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GENOTYPES DISTRIBUTION OF *Plasmodium falciparum*
ERYTHROCYTE BINDING ANTIGEN (*EBA-175*) GENE IN
SUDANESE MALARIA PATIENTS

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B.Sc. MLT-Microbiology, (SUST) 2004

A thesis submitted in fulfillment for the requirements of the degree of Master
in Molecular Biology

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U of K
2010
DECLARATION

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Signature: ..................................................

Date: ......................................................
Dedication

To my Loving Mother and Father

To My Loving Sisters and Brothers

To my Friends
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ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor, Dr. Hiba Salah-Eldin Mohamed who was abundantly helpful and offered invaluable assistance, support and guidance.

Deepest gratitude is due to Dr. Nasruddin H. Abdul-Hadi, for his endless support and encouragement.

My sincere gratitude goes to Prof. Muntasir E. Ibrahim, who supported me throughout my research with his patience and knowledge.

Special thanks also to all my colleagues and staff of the Institute of Endemic Diseases, especially: Ahmed Alnazeer Abdalla Amine and Waleed Hussein Omer for their invaluable assistance.

My thanks extend to all people who agreed to participate in this study from Um Salala, Sennar and Wad Medani.
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<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
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<tr>
<td>ARMA</td>
<td>Atlas du Resque de la Malaria in Africa</td>
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<tr>
<td>BF</td>
<td>Blood Film</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
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<tr>
<td>CD4-T</td>
<td>Cluster of differentiation 4-T</td>
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<tr>
<td>DBL-EBP</td>
<td>Duffy- Binding-like erythrocyte-binding proteins</td>
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<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulation</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double strand DNA</td>
</tr>
<tr>
<td>EBA-175</td>
<td>Erythrocyte Binding Antigen-175</td>
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<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPA</td>
<td>Glycoprotein A</td>
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<tr>
<td>Hb</td>
<td>Haemoglobin</td>
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<tr>
<td>HbC</td>
<td>Haemoglobin C</td>
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<tr>
<td>HbS</td>
<td>Haemoglobin S</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>ICT</td>
<td>Immunochromatography test</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile Range</td>
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K₂HPO₄  Dipotassium phosphate
KCl    Potassium Chloride
KDa    Kilo Dalton
MARA   Mapping Malaria Risk in Africa
NaCl   Sodium Chloride
NaH₂PO₄ Monosodium phosphate
NaOH   Sodium Hydroxide
NK cell Natural Killer Cell
_P. falciparum_  _Plasmodium falciparum_
P. _ovale_  _Plasmodium ovale_
P. _vivax_  _Plasmodium vivax_
p.value  Probability value
PBS    Phosphate buffered saline
PC     Personal computer
PCR    Polymerase Chain Reaction
PfF2   Plasmodium falciparum fragment 2
RFLP   Restriction Fragment Length Polymorphism
Rh     Reticulocyte binding-like homolog
RNA    Ribonucleic acid
TCR    T Cell Receptor
WHO    World Health Organization
_αβ T-cell_  Alpha Beta T-Cell

VI
ABSTRACT

Background: In many *Plasmodium falciparum* (*P. falciparum*), strains the invasion process requires an interaction between merozoite ligands and the sialic acid residues of glycophorin A (GPA) on the erythrocyte surface. It was found that the majority of *P. falciparum* merozoites use the erythrocytic GPA receptor to invade RBCs. The Erythrocyte Binding Antigen (EBA-175) has highly divergent allelic segments (C seg and F seg). There was a study showed that the presence of C segment was not associated with severe malaria but confer a higher risk of fatal disease.

Design: This study was cross-sectional study, hospital and village based.

Setting: General and paediatric Madani Hospitals, Al-Emam Al-bukhari primary school, Sennar malaria centre, and Um-Salala village were surveyed in the time from the 2nd of November to the 1st of September 2007 and 2009.

Objective:

- To determine the frequencies of the dimorphic allele segments of the EBA-175 gene of the *P.falciparum* merozoite among malaria patients in Sudan.
- To investigate whether this dimorphism has a significant association or impact on the clinical manifestation of the malaria disease.

Methods: Blood samples were collected from 339 malaria patients from the three regions. 20 μl Peripheral blood dried on filter paper in addition to 2 ml whole venous blood for thin and thick blood film for parasite identification and count, ICT, Hb, Random Blood Glucose level, and PCR.
Results: The Genotyping results for all samples showed frequencies of different alleles as follow 41.0% for F allele, 51.0% for C allele and 8.0% with mixed infection (allele F+C). F and C alleles were showed significantly different distributions in different geographic areas (p-value= 0.00). The correlation between different malaria phenotype manifestations and genotypes revealed that the severity of the diseases correlated with high frequency of C allele (62.9%), at least in Sennar region.

Conclusion: This study showed that significant differential distribution of F and C fragments in different geographical areas endemic with Malaria which could explain the different pattern of malaria in Um-Salala village in the East and cities in central Sudan.

No significant association was observed between F and C alleles and different Malaria phenotypes, this association was found in Sennar area but the samples size was too small so this results needs to be verified.
المستخلص

المقدمة: في كثير من حالات طفيلة المتصوره المنجلية، تتطلب عملية غزو كرات الدم الحمراء تفاعل بين مستقبلات الميروزويتات (merozoites) وال (GPA) المتواجد على سطح كرات الدم الحمراء.

لقد تبين ان معظم طفيلة المتصوره المنجلية تستخدم في عملية اختراع كرات الدم الحمراء مستقبل يعرف ب EBA (175-185). ويمتلك هذا المستقبل منطقة في غاية التبيان تعرف بالعامل F أو العامل C. هناك دراسة أوضحت أن استخدام طفيلة المتصوره المنجلية للمستقبل C ليس مرتبطة بالاعراض الحادة للملاريا بقدر اربطته بالموت كناتج أخير للملاريا.

التصميم: دراسة لعينه مقطعيه من مستشفيات معينه وقريه ام سلاله.

الإعداد: تم عمل مسح لكل من المستشفى العام ومستشفى الأطفال بود مدني مدرسة الامين البخاري الااعدادي ومركز الملاريا بسنار وقريه ام سلاله. وقد تم اجراء الابحاث المخبريه بمعهد الامراض المتوفرة بجامعة الخرطوم.

الهدف من الدراسة: نظرا لمجموعة كبيرة ومختلفة من مظاهر الملاريا في السودان، فإن هذه الدراسة تهدف إلى التحق من نمط توزيع العاملان الوراثيان لل (EBA - 175) في ثلاث مناطق مختلفة متونهة بالملاريا: سنار، مدني و ام سلاله، وربط هذا النمط الوراثي مع النمط الظاهري لهذا المرض.

أساليب الدراسة: تم جمع عينات الدم من 393 مريضا بالملاريا من ثلاث مناطق كاليلي: 10μL من الدم PCR الطرفي تم تجهيزها على ورق الترشيح لاستخلاص الحمض النووي ومن ثم مضاعفة بتقنية ال لااحقاً، بالإضافة إلى 2 مل من كل الدم الوريدي للمسحة الرقيقة والعميقة على الشريحة الزجاجية للتحقق من وجود الطفيلة وتحديد كميتها، وفحص ال ICT والهيموغلوبين، وتحديد مستوي السكر في الدم.

النتائج: نتائج التحليل الوراثي لل (EBA - 175) لمعرفة توزيع عاملان الوراثي وضحت ان توزعهما في الثلاث مناطق مجتمعه كان على النحو التالي: 41.0% للعامل F ، 51.0% للعامل C ، و 8.0% عدوى مختلطة (C+F). ولقد وجد ان هناك فرقاً معيناً في نمط توزيع العاملين الوراثيين (C+F) باختلاف IX.
المناطق الجغرافية قيد الدراسة (P-value= 0.00). وكشفت العلاقة بين مختلف مظاهر النمط الظاهري للملاريا و هذه الأنماط الجينية أن العامل الوراثي C مرتبط بأرتفاع حدة المرض (62.9 %).

