playing a critical role in addressing the challenge of emerging infectious disease (Rathor, et al., 1996). Also, the development of rapid and specific diagnostic tests to identify individuals infected with malaria is of immense importance in the detection and the control of the severe public health impact of this disease.

Conventional light microscopy examination of blood films is the mainstay of malaria diagnosis but, it becomes very time consuming in relation to high clinical cases and the detection threshold that is highly dependent on experienced personnel. Despite those problems, the Ross' blood smears examination can be considered the most conclusive method in the diagnosis of malaria. To obviate such problems, several other techniques have been tested and a rapid diagnostic tests based on parasite's antigens capture i.e. immunochromatographic tests (ICT) have introduced a new dimension to the diagnosis and treatment of malaria.

1.1 History and geographical distribution of malaria:-

Malaria is a parasitic disease that been known to humanity from the dawn of civilization, references to epidemic fever similar to malaria symptoms can be found in the ancient Chinese and Egyptian manuscripts and also in the literacy sources of ancient Greece and Rome. Owing to its clinical presentation in Hippocratic work (460-377BC) the Italian Lancisi, linked
malaria with poisonous vapors of swamps (mal-aria), bad spoilt air from which the name of the disease (mal-aria) took its origin.

In 1897, Ronald Ross, an British physician, made the important discovery that mosquitoes serve as vectors of avian malaria. The zone of the prevalence of the vector-mosquitoes of the genus Anopheles and the suitable ambient temperature limit the geographical map of malaria occurrence, ensuring the completion of sporogony cycle in it, (Loban, 1989).

Indigenous malaria has been recorded as far as 64°N and as far south as 32°S latitudes. It has occurred in Dead Sea area at 400m below sea level and in Londiani (Kenya) at 2600m above sea level. But within this limitation, there are some areas free of malaria since its transmission depends on local environmental conditions.

Africa is considered to be the most plausible place of malaria origin (Bruce-Chwatt, 1993), it seems to be that man first acquired the malaria parasites from monkeys, following the parasites adaptation to human body malaria spread to the larger part of the globe.

1-2 Biology of malaria parasite:

Malaria parasites are unicellular protozoa which belong to the family Plasmodiidae within the order Coccidiidae, sub-order Haemosporidida which comprises various parasites found in the blood of birds and mammals and man may host four types of them, namely *Plasmodium malariae*, Laveran (1881) *Plasmodiumfalciparum*, Welch (1881), *Plasmodium vivax*, Grassi and Feletti (1890) and *Plasmodium ovale*, Stephens (1922). They are differentiated by their morphological signs, virulence, the incubation period, immunological and epidemiological characters and sensitivity to the impact
of chemotherapeutic drugs. Some authors subdivided the genus Plasmodium into subgenus Laverania i.e. *Plasmodium laverania falcipara*.

**1-3 Life-cycle of human Plasmodia:**

The life cycle of all species of human malaria parasites is essentially the same, the parasites undergo a complex cycle of development with a change of hosts. In female Anopheles mosquitoes occurs the sexual phase (sporogony) while in the vertebrate hosts (man) an asexual phase takes place (schizogony). The sexual multiplication in the Anopheline mosquito (sporogony) starts when the female ingests a blood meal of a human host which contains malaria parasites, all asexual stages are digested while mature sexual stages (gametocytes) undergo further development. Male and female gametocytes develop into gametes, which fuse to form zygote (motionless gloplar body), which elongate, develop and become mobile ookinetes. The ookinete soon forces its way to the stomach outer wall, becomes rounded to form an oocyst which increases in size (forming a sporoblast and sporocyst) and the nucleus divides repeatedly to form elongated sporozoites. These burst the walls of the oocyst and invade the body cavity until they reach the salivary glands of the female Anopheles which now becomes infective.

Following incubation of the sporozoites, during the next blood meal into the circulating blood, they leave the blood stream and enter liver cells within one hour. They develop into liver schizonts and are referred to as pre-erythrocytic schizonts (P.E-), which divide and contain many merozoites. When mature pre-erythrocytic schizonts rupture, thousands of merozoites are released into blood.
**Hyponozoites:** some of the sporozoites of *P. vivax* and *P. ovale* invading liver cells delay their development into pre-erythrocytic schizonts. They become dormant forms called hyponozoites which become active and develop into pre-erythrocytic schizonts only after some months, causing relapses. The liver merozoites infect red blood cells (binding to receptors on the red cells membrane). Entry of the parasites into cells starts a cycle of schizogony in the blood which to complete takes 48 hours for *P. falciparum* *P. vivax* *P. ovale* and 72 hours for *P. malariae*. The youngest stages in the red blood cells are known as ring forms that appear ring-shaped in stained blood films because the ring of cytoplasm stains but not the central food vacule (Wemisdrofer, 1988).

During the schizogony cycle in the blood cells, the intracellular ring forms develop into trophozoites which undergo an asexual dividing process to form schizonts containing 8-32 merozoites (depending on species). Some of these merozoites which are not destroyed by the host's immune system infect new red cells, being a further cycle of schizogony with more red cells being destroyed. Some of the merozoites entering red cells develop into male and female gametocytes, which must be taken by a female Anopheles mosquito in a blood meal for the life cycle to be continued, (Annex 1).

**1-4 Clinical features and pathology of malaria:-**

Pathology processes in malaria are thought to be the result of interactions between the parasite and the human host, particularly erythrocytic cycle. The principal symptoms are fever, headache, chills and sweets but it can present as respiratory or gastrointestinal illness.
Fever is caused by the release of toxins, when erythrocytic schizonts rupture, which stimulate the secretion of cytokines from leukocytes. Also repeated erythrocytic cycles lead to consumption of the red blood cells and depression of erythropoiesis (production of new RBCs) by the effect of toxins causing anemia. Splenomegalcy and jaundice are common features of malaria, particularly falciparum malaria.

-Sequestration (cytoadherence) of P. falciparum:

Parasitized red blood cells with P. falciparum trophozoites contain antigens on their surface which adhere to receptors on endothelial cells in small capillaries in various organs such as kidneys and brain. This leads to dysfunction of such organs with tissue damage causing renal failure and cerebral malaria (Annex 2).

Generally, the degree of illness depends on the parasite species and stages, on the intensity of infection, on the extent of the patients' immunity to malaria and on the impact of antimalarial drugs on the Plasmodium species.

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**Sequestration (cytoadherence) of *P. falciparum***:

Parasitized red blood cells with *P. falciparum* trophozoites contain antigens on their surface which adhere to receptors on endothelial cells lining capillaries in various organs such as kidneys and brain. This leads to dysfunction of such organs with tissue damage causing renal failure and cerebral malaria (Annex 2).

Generally, the degree of illness depends on the parasite species and stages, on the intensity of infection, on the extent of the patients' immunity to malaria and on the impact of antimalarial drugs on the *Plasmodium* species.

### 1.5 Parasitological diagnosis of malaria:

It constitutes one of the four technical elements of the global malaria control strategy by the WHO in 1992. It can be confirmed either directly by the detection of malaria parasites in the blood, even if there is a few number of them, or indirectly by the detection of the parasite antigen, parasite DNA, or antibodies against malaria parasites (Annex 5). The presence of the parasite depends upon their species, their life-cycle periods (prepatency and incubation periods) and prophylaxis or other drug administration, which may reduce parasitaemia to below the detectable threshold examination by inadequately trained technician. Therefore, repeated and examination of serial blood samples is very important (WHO, 1998).

There is another indirect method with relative diagnostic importance, which help in the malaria manifestation and is considered as indicative of the prognosis of the disease, i.e.
1. The presence of malaria pigment in neutrophils and monocytes that reflect the pathogenic burden.
2. The presence of mature trophozoites, malaria pigment and schizonts in the peripheral blood specially in *P. falciparum*.
3. Measurement of haemoglobin or packed cell volume particularly in young children and pregnant women since anaemia is considered to be a major sign of severe malaria.
5. Total white blood cells count (leukocytosis).

1-6 The role of laboratory diagnosis in malaria treatment and control aspects:

Since malaria may be confused with other fevers and other diseases with symptoms similar to malaria infection, such as influenza, typhus, paratyphoid and visceral leishmaniasis, the diagnosis in the laboratory is always a matter of clinical judgement and care (WHO, 1991). Late or misdiagnosis of malaria will have severe impact on both health and economy since it increases morbidity and mortality in the population.

The role of laboratory diagnosis in malaria treatment involves monitoring of the efficacy of the drugs, checking for the clearance of the parasites in follow-up studies, early detection of recrudescence, which is caused by drug-resistant parasites and early treatment to prevent complications and further transmission. Private sector treatment and self-treatment are common in Sudan, thus presumptive treatment is inappropriate, costly and is associated with side effects - and ultimately leads to the development and spread of drug-resistance (Gabir, 1997).
In the control aspect, WHO emphasizes that early diagnosis is a major goal and technical element of the global control strategy because all control programmes are limited by an increased prevalence of drug resistant strains, which can be detected easily with laboratory confirmation and treated with adequate drugs.

Since there is a lack of infrastructure for the disease detection, the WHO, in 1992 recommended that collaborating laboratories form local and regional partnerships. Control and reference laboratories are very crucial as they provide support in many forms such as technology transfer, availability of critical reagents and supplies, and ongoing quality control /quality assurance activities (WHO, 1998).

1-7 **Treatment of malaria:**

The different stages of the life-cycle of malaria parasites like the different species themselves, display varying susceptibilities to chemotherapy so antimalaria drugs may be classified according to the stage of life cycle upon which they act (Bruce-Chwatt, 1996).

The successful chemotherapy depends upon exploitation of differences between host and parasite metabolism. Therefore, there is no known drugs that kill the sporozoites before they enter the liver cells because they are in a resting metabolic state.

In all efforts and measures to prevent and control malaria it is emphasized that early diagnosis and treatment are essential.

In malaria treatment, drugs must be used either to:

1) Treat active infections, particularly, in young children and suppresses infections until they die out.
2) Prevent infections specially in non-immune persons such as businessmen and travelers who frequently visit malarious areas or in persons with reduced immunity such as pregnant women.

Erythrocytic infection can be prevented by drugs which act upon the parasite in the liver (Pre-erythrocytic schizogony). Also such compounds are known as causal prophylactics also they can prevent any further or new infection. So they are referred to as preventative drugs and have been used in immune persons. Some of antimalarial drugs have been used according to their impact on certain stage of the parasite life-cycle (Annex 4).

Recently, effective, affordable and safe treatment of malaria, particularly falciparum malaria, is becoming increasingly difficult as resistance to commonly available quinine and antifol drugs continues to spread throughout tropic.

The problem of resistance of *P.falciparum*, stimulated huge efforts for synthesizing and testing new antimalarial drugs. More than 250,000 compounds were tested in the antimalarial drug development program of the United States Army research and development command. Among these, three drugs (fansidar, mefloquine and halofantrine) are now in use for the treatment of drug resistant falciparum malaria (Boore et al., 1998). Recently artimether was introduced and was found to be an effective drug against *Plasmodium falciparum*.

1-8 Control of malaria disease:-

In areas where many people are at a risk of malaria, it is important to reduce the burden on the family and community by treating those who are ill.
and preventing mosquitoes from biting so the goal is not eradication, but control the cases.

The problem of malaria is getting worse because of the following factors: the old quinine based drugs do not work any more in many places because of drug resistance by the parasite, the more lethal strain of malaria is increasingly prevalent. Earlier malaria control efforts have decayed economic conditions such as increase migration and wars have made malaria control more difficult.

There are certain measures used to prevent and control malaria including:

1) Avoiding mosquito bites.
2) Using drug to treat active infections or prevent infection.
3) Preventing the breeding of mosquitoes.
4) Destroying adult mosquitoes by regular effective spraying with suitable insecticides. Mosquitoes can rapidly develop resistance to a wide range of insecticides used on a large scale therefore, their continuous use to which the mosquitoes have become significantly resistant may reduced the effectiveness of the control programs (Rathor et al., 1996).

With the worsening malaria situation in many parts of the world, the WHO established in 1992 a global strategy which consists of basic elements such as: early diagnosis and adequate treatment; early detection of epidemics; and the regular assessment of a country's malaria situation, specially the ecological, social and economic factors of the disease.
Recently, Roll Back Malaria (RBM) is a global partnership including: WHO, UNICEF, UNDP and the World Bank, which aims to half the global malaria mortality burden by the year 2010 through novel approaches.

1-2 Literature review of diagnostic methods of malaria :-

The diagnosis of Plasmodium species as a blood parasite, since early years has been based upon the demonstration of the parasite in blood smears. Laveran (1880) mentioned that the disease of malaria which is caused by parasites, which developed in the erythrocytes, was diagnosable by the examination of fresh unstained smears of blood, they only show the vague outline of the living parasites.

Then, attempts to color blood smears were made firstly with haematoxyline and methylene blue stains. They were most satisfactory when using fresh and aqueous solution. They color the cytoplasm of the parasite light blue and the chromatin dot red, (Loban, 1989). Romanowsky stains were the early ones made up as an aqueous and alcoholic type, such as Field and Giemsa stains.

Traditional malaria diagnosis has relied on microscopic examination of thick and thin stained blood films for detecting malaria parasites (Bruce-Chwatt, 1993). A thick blood film is one of the numerous concentrated techniques, which improves the efficiency in the diagnosis of malaria. It is concentrated by a factor of 20-30 layers of red blood cells in a small area and it is the best for routine clinical use. The presence of malaria parasites in the thick blood film may be detected when densities are of the order of 10 to 20 parasites pu of blood, so it is a sensitive method.
Cytocentrifugation which uses apparatus for concentrating cells in suspension onto a microscopic slide, haemolysis with distilled water and with saponin were different techniques that allowed us to improve the microscopic examination since they were introduced in 1963 (Loban, 1989).

The only elements that are seen in a thick film are leukocytes, blood platelets and the parasites, the later appearance is sometimes altered because of dehaemoglobinization subsequent to the staining of an unfixed film. also, pigment granules, which are produced from the digestion of haemoglobin by the parasite, and dots or stippling as Maurer's dots of Plasmodium falciparum are being seen in the thick film.

In the thick film, parasites are easily detected but, they may be more difficult to identify by species since the red blood are not visible as a result of haemolysis, so the species should be confirmed by the examination of a thin film.

Examination of the thin films is required to confirm the Plasmodium species; to detect the altered features in parasitized red blood cells; to assist in the identification of mixed infection; to monitor the response to treatment by counting the percentage of parasitized red blood cells before and after treatment; and to give the opportunity to investigate anaemia and white cells abnormalities (Monica, 1998).

Microscopic diagnosis requires a degree of technical precision in the preparation of the blood slides, its handling and staining, in the optical quality of the microscope and the illumination, as well as trained personnel requirements which are not often found in endemic areas as Sudan (Gabir, 1997). It is necessary to emphasize that the interpretation of the
parasites seen in a blood film requires some experience and care should be taken, specially in the thick film, not to confuse artifacts, blood platelets or nuclear fragments with malaria parasites that resemble them in size, shape and fluorescence (Alonso, etaL, 1999). Also, such experience can be acquired by studying first the morphology of parasites in a thin film and then searching for corresponding forms in a thick film.

It advisable to determine the density of parasitaemia, particularly in *P. falciparum* to assess the level of the risk and the response of the parasite to treatment. It has been indicated either by counting the mean number of parasitized red blood cells in relation to number of red blood cells in a thin blood film. Or counting the number of parasites in relation to the leukocyte count by using the simple formula:

\[
\text{No. of parasites/ul blood} = \frac{\text{No. of parasites counted} \times \text{No.of leukocytes/ul blood}}{\text{No.of leukocytes counted}}
\]

The later method is more precise and will give the number of parasites per ul of blood, these being counted in relation to a predetermined number of leukocytes. An average of 8000 leukocytes per ul is taken as the standard. It is normal practice to count all the species present and to include both sexual and asexual parasites together.

Also, there is another simple method of enumerating parasites in thick films, it is the plus system which indicates the relative parasite count and entails using a code of 1-4 pluses, as follows:

\[+
=1-10 \text{ parasites per 100 thick film fields.}
\]

\[++
= 11-100 \text{ parasites per 100 thick film fields.}\]
+++ = 1-10 parasites per one thick film fields.