الخاتمة: تبين هذه الدراسة أن هناك فرقاً معنوية في نمط توزع العاملين الوراثيين (C+F) باختلاف المناطق الجغرافية المختلفة قيد الدراسة (P-value= 0.00). مما قد يفسر تباين نمط الملاريا في كل من قرية إم سلالة في شرق السودان والمدن في وسط السودان.

Appearance.png

المناطق الوراثية المختلفة اظهرت الدراسة أنه لا يوجد فرق معنوي في العلاقة بين توزيع العاملين C و F للملاريا، وبالرغم من وجود هذا الفرق المعنوي في وسط عينات مدينة سنار إلا أن هذه النتيجة لا يعتمد بها وذلك لقلة عدد هذه العينات.
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Chapter One

Introduction & Literature Review

1.1 General Introduction:
In Hippocratic era, malaria was known simply as ‘the fever’, to the Romans as ‘intense burning heat’ (febris ardens) or by its periodicity (accessia). In the modern era, malaria was known to the French by fever and chills and to the English as ‘seasonal fevers’. In Osler’s time, because of its seasonal incidence malaria was also known as the fever of summer fall (Cunha and Cunha, 2008). Of all diseases, malaria is perhaps the one with the most subtle interplay with human history. It has long affected human beings, and has left its genetic mark on modern populations. Thalassaemia, glucose-6-phosphate dehydrogenase deficiency, sickle cell trait, the Duffy antigen, and several other genetic variations owe their prevalence to malaria. The disease acted as a barrier to European imperialism and rendered many areas of the world, even in Europe, largely unproductive. Malaria thus influenced, and its incidence has been in turn influenced by agricultural practices from time immemorial. It is perhaps the most ecologically sensitive of all human diseases. (Fortin et al., 2002; Kwiatkowski, 2005)

Malaria is a mosquito-borne infectious disease caused by an eukaryotic protist of the genus Plasmodium. It is widespread in tropical and subtropical regions, including parts of the Americas, Asia, and Africa. Each year, there are approximately 300–500 million cases of malaria, killing between one to three million people each year, the majority of whom are young children in sub-Saharan
Africa where 750,000 children die annually. Ninety percent of malaria-related deaths occur in sub-Saharan Africa, (Utzinger et al., 2002; Uneke, 2008; and Marangi et al., 2009). It is estimated that in Africa about 74% of all African population live in areas that are highly endemic by malaria, 19% in epidemic areas and only 7% live in low malaria risk areas (WHO, 2006). Malaria is commonly associated with poverty, but is also a cause of poverty and a major hindrance to economic development. In addition of the influence of malaria on human evolution, it is believed that ecological differences associated with malaria prevalence are perhaps the most important reason why some countries today are rich and others poor (Douglas and Christian 2007).

Four species of the plasmodium parasite can infect humans; *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae*. The most serious forms of the disease are caused by *Plasmodium falciparum*.

*Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* causes milder disease in humans that is not generally fatal. A fifth species, *Plasmodium knowlesi*, is a zoonosis that causes malaria in macaques but can also infect humans (Fong et al., 1971).

After mosquito bite, sporozoites rapidly invade hepatocytes in which it develop to mature schizonts containing thousands of merozoites, the hepatocyte ruptures and the merozoites are released in to the blood stream where each merozoite can invade an erythrocyte (fig 1.1) (Tolia et al., 2005) through specific protein-protein
interactions and an intracellular translocation machinery to power the process (Gilberger et al., 2003).

Merozoites develop inside the erythrocyte through the ring, trophozoite and schizont stages (Gaur et al., 2004), to release merozoites that invade new uninfected erythrocytes. This invasion process considered as a central point in the erythrocytic life cycle. The presence of multiple invasion pathways is believed to be a survival strategy of the malaria parasite (Lobo et al., 2004).

Figure 1.1 Diagram depicting the life cycle of human malaria (Asexual phase in human body and sexual phase in the mosquito). (www.net4nets.net).
1.2 Epidemiology of malaria:

Malaria was described for the first time in China around five thousand years ago, but it is thought to have originated in Africa and to have spread subsequently into Asia and the Mediterranean (Douglas et al., 2007). It has been suggested that the origins and spread of the disease in the Old World paralleled the spread of sedentary agriculture (Tishkoff et al., 2001).

Worldwide, Malaria is a major human problem, and its epidemiological patterns are widely different, such information about malaria epidemiology can help programs designers to improve strategic interventions (Himeidan et al., 2005). Over 3 billion people live under the threat of malaria. It kills over a million each year – mostly children (World Malaria Report 2005-WHO). About 90% of all malaria deaths in the world today occur in Africa south of the Sahara. This is because the majority of infections in Africa are caused by Plasmodium falciparum, the most dangerous of the four human malaria parasites. It is also because the most effective malaria vector - the mosquito Anopheles gambiae - is the most widespread in Africa and the most difficult to control. An estimated one million people in Africa die from malaria each year and most of these are children under 5 years old (WHO, 2002).
Figure 1.2: The distribution of the endemic malaria in Africa.

Source: MARA/ARMA (Mapping Malaria Risk in Africa / Atlas du Risque de la Malaria en Afrique).

1.3 Malaria in Sudan:

In Sudan, malaria remains a major public health problem causing about 7.5-10 million cases and 35,000 deaths every year (Himeidan et al., 2005). Malaria risk covers all over the country with considerable differences in its epidemiicity, where in the northern, eastern, and western states, malaria is mainly low to moderate with predominately seasonal transmission and epidemic outbreaks. In southern and central Sudan, malaria is moderate to high or highly intense generally with perennial transmission (Abdalla et al., 2007; Zaroog et al., 2009).
In spite of the presence of all of the four plasmodium species that can cause malaria to a human, \textit{P.falciparum} remains the main malaria parasite in Sudan and is responsible of causing more than 95\% of malaria cases (Elhassan, 1995; Elkhalifa \textit{et al.}, 2008). The main malaria vector in Sudan is the female \textit{Anopheles arabiensis} formerly known as species B of the \textit{A. gambiae} complex. In addition to \textit{An. gambiae}, \textit{An. funestus}, \textit{An. merus}, \textit{An.bwambae}, \textit{An. pharoensis} and \textit{An. nili} are involved in the transmission in localized areas (Elhassan, 1995; Coetzee \textit{et al.}, 2000).

According to another studies, malaria incidence in Sudan was estimated to be about 9 million episodes in 2002 with a case prevalence of 93 per 1000, representing a 25\% of hospital admissions among children. About 44,000 deaths in 2002 were attributed to malaria (Zeidan, \textit{et al.}, 2006; Zaroog, \textit{et al.}, 2009).

\textbf{1.4 Clinical features of malaria:}

Malaria is a parasitic disease caused by one of four malaria parasites. The four kinds of malaria can be sufficiently similar in their symptoms to make species differentiation generally impossible without laboratory studies (WHO, 2003a).

World Health Organization has defined a severe malaria patient as a febrile patient with \textit{falciparum} malaria infection and some complications of no other obvious causes who requires emergency hospitalization treatment (WHO 1991).

Symptoms of malaria include fever, shivering, arthralgia (joint pain), vomiting, anemia (caused by hemolysis), hemoglobinuria, retinal damage and convulsions (Beare \textit{et al.}, 2006). The classic symptom of malaria is
cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting four to six hours, occurring every two days in *P. vivax* and *P. ovale* infections, while every three days for *P. malariae* (Cunha and Cunha, 2008). *P. falciparum* can have recurrent fever every 36–48 hours or a less pronounced and almost continuous fever. For reasons that are poorly understood, but that may be related to high intracranial pressure, children with malaria frequently exhibit abnormal posturing, a sign indicating severe brain damage (Idro *et al.*, 2005). Malaria has been found to cause cognitive impairments, especially in children. It causes widespread anemia during a period of rapid brain development and also direct brain damage. This neurologic damage results from cerebral malaria to which children are more vulnerable (Boivin *et al.*, 2002 and Holding *et al.*, 2001). Cerebral malaria is associated with retinal whitening, (Maude *et al.*, 2009) which may be a useful clinical sign in distinguishing malaria from other causes of fever (Beare *et al.*, 2006).