The interpretation of parasitaemia as an element of diagnosis of severely ill patient should take into a count the following considerations:

I Semi-immune individuals may harbor malaria parasites without symptoms of disease in holo-endemic areas.

II There is a correlation between density of parasitaemia and severity of malaria i.e. when it is more than 5% of red blood cells in a thin film, it should be taken as a sign of severity requiring emergency treatment but, the reverse is not always true in semi-immune persons.

III The presence of *P. falciparum* schizonts in peripheral blood and identification of pigments in monocytes are also signs of severity of malaria infection (Desakon et al., 1997).

Recently, various method have been used to improve and facilitate the conventional microscopic examination for example, centrifugation of heparinized blood specimen; staining of the blood with fluorescence stain such as rhodamine 32 dye; and the use of epifluorescence microscope but, the results were only moderately good in relation to the complexity of the techniques and their high cost (Kawamoto, 2000).

Beside the conventional microscopic method, serological tests for malaria diagnosis have been used since 1962 (Wemsdrofer, 1988). Generally, they reflected the development of immunerponses, in an exposed population, to the erythrocytic stages of malaria infection because malaria antibodies appear in the blood after the second or third attack, undergo
increase and then are gradually reduced in the absence of reinfection and remain at a low level for some years. Serological tests have a diagnostic value including:

(i) Epidemiological purposes such as detection of malaria transmission, endemicity and detection of parasite resistance to antimalarial drugs.

(ii) Diagnosis of hyperactive malaria splenomegaly and anaemia.

(iii) Screening of blood collected for blood banks to prevent transfusion malaria from donors.

(iv) In non-endemic countries they are employed for malaria diagnosis in individuals who have arrived from endemic areas.

In tropical Africa, serology was just one of many methods used to study the epidemiology of malaria and to measure the effects of various control measures. In a rural area of Sierra Leone, parasitological results when compared with serological tests gave an accurate result in epidemiology studies (Beadle et al., 1994).

Serological tests have been developed for the detection of malaria parasitaemia based on the demonstration of malarial antigen and to test the presence of antibodies to Plasmodium species in sera from patients. Human malaria parasites of a given species could be used as a source of homologous antigens from an in vitro-blood culture and erythrocytic schizonts are a most preferable stage, while, heterologous antigens could be obtained from malaria parasites of monkeys *i.e.* *P.* *brazilianum*. Such tests include the following:-
1) Indirect immuno fluorescence antibody test (IFAT) which was described by Sulzer, 1969. It was widely used, it is sensitive and reliable, suitable for the study of large numbers of sera under field conditions but proper equipment are required (Wemsdrofer, 1988).

2) Enzyme-linked immunosorbent assay (ELISA) & a dot-ELISA; they use a soluble antigen, required small quantities of antigen or antibody and can be carried out without expensive equipment. They are highly specific and sensitive, depending only on the purity of the reagents used (Beadle et al., 1994).

3) Also, there are other methods for malana serodiagnosis i.e. radio-immune assay (RIA) and indirect haemoagglutination test (IHAT).

Unfortunately, serological tests are of limited use for the diagnosis of acute malaria since they become positives several days after the disappearance of parasites in the blood. They can not distinguish protective from non-protective antibody, hence a number of new techniques based either on the uptake of labeled aminoacids or by measuring the capacity of merozoites to reinvade erythrocytes have been developed. They are promising for the detection of true protective antibodies (Alonso et al., 1999).

The rise in malaria attributable morbidity and mortality, which is due to emergence of drug-resistant strains and emergence of imported malaria in non-endemic areas, has refocused attention on malaria diagnosis as an element goal in control measures (Taylor et al., 1999).

Thus new diagnostic tests that accurately identify Plasmodium species would be helpful for both clinical and research applications, because the conventional microscopic technique have become less progressively, less
effective, most tedious and time consuming when compared with malaria surveillance.

Many new techniques have been proposed in recent years based on parasite visualization, detection of antigen, detection of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) and amplification of DNA by the polymerase chain reaction (PCR), (Smith, 1999).
Diagnosing malaria using rapid immunochromatographic (ICT) :-

A new generation of easy techniques has been developed for diagnosing malaria rapidly and reliably without the need of a microscope. They are very useful screening or confirming tests specially when there is difficulty in identifying scanty rings in blood films and when more junior, less experienced staff are on duty (Snounou, 2000). For example, several blood tests recently became available for diagnosis malaria, based on the immunochromatographic detection of Plasmodia HRP-2 (histidine rich protein-2) of *Plasmodium falciparum* and *P. vivax*. Another is based on the detection of parasite-specific lactate dehydrogenase (PLDH), both HRP-2 and PLDH are produced by the parasites during their growth and multiplication in the red blood cells (Monica, 1998).

The first immunochromatographic test to be developed was ParaSight™ F dipstick, which was produced by Becton Dickinson, USA. It is an antigen capture assay, based on the detection of the HRP-2 of *P. falciparum*. It uses a water soluble immunoglobuline Gl murine monoclonal antibody, directed against a segment of *P.f.* HRP-2 deposited on anitrocellulose glass fibre dipstick (Emmanuel et al., 1996). The test is fast, simple, requires minimal training and equipment and has a potential for use in the management of malaria. The evaluations have shown that the sensitivity and specificity of the test were 90% and 99.5% respective. Also, it is an indicator of the total parasite load than is the peripheral parasitaemia, this may account for the correlation between the test reading and clinical score of patients, because the HRP-2 being a soluable parasite product may constitutes an important functional part of the malaria toxins. But, this test is
unsuitable for checking the response to antimalarial drugs within 14 days, it does not detect gametocytes and there seems to be little evidence of cross reaction with other Plasmodium species which infect man (Pierri et al., 1998).

PATH falciparum malaria test strip (the Program of Appropriate Technology in Health) is another immunochromatographic test, which detects the HRP-2 antigen of *P. falciparum*. It is contained in a sealed tube to protect the user from potential exposure to blood-bom pathogens, and takes only 20 min. to perform. The results are easy to interpret.

Discrepancies between the results of the test occurred mainly when the test was weekly positive and there was a low level of parasitaemia. The test has equivalent sensitivity and specificity to ParaSight™ F test when evaluated (Gaye et al., 1999).

Also, Determine Malaria *P.f.* (Abbot Labs, Tokyo, Japan) test has been developed recently as an immunochromatographic test which is based on the same principle of the above tests, for the detection of HRP-2 antigen of *P.falciparum*.

An alternative, immunochromatographic assay, which is the most recently developed for rapid diagnosing of malaria, is OptiMAL® test that uses a series of polyclonal and monoclonal, gold-labeled antibodies to capture malaria parasite-specific lactate dehydrogenase (PLDH) in whole blood. It is manufactured by Flow Inc, Portland, OR. It has found to be a sensitive and practical method for the detection of *P.falciparum* and *P.vivax* infections. Differentiation of malaria species in this test is based on antigenic differences between PLDH isoforms (Monica, 1998). It is able to monitor responses to
drug therapy, detect drug-resistant malaria because PLDH reflect the presence of viable malaria parasites in the blood (Gaye et al., 1999).

From previous studies results show that OptiMAL® dipstick compared very well with blood film microscopy for initial diagnosis of malaria, but when used to follow antimalarial treatment, it became negative earlier than the blood films. Either because the level of parasitaemia had fallen below the detection limit of the OptiMAL® or because the parasite had become nonviable and were not producing PLDH. The test is rapid; easy to perform; had a 100% sensitivity on initial diagnosis but, only detected the same level of parasitaemia with 63% sensitivity on treatment follow-up (Moody et al., 2000).

A leading Australian Biotechnology company has developed a new method for diagnosing *P. falciparum* malaria using Amrad ICT® Malaria *P. f.* test that is based on the immunochromatographic detection of HRP-2 antigen of *P. falciparum*. Evaluations so far carried out have shown that the ICT® Malaria *P. f.* (Sydney, Australia) to be sensitive and specific. The test was used in remote villages in India and found to have sensitivity of 100% and specificity of 84.5% (Singh et al., 2000).

It is easy to perform, particularly in field settings, rapid and accurate test, does not require complex equipment and it appears to have highly acceptable levels of detection of the persistence of the HRP-2 antigen in the blood following curative therapy varying from 5-15 days after asexual parasite clearance (David et al., 2001). The test has the potential of enhancing speed and accuracy of the diagnosis of imported, specially in non-endemic
areas where there is a lake of experience on the side of involved laboratory personnel (Jelink et al., 1999).