Severe malaria is almost exclusively caused by *P. falciparum* infection, and usually arises 6–14 days after infection (Trampuz *et al.*, 2003). Consequences of severe malaria include coma and death if untreated, young children and pregnant women are especially vulnerable. Splenomegaly (enlarged spleen), severe headache, cerebral ischemia, hepatomegaly (enlarged liver), hypoglycemia, and hemoglobinuria with renal failure may occur. Renal failure is a feature of blackwater fever, where hemoglobin from lysed red blood cells leaks into the urine. Severe malaria can progress extremely rapidly and cause death within hours.
or days (Trampuz et al., 2003). In the most severe cases of the disease, fatality rates can exceed 20%, even with intensive care and treatment (Kain et al., 1998). In endemic areas, treatment is often less satisfactory and the overall fatality rate for all cases of malaria can be as high as one in ten (Mockenhaupt et al., 2004). Over the longer term, developmental impairments have been documented in children who have suffered episodes of severe malaria (Carter et al., 2005).

Chronic malaria is seen in both *P. vivax* and *P. ovale*, but not in *P. falciparum*. Here, the disease can relapse months or years after exposure, due to the presence of latent parasites in the liver. Describing a case of malaria as cured by observing the disappearance of parasites from the bloodstream can, therefore, be deceptive. The longest incubation period reported for a *P. vivax* infection is 30 years (Trampuz et al., 2003). Approximately one in five of *P. vivax* malaria cases in temperate areas involve overwintering by hypnozoites (i.e., relapses begin the year after the mosquito bite) (Adak et al., 1998).

### 1.5 Immunity to malaria:

Immunity to any disease often results from exposure to that disease. This applies to malaria but not perfectly. Human malarias appear to have evolved the ability to avoid the human immune system, and humans cannot develop a complete immunity to malaria as they can with, for example, smallpox. It is possible, however, to develop a partial immunity to malaria as it is seen in older children and adults, where they can develop essentially complete protection from severe illness and death, although sterile immunity is probably never achieved.
Innate or adaptive immune effector mechanisms can limit the peak of parasitaemia, prevent severe pathology and reduce the load of circulating infected cells. However, they typically fail to eliminate the infection completely, leading to persistent low-grade parasitaemia, which might frequently fall below the limit of detection by microscopy, but which might persist for many months or years (Franks et al., 2001; Stevenson et al., 2004). Children in endemic areas who are born to immune mothers are protected against disease during their first six months by maternal antibodies. This passive immunity is followed by one or two years of increased susceptibility before acquisition of active immunity (Perlmann et al., 2002). In spite of the presence of some studies showing that the immune responses to the pre-erythrocytic stages probably have limited involvement, Immune attack could theoretically be directed at any point in the life cycle from the time of entry of the sporozoite to the formation of the gametocyte (Owusu-Agyei et al., 2001).

The mechanisms by which antibody is effective include blockade of the invasion of RBCs by merozoites, antibody-dependent cellular killing mediated by cytophilic antibodies and binding of antibody to parasite-induced molecules on the RBC surface, leading to greater clearance of infected RBCs. However, the relative importance of each of these mechanisms is still a matter of debate (Langhorne J et al., 2008).

Strategies to limit plasmodium evasion from the human immune system must be based on approaches to induce immunity that do not mimic or closely resemble...
natural encounter with the parasite, because clearly the parasite is adept at avoiding the immune responses that follow (Good et al., 2004).

### 1.5.1 Innate immunity:

Natural (innate) immunity to malaria is an inherent property of the host, a refractory state or an immediate inhibitory response to the introduction of the parasite, not dependent on any previous infection with it (Doolan et al., 2009). Unlike other infections with intracellular pathogens, including viruses, bacteria and some protozoan parasites, in which the role of the innate immune response has been well investigated during the past few years, relatively few studies have addressed the role of innate immunity to malaria in either mouse models or humans. Accumulating evidence, however, also indicates a crucial role for innate immune responses in protective immunity to malaria (Stevenson et al., 2004).

The inhibition of the parasite growth by the human host innate mechanisms is probably the reason for the low parasitemias seen in acute *P. falciparum* (Perlmann et al., 2002). Nevertheless, the potential for innate immune mechanisms to provide rapid protection against malaria has been largely neglected (Stevenson et al., 2004).

In spite of the fact that malaria infection gives rise to strongly elevated blood concentrations of non-malaria-specific immunoglobulin, the importance of the underlying polyclonal B-cell activation for innate immunity is not known. This is also true for the CD4-T cells from malaria-naïve donors responding by *in vitro* proliferation and cytokine production upon exposure to malaria antigens. In
contrast, neutrophils, mononuclear phagocytes and natural killer (NK) cells appear to play a role in innate immunity seen early in malaria infections. In particular, NK cells have been shown to increase in numbers and to be able to lyse *Plasmodium falciparum*-infected erythrocytes *in vitro*. However, NK cells are also potent producers of cytokines such as interferon-γ (IFN-γ) and this capacity, leading to parasiticidal macrophage activation, may be of greater importance for innate malaria immunity than their potential to lyse infected host erythrocytes. Related cell types probably playing a role in innate malaria immunity are the NKT cells which in mice carry both the NK1.1 surface marker and αβ T-cell receptors (TCR). These cells are potent inhibitors of liver-stage parasite replication in mouse malaria systems *in vitro* (Perlmann *et al.*, 2002).

**1.5.2 Adaptive immunity:**

Malaria infection gives rise to host responses which are regulated by three main effectors; innate immune system, adaptive immune system as well as environmental factors. Acquired immunity is both species- and stage-specific. It is rarely sterile, but rather associated with low-grade parasitemia and episodes of clinical disease throughout life (Perlmann *et al.*, 2002). Inadequate understanding of the mechanisms of naturally acquired clinical immunity against plasmodia may be an important factor contributing to the failure to develop a practical vaccine (Doolan *et al.*, 2009). Acquisition of active immunity to malaria is slow and requires repeated parasite exposure to be
maintained (Perlmann et al., 2002). In humans, various types of acquired or adaptive immunity against plasmodia have been defined (Doolan et al., 2009)

(i) Antidisease immunity, conferring protection against clinical disease, which affects the risk and extent of morbidity associated with a given parasite density.

(ii) Antiparasite immunity, conferring protection against parasitemia, which affects the density of parasites.

(iii) Premunition, providing protection against new infections by maintaining a low-grade and generally asymptomatic parasitemia

In term of antibody production, malaria infection induces both polyclonal and specific monoclonal immunoglobulin production. Although antibodies of different isotypes may have protective functions, IgG is most important in this respect. In protected individuals, cytophilic antibodies of IgG1 and IgG3 isotype have frequently been found to be the common isotypes (Perlmann et al., 2002).

1.6 Genetic susceptibility to malaria:
A few years before it was proved that genes are made of DNA, (in the late 1940s) scientists began to suspect that malaria had influenced human evolution in a big way. The clues were that malaria parasites invade human red blood cells, and that diseases of red blood cells such as thalassaemia and sickle-cell anaemia, which are the commonest group of genetic disorders in humans, are mainly found in populations exposed to malaria and their descendants (Kwiatkowski, 2005).
The completion of genome sequences for both *Homo sapiens* and *Plasmodium falciparum* makes this a hugely exciting time to be studying the molecular basis of susceptibility to malaria (Gardner *et al*., 2002). In humans, the influence of genetic factors on onset, progression, pathophysiology, and ultimate outcome of malaria infection has been well documented (Fortin *et al*., 2001; Fortin *et al*., 2002).

The big question, about which we still have relatively little information, is the extent to which susceptibility to malaria is determined by genetic variation in the human immune system.