Previous studies in India and Senegal showed that there was no cross-reactivity with *P. vivax* (Bray et al., 2001). So the ICT malaria *P. f.* test was most preferable than *ParaSight™ F* dipstick assay, which was unable to detect *P. vivax* in the presence of *P. falciparum* due to cross-reactivity (Kodisighne et al., 1997).

ICT Diagnostic (Sydney, Australia) have developed a test that can diagnose both *P. falciparum* and *P. vivax* malaria, and can be performed as easily as the ICT malaria *P.f.* test. With the additional detection of a pan-specific malaria antigen (P-SAg) which is common to both *P. falciparum* and *P. vivax* (David et al., 2001).

AMRAD's ICT malaria *P.f./P.v.* is a rapid, in vitro immune diagnostic test for the qualitative detection and differentiation of circulating *P.f.* and *P.v.* antigens in whole blood. It is based on the same principle of the ICT malaria *P.f.* on detection of *P.f.* HRP-2 in test area (1) in the card and aP-S antigen which is common to both *P. falciparum* and *P. vivax* species in test area (2) through the antigen-antibody capture assay.

MAKROmed® malaria rapid test is another immunochromatographic assay, for the initial detection or confirmation of *P. falciparum* malaria in whole blood, produced by MAKRO medical(PTY) Ltd, Johannesburg, South Africa. The studies in India, Australian Armed forces and many other countries in Tropical Africa proved that the ICT for *t'alciparum* malaria is highly specific, sensitive and reliable.
In a place like Sudan where approximately 50-70% out patients consultation are due to malaria and falciparum malaria is very common (90-95 % of cases), it would be highly useful to see the importance of the ICT test in both treatment and control of malaria programs. Earlier studies revealed that the device is simple, rapid, credit card sized test that can diagnose *P. falciparum* malaria less than 8 minutes without the need for expensive equipment or highly skilled personnel (Baraka et al., 1998).

According to the results of the study carried out in Tropical Diseases Hospital in Omdurman, the sensitivity and specificity of ICT were 100% and 95% respectively.

Several other studies, which were conducted at Omdurman Military Hospital and at laboratory Quality Control in National Malaria Administration (Dafa Allah, 1998) added to that advantage which make the ICT test more reliable these include:

1) The test is easier and faster to perform than the microscopy, which is time consuming.
2) It requires only small amount of unprocessed whole blood (about 1Oui).
3) It does not require electricity, equipment or training, these make it an ideal test.
4) It identifies *P. falciparum* and *P. vivax*, which are the most common and most dangerous of all species of Plasmodium. Facilitating the quick identification and rapid treatment saving many lives and many millions of dollars.
5) It is easily transportable and has a long shelf-life at an ambient temperature.
The ICT tests were also performed on six patients already diagnosed as visceral leishmaniasis and were on the specific treatment (Pentostam). The test was negative in the six patients which excludes cross-reaction between visceral leishmaniasis and malaria (Baraka, 1999).

The most advanced diagnostic test is molecular biological detection assay. The development of this technology has already provided the basis for several new test systems for the diagnosis of malaria in humans i.e. DNA and RNA probes technique, which provides a system of species diagnosis based on hybridization of the DNA in the genes of the organism.

Since 1984, the DNA tests based on the highly repeated sub-telomeric sequences of Plasmodium species have been frequently used in malaria diagnosis, most of the available DNA probes are specific to *P.falciparum*, for example, DNA recombinant method used Rep 20 and oligo nucleotide (P.f-21) unique PCR assay.

Previous studies show that, DNA probes can made specific to a single species and sensitive to as low as 4 asexual parasites/ul blood (Bray et al., 2001).

Ribonucleic acid (RNA) probes have also been evaluated with promising results, the product of RNA polymerase is an RNA copy of DNA that has the same unique sequence of bases as the DNA and when separated into single strand, can be hybridized. Ribosomal RNA is much more abundant than RNA and DNA in a cell but, is less stable and cross-species reactivity could occur.
Further approach to improving the sensitivity of DNA probes is that of the amplification of their defined region. Currently, one or a combination of three amplification methods has achieved this process:

1) Target amplification polymerase chain reaction (PCR) or Transcript amplification system (TAS).
2) Probe amplification ring Q-beta replicates.
3) Signal amplification using enzyme-linked probes.

The polymerase chain reaction (PCR) technique has found wide application in the laboratory, it can amplify selected regions of DNA and RNA rapidly and specifically from a small number of starting copies. It can hybridize even if there is contaminated (non-complementary) DNA sequences, it needs suitable primers for the synthesis of DNA and RNA i.e. those amplify polymorphic region around the center of the gene encoding for Glutamate Rich Protein (GLURP) and CircumSporozoite Protein (CSP), which were referred to as molecular targets for species-specific sequences in the 18 small sub unit ribosomal RNA (ssRNA) gene of the human malaria parasite (Bruce-Chwatt, 1993).

DNA extraction, PCR amplification and electrophoretic analysis are the contents of the PCR assay steps. The amplified products' can be visualized either by ultraviolet fluorescence produced by agarose gel electrophoresis in autoradiographs (radio-labelled probe hybridization RPH) or by the detection of non-isomers of acolorimetric indicator system (Fernandes et al., 2001). Most of the radio active approaches are labor intensive and poorly suited for use in general diagnostic laboratories. Therefore, commercial non-radiometric detection systems for PCR products has recently developed. It uses well
micro-titer plate format in hybridization (MPH), it hybridized PCR products to a single stranded RNA probe. Captured the RNA-DNA on a streptavidin-coated microtiter plate and detected by a calorimetric method using an enzyme-labeled antibody (CR/ELIZA). This format method is rapid, accurate and well suitable for use in routine laboratories.

There are a number of variations in PCR technique, in terms of collection and storage of the specimens, DNA extraction. Selection of primers, amplification conditions and or the analysis of the amplified products for example, sampling may be carried out in culture media, anticoagulants or as dried spot on filter paper. The mode of collection and storage of blood may influence the sensitivity of detection of malaria parasites by PCR i.e. samples collected on filter paper showed a significantly 100 fold lower sensitivity. Also, repeated thawing resulted in loss of sensitivity (Snounbu et al., 2001).

From several previous studies, PCR appears to have the greatest sensitivity (100%) and specificity (99%) than microscopy when used on samples from individuals living under continuous exposure to parasite incubation by mosquitoes in Senegal. PCR revealed a high prevalence of low-grade parasite carriers and most of the microscopically negative samples were found to harbour subpatent levels of Plasmodium parasites which gave support to the idea that malaria infection is more chronic than was previously thought (Emmanuel et al., 1996).

PCR tests have the potential of enhancing accuracy of the diagnosis particularly where low parasitaemia levels, asymptomatic malaria and mixed infection are frequent, inorder to determine the true incidence of each species
to follow-up patients after specific treatment, and in returning travelers (Snounou et al., 2001). It also, can be used as the reference method in most evaluations of new diagnostic assays (Humar 1999).

Studies also evaluated two PCR-based approaches (Colorimetric PCR-based assay & nested PCR). Results indicate that the nested PCR is more sensitive than colorimetric assay but is unsuitable for use in routine laboratories. It requires skills and equipment which are not available in many malaria endemic countries and limits its use to research purposes. Follow-up treatment reports showed that microscopy becomes negative earlier than PCR assay. This is probably due to subjects with subpatent parasitaemia or circulating parasite DNA, therefore it requires long follow-up monitoring studies (Moody et al., 2000).
1-3. Objectives & justifications :-

1-3-1 Objectives of the study :-

1) To evaluate the performance and the usefulness of the immunochromatographic technology (ICT) test as a diagnostic tool for malaria in Sudan.

2) To compare the ICT test and the conventional microscopic blood film (B.F.) examination in terms of sensitivity, specificity and applicability in routine laboratories.

3) To determine the correlation between the depth of the color reaction in the ICT strip and the density of parasitaemia in the thick blood films.

4) To assess the association of the epidemiology factors of the study (sex, age groups, history of fever and recent treatment) with the rate of malaria infection.

1-3-2 Justifications :-

1) The diagnosis of malaria has traditionally relied on microscopical examination of Giemsa-stained blood films. Even for an expert microscopist, this method is time consuming and labrious. The reliability and cost-effectiveness of the microscopic examination are questionable although it may apparently look simple and of low cost.

2) Accurate species identification is now becoming more essential after spread *P.falciparum* antimalarial drug-resistant strains. With microscopic examination of blood films, the identification of species may be difficult specially in patients with low parasitaemia, in those with mixed infection and due to morphological changes induced by
haemolysis during the preparation of the blood films, which hamper the parasite's species identification.

3) In many malaria endemic areas, as the Sudan, owing to a lack of trained microscopists and reliable equipment, the diagnosis of malaria is often presumptively made and is based on clinical diagnosis only. Thus presumptive treatment of malaria without laboratory confirmation is frequently inappropriate, costly and associated with side effects and ultimately contributes to the development and spread of drug-resistant strains.

4) The discovery of HIV virus refocused attention to all blood transfusion-related diseases including malaria, therefore, alternative methods of diagnosis are required specially in blood donation centers, where maximum sensitivity is required.
CHAPTER TWO
MATERIALS & METHODS

Study Area: -

This study was carried out during the period from February to July (2001) in Elkalakia area (South Khartoum), which is located on the east bank of the White Nile. It consists of five small villages inhabited by farmers as well as government and private sector employees. This area is flooded and suitable for breeding of mosquitoes, particularly in agricultural places situated near the White Nile.

Malaria in the study area is unstable, with remarkable transmission in the period between August through December; the major vector of the malaria parasite is the Anopheles gambiae arabjensis. Epidemiological surveys for malaria among the population of the study area revealed that the most important and prevalent species of Plasmodium is P.falciparum, and the phenomenon of the drug resistance is rare (Local council report, 2000).

2-1 Materials & reagents: -

Materials that are utilized in this study to run the selected tests were as follows: -

1. Sterile disposable microlancets (LANZ, PLUTENATAHME-LANZETTEN, zum EINMAL-Gebrauch).
2. Staining jars and racks for drying slides.
4. Distilled water.
5. 100 microscopic glass slides.
6. Immersion oil.
7. Methyl alcohol and cottons.
8. Microscope.
9. Marker for labelling.
10. Kits for the immunochromatographic test (which were obtained by local purchase) was :-

- 1CT™ Malaria P.//P.v., AMRAD operations (Pty)Ltd . CAN, French forest Australia, product code: ML02.LOT No.0100510502. Each kit consisted of the following: -
  1) 25 individuals packaged test cards.
  2) Malaria reagent (A).
  3) 25 capillary tubes (coated with EDTA).
  4) 1 product insert.

2-2. Methodology :-

2-2-1. Samples collection :-

Ninety two patients, who presented to the Turkish Hospital in the study area (Elkalakla), with clinical symptoms similar to malaria, were included in this study, blood was obtained from the patients by finger prick for both thick and thin blood films microscopical examination, and for the immunochromatographic test.

-Specific data of the patient population :-

Patients were questioned during the sample collection about their residence, age, sex, presence or absence of fever and recent administration of antimalarial drugs.
Also, 10 control volunteers, who were normally healthy without any recent histories of fever or antimalarial treatment, were included in the study as controls.


To prepare thick and thin blood films, perfectly clean microscope slides (7x 2.6cm) which were free from grease or scratches, were used. Blood was obtained from the second or third finger of the left hand, the skin was cleaned with ether and dried before puncture with a sterile disposable lancet (WHO, 1991). The finger was squeezed gently to obtain a large drop of blood, and gently touched to the slide, then spread evenly with a corner of another slide until it was about 10-15 mm in diameter and a thickness through which it is possible to see print. The slides were kept horizontal on a rack while drying in a safe area.

For a thin blood film preparation, the drop of blood which was obtained as explained previously, was smaller than the drop for thick films and it was spread immediately by the smooth edge of another clean slide (spreader), which was applied to the blood at an angle 45° and the spreader stopped until the blood spread along the edge. Then pushed forward at the same angle and the film was dried out (WHO, 1991). Thick and thin films were taken on the same slide and the serial number of the patients was written on it with a marker pen.

2-2-3 Method for staining thick and thin blood films :-

All blood films were stained later after completed sampling. They were stained by Giemsa stain as the follows :-
Before starting the stain method all dried thin films were fixed with absolute methyl alcohol by putting a few drops of alcohol on the films by apipette for many seconds. Then 3% solution of Giemsa stain was prepared freshly from stock solution by diluting 1.5 ml of the stock with 50 ml of distilled water. Then all slides (including thick and thin films) were stained in the staining jar for 30 minutes. The films were put in an upright position, that allowed any debris to fall to the bottom of the jar. Then they were gently flushed with tap water (taking care not to wash the blood away), they were stood upright in the rack to dry.

2-2-4 Microscopic examination of blood films (WHO, 1991):

Blood films were examined under the microscope; the thick films were examined firstly using an oil immersion lens. They were tested for malaria parasites and their density determined in at least 100 microscopic fields (in accordance with the WHO criteria before negativity was declared).

The parasite presence was confirmed by the examination of thin films, which identification other Plasmodium species. Positive slides were classified after finding the parasite (one or more stages) in the blood films while, the negative ones were not classified before examining the parasite asexual and sexual stages in at least 100 microscopic fields.

2-2-5 Malaria parasites count :-

Parasites density was calculated for each positive slide by the following plus sign scheme (WHO, 1991):

Parasites

1-10 Per 100 high power fields +
11-100 Per 100 high power fields ++
2-2-6 Method for the immunochromatographic test :-

All collected specimens were tested by the immunochromatographic test (ICT) strips for the rapid detection of circulating *Plasmodium falciparum* (P./) specific histidin rich protein-2 (HRP-2) and for the detecting *Plasmodium vivax* (P.v.) parasitaemia by the detection of Pan-specific malaria antigen (P-Sag) which is common to both *Plasmodium falciparum* and *Plasmodium vivax*.

ICT Malaria *P.f./P.v.* kit's card contains two antibodies which have been immobilized as two separate lines across a test card. One antibody (test area 1) is specific for the HRP-2 antigen of *P. falciparum*, while the other (test area 2) is specific for malaria antigen P-Sag for both *P.falciparum* and *P. vivax*.

According to the manufacturer-Is instructions the ICT card was opened immediately prior to conducting the test, from the same finger prick for the first test (microscopic examination of blood films). The finger was cleaned with an alcohol cotton swab and allowed to dry, then squeezed gently to obtain a good-sized drop of blood. One end of the EDTA coated capillary tube was touched to the blood on the finger prick, then about 15ul of collected blood were applied to the sample pad impregnated with colloidal gold labeled antibody, which was directed against the two malarial antigens.

The blood was flushed along the strip by adding one drop of malarial reagent (A) above the blood, then two drops were added also below the blood which allowed blood spreading through the pad. Lastly, 4 drops of the
reagent (A) were added to the left side of the test card which was closed, then the lysed blood was returned back and the strip cleared leaving a white background for viewing the results.

When a positive sample was applied, malarial antigens were bound to the gold coupled antibody in the pad, then the immune complexes formed compounds which migrate along the test strip on which they were captured by the second immobilized antibody. Then a pink lines were formed in area land/or 2 of the test window.

On the other hand, in a negative sample these lines do not appear. A procedural control line appears in area (C) of the test window in both negative and positive samples to reveal the correct performance of the test.

2-2-7 Interpretation of the results and statistical data analysis:

In the microscopic examination of the blood films, a thick blood film was considered negative when at least 100 microscopic fields were free of malaria parasites. While it was considered positive when at least one asexual or sexual form of malaria parasites was found (alone or with both forms).

-In the ICT test results were interpreted as :-

(A)
Positive test result was indicated by any visible line in area (1) and/or (2), together with a line in area (C) even the test line was lighter or darker than (C) line.

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<tbody>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

(B)
- Negative test results when only (C) line appeared.
- Results with no control band were excluded and considered as invalid results, they were repeated again using a new card.

**Statistical data analysis :-**

Data were analysed using the statistical package for social science (SPSS). Chi-square test was used for comparison of proportions. To normalize the distribution of abnormal data, log transformation was used and the means were compared using one way analysis of variance (ANOVA) or the t-test.