This is a hugely important topic because the malaria vaccine effort is being slowed down by our poor understanding of the molecular basis of natural immunity, and one of the most effective ways of identifying critical immune mediators may be to determine how genetic variation in the corresponding genes affects susceptibility to malaria in the populations of malaria-endemic areas (Mackinnon *et al*., 2005). In order to get an answer for this very important question, Mackinnon and his colleagues studied two populations of children from a malaria-endemic area in Kenya for which they could obtain the necessary data; they found that host genetic factors accounted for approximately one quarter to one-third of the total variation in susceptibility in the populations to malaria (Figure 1.3). Of this percentage, only a small proportion could be attributed to the best known malaria resistance genes. This is consistent with other studies that suggest that malaria susceptibility is
under the control of many different genes, with each individual gene having a relatively small epidemiological effect (Mackinnon et al., 2005).

**Figure 1.3: The effect of different factors on the susceptibility for malaria disease** (Mackinnon et al., 2005).

It is remarkable how many different types of genetic variation of human red blood cells appear to have evolved due to natural selection by malaria (Kwiatkowski, 2005). They include thalassaemia, a common group of diseases arising from disordered regulation of haemoglobin production; HbC, another variant form of haemoglobin that protects against malaria but has fewer harmful consequences than HbS; variations in a red blood cell enzyme called glucose-6-phosphate
dehydrogenase; and ovalocytosis, a defect of a structural protein that helps to maintain the normal shape of the red blood cell (Fortin et al., 2002; Kwiatkowski, 2005).

Special mention must be made of a genetic factor possessed by many Africans that suppresses the Duffy antigen (a human protein on the surface of red blood cells that in part determines blood group) and thereby protects them from developing *Plasmodium vivax* infection (Handel et al., 2010).

In spite of the rapid growth in the last few years in the number of reported genetic associations with susceptibility and resistance to malaria—many of which involve immune system and inflammatory genes, still little is known about the effects of malaria on the evolution of the human immune system, possibly because the phenotypic consequences are more subtle than those of the classic erythrocyte variants; for example, alteration of a splenic dendritic cell receptor is not as easy to visualize as a sickling red cell (Kwiatkowski, 2005).

### 1.7 Malaria vaccines:

Among the potential control measures that have been given high priority by national and international health organizations, the development of a vaccine against malaria is recognized as one of the most promising and cost-effective addition to the arsenal of current malaria-control measures. Thus malaria vaccine development has been an active field of research for over two decades, with a primary focus on *Plasmodium falciparum*, the deadliest of the four species of Plasmodia parasites that infect man (Ballou et al., 2007). On the other hand, the
complexity of the immune system presents particular challenges to the development of malaria vaccines. The current understanding of malaria immunity was significantly predated by the cloning of malaria antigens in 1983. These events ignited enormous interest in malaria vaccine research, as the only approach otherwise available at the time was to culture parasites *in vitro* in human RBCs and extract plasmodial antigens in a strategy that would involve significant logistic and regulatory challenges (Good *et al.*, 2004). It is clear that the challenges to developing a malaria vaccine are very significant, given that there is no vaccine 27 years after blood-stage antigens were first cloned, and most optimistic predictions would say a vaccine is still at least 10 years away. Many of the challenges are immunological in nature (Good *et al.*, 2004). Although Scientists have recently confirmed that it is possible to develop a malaria vaccine, there are many challenges facing this development process, including: scientific unknowns such as the lack of full understanding of mechanisms of malaria infection, disease and immunity, inadequate resources, limited private-sector involvement, and uncertain mechanisms for procuring and distributing a successful vaccine (WHO/MVI, 2006).

There is increasing evidence that the parasite’s erythrocyte-invasion ligands, including the erythrocyte binding antigen-175 (EBA-175) and/or the rhoptry (Rh) proteins, may be suitable antigens for a blood-stage vaccine. Three doses of a DNA plasmid expressing EBA-175 region F2 followed by a single dose of the EBA-175 F2 protein were found to protect three Aotus monkeys, out of the four
vaccinated, against *P. falciparum* (Goodman *et al.*, 2010). The challenge for the next decade is to build the global epidemiological infrastructure required for statistically robust genome wide association analysis, as a way of discovering novel mechanisms of protective immunity that can be used in the development of an effective malaria vaccine (Kwiatkowski, 2005).

RTS,S (also known as RTS,S/AS) is the most clinically advanced malaria vaccine candidate up to date. In clinical trials, it was the first to demonstrate that it can protect young children and infants in malaria-endemic areas against infection and clinical disease caused by *Plasmodium falciparum* (Bejon *et al.*, 2008; Goodman *et al.*, 2010).

**1.8. Erythrocyte Binding Antigen- 175 (EBA-175):**

Unlike many other members of the phylum Apicomplexa, malaria parasites limit their infection of host cells to the restricted population of erythrocytes in the bloodstream. The invasion of erythrocytes and the subsequent cycles of growth, replication, and rupturing of infected cells are responsible for the majority of symptoms relating to malaria disease, with severe parasite infections giving rise to rapid hemolysis and metabolic acidosis. This makes the blood stage of the parasite life cycle a primary target for novel interventions to prevent invasion and combat malaria disease (Baum *et al.*, 2005).

Merozoites develop inside the erythrocyte through the ring, trophozoite and schizont stages (Gaur *et al.*, 2004) to release and invade new uninfected erythrocytes. This invasion process considered as a central point in the
erythrocytic life cycle, so, the presence of multiple invasion pathways is believed to be a survival strategy of the malaria parasite (Lobo et al., 2004).

The availability of the P.f genome sequence has allowed the identification of five major invasion pathways, two of them are well characterized, the first involving glycophorin A (GPA) and the erythrocytic binding antigen (EBA-175), the second one involve interaction between glycophorin C and a 140 KDa paralogue of EBA-175 (Lobo et al., 2004).

Natural selection maintains allelic variation in some antigens of the malaria parasite Plasmodium falciparum. Analysis of allele frequency distributions could identify the loci under most intense selection (David et al., 2000).

Some P.f strains mainly use ligands that bind to sialated receptors of erythrocyte, other strains use ligands that bind to receptors independently of sialic acid. However, switching from sialic acid dependent to independent invasion is reversible and depends on parasite ligand used (Stubbs et al., 2005).

One of the most important ligands mediating invasion of erythrocytes depending on sialated RBCs receptor is the (EBA-175), that bind to (GPA), the major glycophorin found on human erythrocytes and is heavily sialated(Cramer et al., 2004, Tolia et al., 2005) but it can not bind to glycophorin B (Sim et al., 1994).

This antigen is located in the microneme organelles at the apical end of merozoites (Binks et al., 2001; Cramer et al., 2004) and belong to a family of parasite adhesion molecules, the Duffy- Binding-like erythrocyte-binding proteins (DBL-EBP) (Cramer et al., 2004).
EBA-175 consists of 7 regions, in region II at the N terminus there are two cystine rich segments (F1, F2) responsible for the binding to the (GPA) (Figure 1.4), (Toure et al., 2006).

Disruption of these binding through modifying of erythrocytic surface or by gene disruption has shown that the merozoites can use other invasion pathways (Gilberger et al., 2004). The genetic bases of switching from sialic acid-dependent to sialic acid-independent invasion are unknown (Gaur et al., 2004; Stubbs et al., 2005).

Figure 1.4: Model for *P. falciparum* EBA-175- RII binding to the Red Blood Cell Receptor (GPA). The *P. falciparum* membrane is shown on the top and the erythrocyte membrane on the bottom. The receptor binding domain of EBA-175, RII, is shown as a surface representation with F1 in green and F2 in purple and the linker in gray. Blue lines represent portions of EBA-175 backbone not included in
the crystal structure. GPA is shown in red with the membrane-spanning region in detail using the NMR structure and the extracellular domain drawn as a schematic flexible line (Tolia et al., 2005).

It is well established that the gene that encodes the EBA-175 has a highly divergent dimorphic segments of sequences in region III (Binks et al., 2001, Toure et al., 2006), the first one detected in the FCR3 strains of P.f “referred to as the F loop” and the second in CAMP strains “referred to as the C loop” (Toure et al., 2001; Binks et al., 2001; Cramer et al., 2004).

F and C segments which are inserted at different positions in the coding sequence of exon 1 (Cramer et al., 2004) encoding 141 and 114 amino-acid respectively (Binks et al., 2001). These two divergent segments are conserved in all P.f examined to date (Kain et al.,1993, Ware et al.,1993) and since merozoites are haploid; each parasite has C or F segment, but not both or neither (Binks et al., 2001; Toure et al., 2006).

In spite of the role of this dimorphism in host-parasite interaction is unclear (Toure et al., 2001, Cramer et al., 2004), a study shows that the initial interaction of merozoite invasion involves binding of F or C segment to the glycophorin A backbone after binding of region II “of EBA-175” to sialic acid residues of glycophorin A (Binks et al., 2001; Toure et al., 2001; Toure et al., 2006), furthermore, it has been reported that the majority of P.f merozoites use the erythrocytic GPA receptor in the invasion process (Baum et al., 2003).
Study to determine distribution of F and C segment dimorphism among different five African parasite populations (Sudan is one of them) shows that the inspection of the allele frequencies at each polymorphic site reveals that Sudan is the most divergent population and Sudanese allele frequency of the C segment of EBA-175 is 73% (Binks et al., 2001). Another study among Ghanaian children explains that the C segment is not associated with severe malaria but confers a higher risk of fatal disease (Cramer et al., 2004).
Rationale and Objectives

The Erythrocyte Binding Antigen-(EBA-175) is a member of the erythrocyte binding protein (EBP) family of related multidomain transmembrane proteins (Cramer et al., 2004), which in turn used by the merozoite as part of a cascade of contact molecules involved in the multi-protein, multistep invasion process that ultimately sees the parasite gain access to the erythrocyte cytosol and establish a vacuole (Tolia et al., 2005).

There is increasing evidence that the parasite’s erythrocyte-invasion ligands, including the erythrocyte binding antigen-175 (EBA-175), may be suitable antigen for a blood-stage vaccine. Three doses of a DNA plasmid expressing EBA-175 region F2 followed by a single dose of the EBA-175 F2 protein were found to protect three Aotus monkeys, out of the four vaccinated, against *P. falciparum* (Goodman et al., 2010).

Furthermore, this cystine rich segment (F2) that shares the responsibility for the binding to the glycophorin A on the RBCs surface is homologous to the binding domains of other erythrocyte binding proteins such as *Plasmodium vivax* Duffy binding protein. Immunization with refolded PfF2 yields high titer antibodies that efficiently inhibit *P. falciparum* invasion of erythrocytes in vitro. Importantly, antibodies rose against PfF2 block invasion by a *P. falciparum* field isolate that invades erythrocytes using multiple pathways (Pandey et al., 2002; Toure et al., 2006).
The safety and immunogenicity of aluminum phosphate-adjuvanted, recombinant EBA-175 vaccine in healthy young adults living in the US has been evaluated and shown to be safe and immunogenic in malaria-naïve subjects (El Sahly et al., 2010).

Human sera from subjects exposed to *P. falciparum* malaria contain IgG which reacts with the Cseg and Fseg sequences in recombinant proteins (Okenu et al., 2000), if such naturally acquired antibodies were protective, then the allelic forms might be selectively maintained in the parasite population by a frequency-dependent process (Binks et al., 2001).

Therefore the study was designed to study the distribution of the divergent dimorphic alleles of *P. falciparum* EBA-175 (F and C) in three different geographical areas and the possible association of this dimorphism with the severity of the disease.

**Specific Objectives:**

1- To determine the frequencies of the dimorphic allele segments of the *EBA-175* gene of the *P.falciparum* merozoite among malaria patients in Sudan.

2- To investigate whether this dimorphism has a significant association or impact on the clinical manifestation of the malaria disease.
Chapter Two

Materials and Methods

2.1. Study area and populations:

This study was conducted in three regions; in Eastern and Central Sudan (fig 2.1). The selected study areas were; Um-Salala village in Gedarif State - Eastern Sudan, Madani in Geizera state and Sennar in the Blue Nile state, both lies in the central Sudan. Um-Salala village is characterized by stable malaria transmission. Madani and Sennar located Eastern to Khartoum and are inhabited by different tribes and characterized by unstable malaria transmission (Himeidan et al, 2005).

Um-Salala village:

This village is located on the eastern bank of the River Rahad (Gedarif State) 400 km south-east of Khartoum. It was founded in 1969 by a groups migrated from western Sudan (near El-Geneina town, Darfur state which borders with Chad) and belongs to Masalit tribe. They are primarily farmers and they obtain additional income by selling animals, tanning hides, sewing and transporting goods. In addition to farming, the Masalit raise cattle, sheep and goats. Most of the Masalit live as nuclear families in village settlements. In Sudan the population of Masalit was 173,810 (2000 survey) and they live in Geneina, Mistere, Habila, Kajangise, Dar Fur, Dar Masalit, Nyala and Northern Sudan, and as scattered colonies in Dar Fongoro and south and east of Gedarif state. Um-Salala village is characterized by stable malaria transmission. (El Hassan et al., 2002; Khalil et al., 2002; Salih et al.,2010 ).
Madani:

Madani is the capital of Geizera state in east-central Sudan with population of 452,628. It lies on the west bank of the Blue Nile, nearly 85 miles (136 km) southeast of Khartoum the capital of Sudan. It is linked by railway to Khartoum and is the center of a cotton-growing region. Madani is a commercial centre of the Gezira agricultural district and is mostly residential. The city's facilities are more modernized than most places in the Sudan.

The peak of Malarial transmission occurs just after the rainy season, in October–November, and constitutes the main health problem in the area. The predominant species that causes the malaria infection in Madani is *Plasmodium falciparum* (Ahmed, 2005; Hassan *et al*., 2009).

Sennar:

Sennar is the ex-capital of the state of Sennar, located on the Blue Nile. For several centuries it was the capital of the Funj Kingdom of Sennar. The modern town lies 17km south-southeast of the ruins of the ancient capital. It is located close to Sennar Dam, which spurred agricultural activity when it was created in 1925 for crop irrigation. Sennar has an estimated total population of 1.2 million people, the majority of whom are farmers (Zeidan *et al*., 2006).

Today there are two towns bearing the name Sennar. Sennar Junction, the older one, which represents an actual continuation to the ancient Sennar, both in time and geographical location. The junction element came from the fact that many railways intersect there, it had been the heart of the country, which accompanied
the invasion of Sudan during the British colonization at the end of eighteenth century. The other is Sennar Al-Madeena (i.e. Sennar Town), 5km ESE of the Junction, located beside the Sennar Dam. It was built over the ruins of a small village called Mok-waar which was notable in the former cultures of the tribes of African origins who lived there before the arrival of Arab tribes to the Sudan.

Sennar is an endemic area of malaria but the transmission is highly seasonal and reaches the peak just after the rainy season i.e. September-October.

Figure 2.1: Map shows the study areas (Um-Salala, Sennar, and Madani) with red dots.
2.2. Ethical consideration of the Study:

The study was approved by the Ethical committee of the institute of endemic diseases, University of Khartoum. Informed consent was obtained from adults or parents of children before they were enrolled in the study. Questionnaires were filled including information about the family and clinical picture (Appendix-1).

Study design:

This study was cross-sectional study, hospital and village based. General and paediatric Madani Hospitals, Al-Emam Al-bukhari primary school, Sennar malaria centre, and Um-Salala village were surveyed in the time from the 2nd of November to the 1st of September 2007 and 2009.

2.3. Sample collection:

Peripheral blood samples were collected from a total of 339 microscopically confirmed *plasmodium falciparum* infected malaria patients-from the three different geographic aeries: Um-Salala 57 samples, Sennar 166 samples, Madani 116 samples. Mixed infections with other malaria species rather than *Plasmodium falciparum* were excluded, infection were confirmed by the ICT. Symptomatic severe malaria, symptomatic uncomplicated malaria, and asymptomatic malaria of all ages were involved.
Severe falciparum malaria has been defined by the WHO as:

(1) Asexual parasitemia with *Plasmodium falciparum* (although smear-negative cerebral malaria may occur).