The test was considered as significant when P-value are < 0.05.
CHAPTER THREE

RESULTS

General observation :-

This study was carried out in the period from February to July (2001) to determine the relative accuracy of an advanced diagnostic technique, the immunochromatographic test (ICT) as compared to the conventional microscopic examination of Giemsa-stained blood films for the diagnosis of *P. falciparum*. Measures of diagnostic accuracy of the ICT were calculated in relation to the results of microscopic examination of blood films, in terms of percentage of positive and negative results, of the selected samples and the relative sensitivity and specificity of the ICT.

Results of all selected control samples (without symptoms of fever and previous treatment with antimalarial drugs) were found to be negative with both blood films and ICT carried according to our described procedures.

During this study, ninety two (92) patients clinically classified as malaria-patients were enrolled in the study. The results of positivity and negativity were obtained by both techniques: ICT and the microscopic examination of blood films for the samples collected from those patients were shown in Table 1.

Of the ninety two patients whose blood specimens were examined with both films examination and the ICT techniques: 54 samples (58.7%) showed malaria parasites *P. falciparum* in their peripheral blood, while 38 (41.3%) were found to be negative; i.e. no malaria parasite detected in at least 100 thick and thin film fields when diagnosed by the microscopic examination of Giemsa-stained blood films.
On the other, 65 of ninety two patients (70.7%) were found to be positive by the ICT. while 27 (29.3%) were negative; i.e. no colour reaction in test area, Figure 1.

III.1. Comparison of microscopic examination of blood films and the ICT:-

Results findings showed that both diagnostic techniques were concordant in 54 samples (B.F. positive slides and positive ICT).

Out of 54 concordant samples, microscopic examination of Giemsa stained blood films detected both sexual and asexual stages in 18 samples and only asexual stages in 29 samples and only sexual stage (gametocyte) in 7 samples. Furthermore, none of the samples that were found to be positive microscopically was negative by the ICT but the reverse was true in 11 samples which were positive by the ICT and negative on microscopic examination of Giemsa-stained blood films (Table 1).

ICT positive results were graded accordingly to the control band intensity into three groups; faint, equal or darker test line group (Table 2). Then each group was compared with the parasite density calculated out in each positive blood film.

Results from statistical analysis showed that there was no significant correlation between the intensity of colour reaction (test line intensity) and the density of peripheral parasitaemia that was expressed as crosses of the WHO system (Table 2, P>0.05).

III.1. Sensitivity and specificity of the ICT:-

In this study, the sensitivity and specificity of the immunochromatographic test (ICT) for the detection of Plasmodia infection
were calculated using the results of microscopic examination of Giemsa stained blood films as reference standard, and the sensitivity and specificity were found to be:

1 - Sensitivity  = \( \frac{\text{true positive}}{\text{true positive} + \text{false negative}} \)  
  = \( \frac{54}{54+0} \) x 100 = 100%

True positives  = positive results by both microscopic examination of blood films and the ICT.

False negative  = negative results by the ICT but, positive by microscopic examination of blood films.

2 - Specificity  = \( \frac{\text{true negative}}{\text{true negative} + \text{false positive}} \)  
  = \( \frac{38}{38+1} \) x 100 = 77.6%

True negative  = negative results by both microscopic examination of blood films and ICT.

False positive  = positive results by the ICT but, negative by microscopic examination of blood films.

Direct comparison of the ICT with the blood films examination indicated that the ICT had a sensitivity of (100%) and specificity of (77.6%), Table 3.

III.2. Specific data of the patients population :-

The sex and the age of the participants were considered in the present study to determine their association with the rate of infection by *P.falciparum*.

III.2J. Sex ratio of the patients in association with the rate of malaria infection :-
Out of ninety two patients, there were 50 (54.3%) males and 42 (45.7%) females. They were examined with both ICT and the microscopic examination of Giemsa stained blood films. As showed in (Table 4). The differences in the sex-ratio (male:female) with the rate of malaria infection in all samples were statistically insignificant (P>0.05).

Figure (2) showed the general distribution of the sex in the samples.

111.2.2 Age groups of patients in relation to the rate of malaria infection:

The rate of infection with malaria parasites differed significantly between the different age groups being high in children (0-5 years). Tables (5&6), figure (3) showed the distribution of the age groups in all samples.

111.2.3 The symptom of fever in relation to the rate of malaria infection:

Results of fever complaint were compared with positivity of both blood films and the ICT results in Tables (7&8), which revealed that 60 (65.2%) of ninety two patients had fever, a few days before taking their samples, while 32 (34.8%) of them were without fever. Statistical analysis showed that there was a significant correlation between the existence of fever and the high parasitaemia in the blood films and the positivity of ICT.

111.2.4 Pre-treated patients in association with the rate of malaria infection:

Out of ninety two patients, 35 (38.04%) of them had received antimalarial treatment (i.e. oral or injectable chloroquine) shortly before they were examined in this study for malaria diagnosis (pre-treated) while 57 (61.96%) of them were not treated before they were examined (non-treated).
Then results of the samples obtained by both ICT and the blood films examination were compared, microscopy of blood films showed that 21 (60.0%) of pre-treated patients and 33(57.9%) of non-treated ones were positive. While, 14(40.0%) of pre-treated patients and 24 (42.1%) of non-treated ones were negative, (Table 9).

On the other hand, ICT results showed that there were 10 (27.1%) pre-treated patients with negative results and 25 (38.5%) others with positive results. While, 17 (62.9%) patients, whom did not received treatment (non-treated) showed negative results and 40 (61.5%) of them tested positive (Table 10).

Statistical analysis of these data showed that there was a significant correlation between treatment and the results of both blood films and ICT tests at the levels 0.01 and 0.05 respectively. There was a high percentage of pre-treated patients in the positive blood films group (60.0%) and the positive ICT group (38.5%) than in those with negative samples of either blood films or ICT, (24.4%) and (27.1%) respectively, figure 4.
Table 1. Comparison between the microscopic examination of Giemsa-stained blood films and the detection of *(P.f. HRP-2)* by the ICT for the diagnosis of malaria.

<table>
<thead>
<tr>
<th>Total number examined</th>
<th>Microscopy examination of blood films (B.F.)</th>
<th>ICT test</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Positive B.F.</td>
<td>Negative B.F.</td>
</tr>
<tr>
<td>92</td>
<td>54 (58.7%)</td>
<td>38 (41.3%)</td>
</tr>
</tbody>
</table>

*Association is significant at 0.01 accordingly to chi-square test.*
Table 3. Sensitivity and specificity of the ICT in relation to the microscopy examination of blood films.

<table>
<thead>
<tr>
<th></th>
<th>Blood films</th>
<th>ICT</th>
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<tbody>
<tr>
<td>Positive result</td>
<td>54</td>
<td>65</td>
</tr>
<tr>
<td>Negative result</td>
<td>38</td>
<td>27</td>
</tr>
<tr>
<td>Sensitivity %</td>
<td>-</td>
<td>100%</td>
</tr>
<tr>
<td>Specificity %</td>
<td>-</td>
<td>77.6%</td>
</tr>
</tbody>
</table>
Table 4. Comparison between the sex-ratio of the patients and the malaria infection rate in the microscopy examination of blood films and the ICT.

<table>
<thead>
<tr>
<th>Sex of the patients</th>
<th>Total No. of sex</th>
<th>No. of positive B.F.</th>
<th>No. of positive ICT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>50</td>
<td>23</td>
<td>39</td>
</tr>
<tr>
<td>% with test result</td>
<td>42.6%</td>
<td>60%</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>42</td>
<td>31</td>
<td>26</td>
</tr>
<tr>
<td>% with test result</td>
<td>57.4%</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>92</td>
<td>54</td>
<td>65</td>
</tr>
</tbody>
</table>

** Association is not significant since P>0.05 accordingly to chi-square test.**
Table 5. The association of the patients age with the density of parasitaemia in positive blood films.

<table>
<thead>
<tr>
<th>Patients age group (years)</th>
<th>Density pluses</th>
<th>1.00</th>
<th>2.00</th>
<th>3.00</th>
<th>4.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 count % with density</td>
<td></td>
<td>6</td>
<td>9</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>41.7%</td>
<td>35.4%</td>
<td>33.3%</td>
<td>60%</td>
</tr>
<tr>
<td>6-15 count % with density</td>
<td></td>
<td>6</td>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>58.3%</td>
<td>16.7%</td>
<td>64.5%</td>
<td></td>
</tr>
<tr>
<td>&gt;15 count % with density</td>
<td></td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48.7%</td>
<td>33.3%</td>
<td>50.0%</td>
<td>40.4%</td>
</tr>
</tbody>
</table>

**Association is significant at 0.05 level accordingly to chi-square test.**
Table 6. The association of the patients age with malaria infection rate by the ICT

<table>
<thead>
<tr>
<th>Patients age group (years)</th>
<th>Results of ICT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>0.5 count % with ICT result</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>33.9%</td>
<td>55.6%</td>
</tr>
<tr>
<td>6-15 count % with ICT result</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>30.8%</td>
<td>29.6%</td>
</tr>
<tr>
<td>&gt;15 count % with ICT result</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>35.3%</td>
<td>14.8%</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>27</td>
</tr>
</tbody>
</table>

*** Association is significant at 0.04 level accordingly to chi-square test.
Table 7. The association of the symptom of fever with both positive & negative blood films.

<table>
<thead>
<tr>
<th>Results of blood films</th>
<th>History of fever</th>
<th>Total BF results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Positive count % with BF result</td>
<td>33</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>61.1%</td>
<td>38.9%</td>
</tr>
<tr>
<td>Negative count % with BF result</td>
<td>27</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>71.1%</td>
<td>28.9%</td>
</tr>
<tr>
<td>Total history of fever</td>
<td>60</td>
<td>32</td>
</tr>
</tbody>
</table>

*Association is significant since P > 0.05 level accordingly to chi-square test.*
Table 8. The association of the symptom of fever with both positive & negative ICT results.

<table>
<thead>
<tr>
<th>Results of ICT assay</th>
<th>History of fever</th>
<th>Total of ICT results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Positive count</td>
<td>42</td>
<td>23</td>
</tr>
<tr>
<td>% with ICT result</td>
<td>64.6%</td>
<td>35.4%</td>
</tr>
<tr>
<td>Negative count</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>% with ICT result</td>
<td>56.7%</td>
<td>33.3%</td>
</tr>
<tr>
<td>Total history of fever</td>
<td>60</td>
<td>32</td>
</tr>
</tbody>
</table>

*/* Association is significant since $P<0.05$ level accordingly to chi-square test
Table 9. The association between results of blood films examination and a history of antimalarial treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Results of blood films</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Yes count</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% with BF result</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>60.0%</td>
<td>40.0%</td>
</tr>
<tr>
<td>No count</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% with BF result</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>57.9%</td>
<td>42.1%</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>38</td>
</tr>
</tbody>
</table>

*** Association is significant at 0.01 level accordingly to chi-square test.
Table 10. Comparison between results of the ICT test for malaria in treated and non-treated groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Results of ICT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Yes count</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>% with ICT result</td>
<td>28.6%</td>
<td>71.4%</td>
</tr>
<tr>
<td>No count</td>
<td>17</td>
<td>40</td>
</tr>
<tr>
<td>% with ICT result</td>
<td>29.8%</td>
<td>70.2%</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>65</td>
</tr>
</tbody>
</table>

*** Association is significant at 0.05 level accordingly to chi-square test.
Fig. 1. Comparison between the microscopic examination of blood films and the ICT for the diagnosis of malaria
Fig. 2. Distribution of the patients' sex in positive B.F.
Fig. Distribution of the patients' age groups in positive B.F.

- <=5 years: 34%
- > 15 years: 20%
- 6 to 15: 46%
Fig. 4. Comparison between results of the blood films examination and the ICT for malaria in treated and non treated.

- BF (with treatment): 60% (Positive), 40% (Negative)
- BF (without treatment): 58% (Positive), 42% (Negative)
- ICT (with treatment): 71.40% (Positive), 28.60% (Negative)
- ICT (without treatment): 70.20% (Positive), 29.80% (Negative)
CHAPTER FOUR
DISCUSSION

IV.DISCUSSION:-

Malaria is a major health problem and it plays a great role affecting the economic and social status of the community. The rapid diagnosis and early treatment of clinical cases are central measures towarded the reduction of malaria attributable morbidity and mortality. Early diagnosis is a major principle of the global malaria control strategy; thus delayed diagnosis and treatment of patients specially with \textit{P.falciparum} may lead to the development of severe and complicated malaria.

Basic requirements relating to the detection and diagnosis of malaria include sensitivity, specificity, rapidity, simplicity and availability as shown by many authors as Kodisighne(1997) and Singh (2000) who viewed that such requirements are crucial in malaria diagnosis.

Traditionally, microscopic examination of thick and thin blood films which was reported by Ross (1903) is the most widely used routine method for malaria diagnosis and it still remains the golden standard test in clinical practice. However, because of the amount of time involved on preparation and examination of blood films, this method becomes impractical with high sample loads. Furthermore, it is laborious intensive, requires skilled microscopists and a centralized nature of services(Smith et al.,1999) and (Snounou, 2001).

In Sudan, microscopic diagnosis of malaria was found frequently to be of low sensitivity, due to several factors including: general conditions related to microscopic service as : electricity ; type and quality of stains and
proficiency of the microscopists (Ali, 2001). This was proved to be true as reported by (Gabir 1997) and (Baraka, 1998) who showed that the sensitivity of microscopic examination depends on well-trained personnel; time needed and has significant startup and maintenance costs.

Hence a major disadvantage of microscopic of blood smears is that it needs a relatively prolonged time for examination of blood films to achieve high sensitivity in diagnosis. Since time constraint leads to have an appreciable failure rate particularly at low parasitaemia. These findings are in agreement with that obtained by Beadle et al. (1994); WHO, (1991); Moody (2000) and Bray (2001) who reviewed that the time factor in relation to high numbers of clinical cases of malaria is one of main problems encountering the microscopic diagnosis of malaria because there is a significant change in endemic areas in the clinical and parasitological features of malaria infections as a result of drug-resistant malaria. They suggested the use of alternative method such as antigen detection test for example, OptiMal dipstick and ParaSight tests.

In contrast, Loban (1989) reported that the microscopic examination is the mainstay of malaria diagnosis. Correspondingly he showed that thick film examination is more sensitive, allows estimation of parasitaemia, distinguishing between parasite growth stages and it covers all species. Furthermore, it succeeded in determining the efficacy of therapy.

Therefore, this study was carried out as an attempt to evaluate other advanced techniques leading to more accurate and reliable results for the diagnosis of malaria. In this study microscopic examination of blood films
for diagnosis of malaria was used as the gold standard to which an alternative
test, the immunochromatographic test (ICT) was compared.

The main purpose of this study is to determine the sensitivity and
specificity of the ICT compared to the conventional microscopic method.

In the present study, data analysis showed that the ICT appeared to
have considerable accuracy level when compared with the microscopy for
initial diagnosis of malaria which is in agreement with Pierri (1998) and
(2001) but, there was insignificant correlation between the intensity of the
colour reaction in the positive ICT and the peripheral parasitaemia in blood
films (P>0.05), table 2. This result was similar to those which were reported
in previous studies by Boore (1998) and David (2001).

In this study the sensitivity and specificity of the ICT were found to be
100% and 77.6% respectively compared to the microscopic diagnosis of
Giemsa-stained blood films. Similar results were also be noted by Alonso
(1999), Sharma, Tariemo et al., (2001) and David (2001). But these results
were in contrast to some previous studies by earlier authors, who noted that
the ICT has greater specificity e.g. Desakon et al., (1997) found that it was
99%; Tiemoko (2000) found it was 99.5%. Low specificity of the ICT may
be due to the low specificity of parasite antigens which give positive results
to _P. falciparum_ and to some extent positive results to other _Plasmodium_
species antibodies.

Generally, in the present study, both tests were discordant in 11
samples which were positive when detected by the ICT and negative by the
microscopy. They were considered as false positive results for the ICT. There
are several explanations for these differences; first it may occurse as a result
of persistence of a residue of circulating P./HRP-2 antigen after parasite clearance and elimination of infection by successful treatment. Secondly, those patients may be having a low-level parasitaemia below the detectable limit of the microscope examined blood films.