(2) One or more of the defining criteria (Appendix 2).

Risk factors for development of severe falciparum malaria include:

(1) Splenectomy.

(2) Pregnancy, especially primigravid.

(3) Immunosuppression.

(4) Low immunity states: non-immune (lack of previous exposure), especially in small children, or lapsed immunity (due to living away from malarious area for several years).

2.4. Diagnosis of Malaria:

Two ml whole blood samples were collected in heparinized tubes from all confirmed malaria cases who were showed positive blood film (BF) for malaria. Another blood film and Immunochromatography test (ICT) were performed not only to confirm the infection, but also to differentiate between malaria plasmodium species, as *Plasmodium falciparum* is the only species under study. Thin and thick blood smears were air-dried in the field, transported to the central laboratory at Sennar for microscopic examination by experienced technicians. For those who were found to be infected with malaria parasites, the approximate
number of parasites per field was determined. For quality control, 10% of the slides were retested.

About 2ml of the collected blood sample was used to investigate different parameters: glucose level-and hemoglobin concentration- which help in defining disease severity more accurately.

2.5. DNA extraction:

Chelex extraction method was used for DNA extraction from the whole blood spotted and dried on filter papers.

In order to disinfect the working bench, it was wiped firstly with HCL, then with NaOH 5M, and finally with distilled water, Parasite DNA was extracted from the blots.

Each filter paper punch was incubated overnight at 4°C in 1 mL of 0.5% saponin in phosphate-buffered saline (PBS) (8 g of NaCl, 0.2 g of KCl, 1.15 g of NaH₂PO₄, 0.24 g of K₂HPO₄ per liter, pH 7.4). The punches were washed for 30 minutes in PBS at 4°C, transferred into new tubes containing 25 µL of 20% Chelex-100 and 75 µL of distilled water, and vortexed for 30 seconds. The tubes were heated at 99°C for 15 minutes to elute the DNA, vortexed, and centrifuged at 10,000 x g for 2 minutes.

The supernatants, which contained the DNA, were carefully removed to avoid transfer of the Chelex resin and transferred into new tubes.
2.6. DNA Quantification using NanoDrop:

DNA concentrations were measured with a NanoDrop (ND-1000) spectrophotometer and adjusted to 40 ng/μl.

The NanoDrop ND-1000 spectrophotometer uses a patented sample retention system that holds 1 μl of sample without the use of traditional containment devices such as cuvettes and capillaries providing a calculated DNA concentration (2 ng/μl to 3700 ng/μl for dsDNA). It essentially eliminates the need to perform dilutions and provide results in purity ratios in less than 10 seconds. This feature has helped to increase the workflow efficiency by removing time consuming traditional spectrophotometers. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. A ratio of ~1.8 was generally accepted as “pure” for DNA; a ratio of ~2.0 was generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

One μl sample was pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap was controlled to both 1mm and 0.2 mm paths. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear CCD array was used to analyze the light after passing through the sample.

The instrument was controlled by special software run from a PC, and the data was logged in an archive file on the PC.
2.7. Detection of *Plasmodium falciparum* infection using nested PCR:

The 339 blood samples were confirmed as +ve *P. falciparum* using nested PCR using the following program:- Pre heating at 95 °C/5 min just once at the beginning of the trial, followed by thirty Cycles of three multiple temperatures: Denaturation 95 °C /1 min, annealing was 54 °C /1 min, extension 70 °C /2 min, and one time final extension at 70 °C /5 min and incubation at 4 °C /1 hr using specific primers of the following DNA sequences:

For the first nested PCR:

Inner PCR forward Primer (rPLU1) : TCAAGATTACAACATGCAAGTGA

Inner PCR reverse primer (rPLU5) : CCTGTTGTGCCTAAACTTC.

For the second nested PCR:

Outer PCR forward Primer (rFAL1) :

TTAAACTGGTTTGGGAAAACCAAATATATT- Outer PCR reverse primer (rFAL2) : ACACAATGAACCTCAATCATGACTACCCGTC.

For all species detection PCR amplifications, the volume used for each reaction is 20 μl. A master mix containing all the reagents, except for the DNA, is prepared and aliquoted into the reaction tubes. The DNA template is always added last. The master mix for the first and nested PCR contains the same following reagents for one single test tube except for the set of the primers:

MgCl = 1μl, 10x buffer =2μl, dNTPs =0.4μl, Primer1 =1μl, primer2 =1μl, DNA Taq polymerase =0.5μl, and sterile D.W =12.1μl.
2.8. Genotyping of EBA-175:

In the past, the genotyping of EBA-175 was determined by polymerase chain reaction (PCR) followed by hybridization (Kevin & David, 1991). However, this method requires the use of specific primers and probes for each genotype during amplification and Southern blot analysis. Here, we used a nested PCR method which improves EBA-175 genotyping of blood samples from infected individuals (Toure et al., 2001). DNA samples confirmed as +ve P.f with nested PCR were genotyped for C seg/ F seg dimorphism of the EBA-175 with a nested PCR using the following condition for both, the first and the second round of the nested PCR: Hot start at 95°C/5 for 5 min, thirty cycles of: Denaturation at 95°C for 1 min, Primer annealing at 58°C for 1 min, Extension at 70°C for 2min, and final extension at 72°C/2min.

The volume used for each reaction is 25 μl. A master mix containing all the reagents, except for the DNA, is prepared and aliquoted into the reaction tubes. The DNA template is always added last. The master mix for the first and nested PCR contains the same following reagents for one single test tube except for the set of the primers:

MgCl = 1.5μl, 10x buffer =2.5μl, dNTPs =1μl, Primer1 =1μl, primer2 =1μl, DNA taq polymerase =0.2μl, Bovine Serum Albumin (BSA) =1μl, and sterile D.W =14.8μl.

The following primers have been used to amplify the gene encoding EBA-175 of *P. falciparum* (Genebank database with accession number L7755): EBA1 forward
(nucleotides 2336-2356) 5’caagaagcagttctgaggaa3’ EBA2 reverse (nucleotides 3060-3083) 5’tctcaacattcatattaacattc3’ for the first amplification (PCR); EBA3 forward (nucleotides 2351-2364) 5’gaggaaacactgaaatagcacad3’ and EBA4 reverse (nucleotides 3042-3065) 5’caattctccagctgttgaacat3’ for the second amplification (nested PCR).

2.9. Gel electrophoresis

In order to visualize the nested PCR end products, gel electrophoreses was prepared as follow: 1.5g agarose powder was mixed in a flask or a bottle with 75 ml 10 X TBE (Appendix 3). The mixture was heated till the agarose was completely dissolved. The agarose solution was cooled. Then, 1.5µl of 10 mg/ml of Ethidium promide stain was added and the solution was shaken and poured onto a plastic plate in a gel –casting stand with a comb in place. The gel was allowed to polymerize for about 20 minutes. Finally, the comb was removed, and the solid gel was transferred to a gel tank and 1% of TBE buffer was added to cover the gel. One Kb DNA molecular weight marker was loaded along with the nested PCR end products in the same gel. The gel was examined under UV light to visualize the DNA. The size of band was measured according to the migration of DNA molecular weight marker and was photographed in a gel documentation system (BioDoc-It Imaging System, Cambridge, UK).

The band size in plasmodium species detection was 206bp for plasmodium falciparum.
EBA 175 genotyping was revealed in 714bp for the divergent segment C loop and 795bp for the F loop. This PCR product size was obtained after addition of a conserved region of 372 bp to F and C segments as the original or the actual size of C and F alleles are 342bp and 423bp respectively.

2.10. Statistical analysis:
In most isolates only a single allele was present, either C or F. Other isolates contained both alleles (due to the infection with more than one *P.falciparum* genotype, since *plasmodium falciparum* are haploid) (Toure *et al.*, 2006).