In the present study, also there were 7 Patients with only gametocyte stage in their peripheral blood. They were considered to be positive results although such patients usually showed mild or no symptoms of malaria. This in agreement with the findings of Taylor (1999), Kawamoto (2000) and Snounou (2001). Results showed that some of these gametocyte samples were found to be positive by the ICT, so it was consistent with previous reports which showed the association between the ICT results and the presence of gametocyte in the blood. This can be explained by the fact that P./HRP-2 antigen and gametocyte took longer time in the blood after distinction of asexual stages. This confirms many previous reports reviewed by Emmanuel (1996), Humar (1999) and Femandes et al. (2001).

The present study, clearly demonstrated that the ICT appeared to have an acceptable level of sensitivity and specificity as has been shown by Beadle (1994), Sharma (1999) and Tiemoko (2000) and it has several advantages over microscopy which include: the ease to use with minimal training; speed of performance which suggests that it maybe suitable for wide clinical use; accurate and relatively inexpensive since there is no need for high skilled technician and equipment.

Also, similar views were reported in Sudan by Elkadaro and Baraka (1999) who noted that reliable microscopic diagnosis of malaria is often not available, specially in remote areas. Therefore, the ICT will greatly assist in
making an early diagnosis of malaria with good consequences for the health of individuals and minimizing unnecessary treatment.

The association of sex and age of the patients were considered in the present study with the rate of malaria infection by both techniques. Results showed that differences in the sex-ratio (male: female) in all samples were statistically insignificant (Table 4, P>0.05). The same finding was observed by Taylor (1999) who found that there was no differences in the sex ratio of infected population. In contrast, TajElsir (1999) in a previous study reported that the malaria infection rate was higher in males than in females in Altaragma area, Sudan.

Results in this study also found, as have other (David et al., 2001) a significant difference in parasite positivity rate between different age groups of the patients with increase in children under five years of age (Tables 5 & 6). This was explained by the fact that older age may reflect higher immunity reducing parasite densities.

The symptom of fever as an indicator of malaria was considered in this study. Its prevalence was compared with both microscopic examination of Giemsa-stained blood films, with the levels of parasitaemia, and with the ICT. It was found that there was a significant correlation between fever and positivity rate of malaria since there was significant association with the higher parasite densities in blood films and fever (Tables 7 & 8). These findings confirm reports in previous studies carried out by Moody (2000), Bray et al., (2001) and Ali (2001) who revealed that there was a significant association between fever and infection; but there is a need to document temperature and pallor correctly in the integrated management of malaria.
disease. Also fever alone encourages patients to self-treating behavior without medical supervision (even in absence malaria), and this leads to further spread of drug-resistant malaria.

In this study, pre-treatment by patients for malaria was commonly observed. Whether the patients had received treatment or not before taking sample, or were in follow-up mode, results showed that a high percentage of pre-treated patients was found in the positive group by both blood films examination and ICT (figure 4). This can be explained several factors: first, the study conditions (i.e. sample size, treatment follow-up not available). Secondly, high positivity rate with blood films examination in pre-treated patients may be due to recrudescence or treatment resistance of a first degree.

On the other hand, the ICT positivity rate with pre-treated patients may be due to high level of P./HRP-2 antigen in drug-treated patients (damaged parasites release the antigen) than in non-treated ones. Also, this may reflect a residue of circulating P./HRP-2 after parasite clearance.

Most previous follow-up studies, which were conducted to assess the use of the ICT for posttreatment diagnosis, were in agreement in the fact that the time for a patient to become negative by the ICT test reaches up to 14 days from the start of treatment (later than microscopy diagnosis), Beadle (1994), Emmanuel (1996), Sharma (1999), and David (2001).

Unfortunately, the ICT test is not an ideal tool that can be used to determine the response of the patient to anti-malarial drugs because it has a problem of false positive results after successful treatment and delayed return to test negative due to persistent antigenaemia. Also, in the presence of
gametocyte stage the ICT test gives positive results due to the presence of P./HRP-2 antigen of gametocytes after the elimination of asexual stages of the parasite.

IV.2 Conclusion and recommendations :-

Finally, this study has concluded that the ICT has an appreciable level in terms of accuracy, sensitivity and specificity when compared with the microscopic examination of Giemsa-stained blood films for malaria diagnosis (Table 3), and has the potential to transform the diagnosis of acute malaria and represent a major advance over the microscopical examination of blood films.

However the antigen detection assay e.g. ICT can't replace conventional microscopy in malaria diagnosis. It has many advantages including the following: it is quick to perform, easy to interpret, requires no electrical equipment or laboratory facilities. But, if it is to replace microscopy further development is required to address problem such as: false-positivity, treatment follow-up, reliable species diagnosis, determination of the real parasite load and the cost of the test.

Generally, an ideal diagnostic tests for malaria diagnosis must be simple, rapid, easy to interpret and capable of differentiating between Plasmodium species. In addition, it should be affordable in the many developing countries where the disease is prevalent. Although the ICT test was simple, rapid, and easy to interpret, it was not considered as an ideal diagnostic method because it has many problems as mentioned above.

At last, ICT is recommended to be used in all laboratories for malaria diagnosis plus blood films.
CHAPTER FIVE

References


Desakon, A. (1997). Polymerase chain reaction and aliquant-phase, non-isotopic hybridization for species and sensitive detection of malaria


**Internet communications:**


http://vvww.WHO.In/healthtopics/malaria/Htm Author: Sonounu (2001)


Annex 1. Life-cycle of human malaria parasite

1. Sporozoites in salivary gland.
2. Oocysts in stomach wall.
3. Male and female gametocytes.
4. Liver phase.
5. Release of merozoites from liver. These enter red cells where both sexual and asexual cycles continue.

**Diagnostic points:**

1. Red cells are not enlarged.
2. Rings appear fine and delicate and there may be several in one cell.
3. Some rings may have two chromatin dots.
4. Presence of marginal or appliqué forms.
5. It is unusual to see developing forms in peripheral blood films.
6. Gametocyte have a characteristic crescent shape appearance. However, they don’t usually appear in the blood for the first four weeks of infection.
7. Maurer's dots may be present.
Annex 7. Differential diagnosis of *Plasmodium vivax* in thin blood film

**Diagnostic points:**

1. Red cells containing parasites are usually enlarged.
2. Schuffner's dots are frequency present in the red cells as shown above.
3. The mature ring forms tend to be large and coarse.
4. Developing forms are frequency present.
Annex 8. Differential diagnosis of *Plasmodium ovale* in thin blood film

**Diagnostic points:**

1. Red cells enlarged.
2. Comet forms common (top right).
3. Schuffner's dots, when present, may be prominent.
4. Mature schizonts similar to those of *P. malaria* but larger and more coarse.

**Diagnostic points:**

1. Ring forms may have a squarish appearance.
2. Band forms are a characteristic of this species.
3. Mature schizonts may have a typical daisy head appearance with up to ten merozoites.
4. Chromatin dot may be on the inner surface of the ring.
Vector

→ Sporozoites in salivary glands → Circulating

→ Oocyst

→ Ookinere

→ Zygot

→ Gamet

→ Gametocytes → Circulating

→ Man Vector

→ Schizogony in the liver → Toxins → Cytokines

→ Circulating

→ Merozoites

→ Sequestered PRBCs (Tissues damage)

→ PRBs

Veeter

Man Vector Contact

- Sporozoites in salivary glands
  - Oocyst
    - Ookinet
      - Zygot
        - Gamet
          - Gametocytes
            - Circulating PRBCs
              - PRBCs

- Circulating
  - Sequestered PRBCs

- Schizogony in the liver
  - Merozoites
    - Circulating PRBCs

- Mature Schizogony
  - Transmission – Blocking IR'S

* In each natural immune response points occurred potential vaccines could be expected with different impacts.

**Vector**

**Man Vector Contact**

**Man**

**Proguani**

Sporozoites in salivary glands

- Man Vector Contact

- Circulating

- Schizogony in the

- Mature Schizogony

- Sequestered PRBCs

- Chloroquine amodiaquine
- Quinine tetracyclines

- Mefloquine, halofantrine
- Artemisinine


* Exception gametocytes of *P.falciparum*
* In each natural immune response points occurred potential vaccines could be expected with different impacts.