The allele’s frequency F and C segments were scored separately. Allele frequencies among population were calculated, first collectively in all population, then secondly in each of the three different geographical study areas separately. Comparison between genotypes and phenotypes were carried out using Fisher exact test to determine the possible association between allele distributions and severity of the disease. The same test was used to detect the significance of the distribution of different alleles among the overall population and secondly among the population of central Sudan: Madani and Sennar.
Chapter Three

Results

3.1 Demographic Data:

The age of patients from all three study areas ranged from 1 year to 85 years, figure 3.1 shows the distribution of the age groups where 80% of the patients were less than 20 years old.

![Age distribution of all malaria cases](image)

**Figure 3.1: The distribution of age groups among the overall malaria patients from the three regions.**

Sex ratio was 1:1. The haemoglobin levels ranged from 48%–83% with an average of 59%, and glucose levels ranging from 70–240 mg/dl, with an average of 115mg/dl.
3.2- Genotyping results:

3.2.1- Species Specific identification of *Plasmodium falciparum*:

All 339 samples from different geographical study areas were confirmed as *P. falciparum* positive using a nested PCR (species detection). DNA band of 206bp in size representing *P. falciparum* (Figure 3.2).

![Figure 3.2](image)

**Figure 3.2** Representative a gel electrophoretic profile of the species detection of *P.falciparum* (206bp size band) using nested (PCR) products.

Lane 1 represents 100bp DNA molecular weight marker ladder.

Lane 8 represents a nested PCR negative control.

Lane 2, 4-7&9-15 represents nested PCR positive samples for *P.falciparum*.

Lane 3 represents a PCR failed sample.
3.2.2- Distribution of FCR3 and CAMP genotypes in study areas:

Nested PCR was used to detect both FCR3 and CAMP alleles. There were two bands of different sizes, whereas the allele specific primers were amplified to give 795 bp and 714 bp for F and C alleles respectively (Figure 3.3).

Figure 3.3: Representative electrophoretic profiles of the *Plasmodium falciparum* erythrocyte binding antigen (EBA)-175 nested (PCR) products.

Lane 18 represents 100 bp DNA molecular weight marker ladder.

Lane 17 represents nested PCR negative control.

Lane 1 and 16 are positive controls for F and C alleles respectively.

Lanes 9 and 15 are negative samples.

Lanes 2, 4, 6, 7, 13 and 14 are positive samples for FCR3 genotype.

Lanes 3, 5, 10, 11, and 12 are positive samples for CAMP genotype.

Lane 8 represents a sample with mixed infection (FCR3 and CAMP).
The overall allele frequencies of 339 isolates were; 41.0 % FCR3, 51.0 % CAMP and 8.0 % were mixed infection with CAMP and FCR3 strains. The distribution of FCR3 and CAMP genotypes in the three study areas are shown in Table 3.1 and figure 3.4.

In Sennar city, out of 166 samples, 95 samples (57.23 %) were detected as CAMP, 55 samples (33.13 %) FCR3 and 16 samples (9.64 %) as mixed infection.

In Madani, out of 116 samples, 66 (56.90 %) were detected as CAMP, 43 (37.07 %) FCR3 and 7 samples (6.03 %) as mixed infection.

The total number of samples from Um-Salala village was 57 samples all of them with mild malaria as the severe malaria is not common in this village, out of 57 malaria patient formerly confirmed as positive *P. falciparum*, 12 samples (21.1%) were detected as CAMP, 41 (71.9 %) as FCR3, and 4 samples (7.0 %) as mixed infection. In order to analyze this data Fisher exact test was used using the software (R statistics, version 1.2.11) to detect the significance of the distribution of different alleles firstly among the overall population and secondly among the population of central Sudan: Madani and Sennar. The difference of the FCR3 and CAMP genotypes distribution among the different study areas collectively was significant (p-value = 0.00). No significant differences in the distribution of genotypes in central Sudan-Sennar and Madani (p-value = 0.4983).
Table 3.1: Distribution of FCR3 and CAMP genotypes in study areas:

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<tr>
<th>Areas</th>
<th>Um-Salala (n=57)</th>
<th>Sennar (n=166)</th>
<th>Madani (n=116)</th>
<th>Total (n=339)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F allele</td>
<td>41 (71.9%)</td>
<td>55 (33.13%)</td>
<td>43 (37.1 %)</td>
<td>139 (41.0%)</td>
</tr>
<tr>
<td></td>
<td>(n=139)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C allele</td>
<td>12 (21.1 %)</td>
<td>95 (57.23 %)</td>
<td>66 (56.9 %)</td>
<td>173 (51.0%)</td>
</tr>
<tr>
<td></td>
<td>(n=173)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F/C allele</td>
<td>4 (7.0 %)</td>
<td>16 (9.64 %)</td>
<td>7(6 %)</td>
<td>27 (8.0 %)</td>
</tr>
<tr>
<td></td>
<td>(n=27)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
<td></td>
<td>0.00</td>
</tr>
</tbody>
</table>

Figure 3.4: The distribution of F and C genotypes among the selected population from the three study areas.
3.3- Distribution of the genotypes with the phenotypes:

Malaria phenotypes of all patients were categorized into three main groups: asymptomatic, mild, and severe malaria. All samples from Um-Salala were mild cases; other phenotypes were found in Sennar and Madani samples. Table 3.2 and figure 3.5 show the distribution of the F/C genotypes with the phenotypes in all three study areas.

Table 3.2: Distribution of the malaria clinical phenotypes with the genotypes

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>F</th>
<th>C</th>
<th>F/C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>13 (35.1)</td>
<td>21 (56.8)</td>
<td>3 (8.1)</td>
<td>37</td>
</tr>
<tr>
<td>Mild</td>
<td>117 (43.8)</td>
<td>130 (48.7)</td>
<td>20 (7.5)</td>
<td>267</td>
</tr>
<tr>
<td>Severe</td>
<td>9 (25.7)</td>
<td>22 (62.9)</td>
<td>4 (11.4)</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>139 (41.0)</td>
<td>173 (51.0)</td>
<td>27 (8.0)</td>
<td>339</td>
</tr>
</tbody>
</table>
Figure 3.5: The distribution of C and F alleles among all three phenotypic of the overall samples.

To determine the possible association between genotypes distributions and severity of the disease, Fisher exact probability test was used for all three groups collectively. There was no significant differences in the distribution of FCR3 and CAMP among asymptomatic, Mild and severe clinical (p= 0.2503).

3.3.1: Sennar region:

Most of Sennar cases (161 cases) were mild with age range 2-85 and an average of 10.49 years. The distribution of the age groups is shown in figure 3.6. The Hb average among Sennar cases was 64.95 %, Random blood glucose ranged from 43 - 95mg/dL, with an average of 64.43mg/dL, the body temperature ranged from 36.3-
41.2 °C with an average of 38.7°C, and the parasite count ranged from 1120 μL-100000 μL with an average of 40309.6 μL. Sex ratio was 1:1.

The genotype distribution among mild and severe cases was shown in table 3.3 and figure 3.6. 34.2% F allele, 57.1% C allele and 8.7% mixed infection for mild cases. F allele was absent among severe cases. C allele was found in three severe cases out of five and two showed mixed infection. When the distribution of genotypes among different malaria cases was examined in different study areas separately significant distribution of genotypes among mild and severe malaria cases was detected in Sennar, the C segment seems to associate with severe malaria (p value = 0.04).

Table 3.3: The distribution of the genotypes with the phenotypes among Sennar samples

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>C</th>
<th>F/C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td>N</td>
</tr>
<tr>
<td>Sennar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>55 (34.2%)</td>
<td>92 (57.1%)</td>
<td>14 (8.7%)</td>
<td>161</td>
</tr>
<tr>
<td>Severe</td>
<td>0</td>
<td>3 (60%)</td>
<td>2 (40%)</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>55 (33.1%)</td>
<td>95 (57.2%)</td>
<td>16 (9.6%)</td>
<td>166</td>
</tr>
</tbody>
</table>
Figure 3.6: The distribution of the genotypes with the phenotypes among Sennar samples

3.3.2- Madani region:

The age of malaria cases included ranged from 1.3-68 years and the distribution of the age groups is shown in figure 3.7, with an average of 13.19 years. Hb ranged from 35% -111% with an average of 71.94%, Random blood glucose ranged from 53-205mg/dl, with an average of 109.42 mg/dl, the body temperature ranged from 36.3-40 °C with an average of 38.36°C and the sex ratio was 2:1.2 male to female.

Temperature, glucose, and Hb were at a better situation in Madani compared to Sennar, although Sennar cases were mostly of mild malaria.
Figure 3.7: The distribution of age groups among malaria patients from Madani region.

The distribution of FCR3 and CAMP genotypes among malaria cases with different manifestation of the diseases was showed in table 3.4 and figure 3.8 (p-value=0.76). Out of 116 malaria cases involved from Madani, 37 were asymptomatic, 49 mild, and 30 severe cases.
Table 3.4: The distribution of the genotypes with the phenotypes among Madani samples.

<table>
<thead>
<tr>
<th>Madani</th>
<th>F</th>
<th>C</th>
<th>F/C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic</td>
<td>13 (35.1%)</td>
<td>21 (56.8%)</td>
<td>3 (8.1%)</td>
<td>37</td>
</tr>
<tr>
<td>Mild</td>
<td>21 (42.8%)</td>
<td>26 (53.1%)</td>
<td>2 (4.1%)</td>
<td>49</td>
</tr>
<tr>
<td>Severe</td>
<td>9  (30%)</td>
<td>19 (63.3%)</td>
<td>2 (6.7%)</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>66</td>
<td>7</td>
<td>116</td>
</tr>
</tbody>
</table>

Figure 3.8: The distribution of the genotypes with the phenotypes among Madani samples.
3.3.3- Um-Salala Region:

In Um-Salala region only mild malaria cases were detected. The total number was 57 cases. the genotypes distribution was 41 F allele (72%), 12 C allele (21%) and 4 (7%) with mixed infection C and F were detected.

Age group ranged from 1-62, with an average of 18.5, figure 3.9 shows the distribution of the age groups. The body temperature ranged from 41-36.4 with an average of 37.98, sex ratio was 1:1.6 male to female.

Figure 3.9: The distribution of age groups among malaria patients from Um-Salala region.

3.3.4. Distribution of the F and C alleles among sickle cell trait subjects:

Eleven asymptomatic malaria patients from Madani region show positive result for sickle cell test. The distribution of the F and C alleles among sickler was shown in
figure 3.10, where 4 out of 11 samples of sickler showed F genotype and 7 were C genotypes.

![Distribution of the F and C alleles among sickle cell trait subjects](image)

Figure 3.10: The distribution of the genotypes among sickler samples.

3.4. The correlation between F and C alleles’ distribution and parasitemia:

![Parasitemia by Genotype](image)

Figure 3.11: The correlation between F and C alleles’ distribution and parasitemia
As the parasitemia level of malaria parasite in the patient blood considered one of the parameters that determine the severity of the disease. The parasitemia of 49 samples were correlated to F and C genotypes (Figure 3.11 and Table 3.5), it was found that parasitemia in cases infected with parasites showed C alleles ranged from 1200 -/μl -100000 -/μl with an average of 40615.8 -/μl, while the parasitemia among F alleles carriers was ranged from 1120 -/μl -100000 -/μl with an average of 33042.4 -/μl with no significant p value of=0.059.

Table 3.5: The correlation between F and C alleles’ distribution and parasitemia

<table>
<thead>
<tr>
<th>Parasitaemia median (IQR)</th>
<th>C</th>
<th>F</th>
<th>Test stat</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parasitaemia</td>
<td>46400</td>
<td>15540</td>
<td>Mann-Whitney</td>
<td>0.059</td>
</tr>
<tr>
<td></td>
<td>(20000,88000)</td>
<td>(5570,55092.5)</td>
<td>Ranksum test</td>
<td></td>
</tr>
</tbody>
</table>
Specific ligand–receptor interactions are required for the invasion of *P. falciparum* merozoites into human erythrocyte. One of the most important ligands that mediate invasion through the sialated receptor Glycophorin A is the plasmodium falciparum ligand-Erythrocyte binding antigen 175 (EBA-175) (Gramer *et al.*, 2004, Tolia *et al.*, 2005).

The importance of the erythrocyte binding antigen (EBA-175) has been demonstrated by parasite gene knock out studies and invasion assays using erythrocytes treated with enzymes that cleave different receptors, showing that the EBA-175 is functional in parasites that invade by using either sialic acid-dependent or -independent pathways (Duraisingh *et al.*, 2003). Confirming the importance of the EBA-175, another study shows that the truncation of EBA-175 in W2mef strain was associated with switch toward a sialic acid-independent pathway of invasion. Furthermore, removal of the 3’ cysteine-rich region, cytoplasmic tail, and transmembrane region of EBA-175 is equivalent to lack of expression of the protein, and these regions are essential for EBA-175 function in both W2mef and 3D7 (Reed *et al.*, 2000).

The Erythrocyte binding antigen 175 (EBA-175), one of the most important malaria vaccine candidates (Cramer *et al.*, 2004; Toure *et al.*, 2006), has been studied extensively over the last decade in different malaria endemic areas in Asia.
and Africa (Toure et al. 2001, 2006; Dittrich et al., 2003; Cramer et al. 2004; Heidari et al., 2009 Soulama et al., 2010).

Within the context of the global interest, a nested PCR method was used in this study in order to detect two alleles of EBA-175 (F-fragment and C-fragment) and to map their distribution in three different geographical areas: Um-Salala (Eastern Sudan) and Madani and Sennar (Central Sudan). The study also aimed to determine the possible association of this dimorphism to the clinical outcome of the disease.

With a combined pool of isolates derived from 339 patients, both fragment types had nearly the same distribution within the population of Madani and Sennar. The frequency of the C allele was slightly higher than that of the F allele in Madani (37.1% and 56.9%, respectively) and Sennar region (33.13%, 57.23%, respectively) while the percentage of the co-infection with both alleles C/F were 9.64% and 6% for Sennar and Madani respectively.

The geographical proximity of the two groups of Madani and Sennar, which results in their contact and intermarriage between the groups, seems to be main factor in the similar allelic distribution as this can cause gene transference if we assume that the distribution of the dimorphic alleles is affected by the genetic background of the human host. On the other hand, the important finding is that, in the third region (Um-Salala, Eastern Sudan), the distribution of F allele was markedly higher than that of C allele. The latter finding disagrees with previous studies by Binks et al (2001) that took place in Eastern Sudan (Daraweesh and
Asar regions) and other four different African countries, where they reported that the distributions of C in Eastern Sudan was markedly larger than that of F allele. This contradiction might explained by the different genetic background of the two populations of Um-Salala on side and Daraweesh and Asar villages on the other side, given the probability that the pattern of the F and C alleles’ distribution might be affected by the genetic make up of the infected human host. It is worth mentioning that all malaria patterns in Um-Salala is characterized by asymptomatic form of malaria while in Madani the asymptomatic cases are relatively few but almost non for Sennar where most of the cases were of mild and severe malaria. However the pattern of the EBA-175 genotypes distribution in Um-Salala showed that F was more distributed than C. This result is similar to that obtained in the Lao PDR and Iran in Asia, Gabon and Burkina Faso in Africa (Dittrich et al., 2003; Toure et al., 2006; Heidari et al., 2009; Soulama et al., 2010). A study that took place in four malaria endemic areas in Burkina Faso among children under five years age, the first study to assess the impact of low and high malaria transmission seasonal variation on the distribution of the eba-175 allelic forms, showed that F- alleles were more prevalent than C-alleles in the low [0.66 vs. 0.34 (P < 0.0001)] and high transmission season [0.67 vs. 0.33 (P < 0.0001)]. This implies that the distribution of F and C alleles is not affected by seasonal variation. When they applied Sewall Wright rules on their data they found that the population pair wise FST values, between different areas and different seasons indicate a moderate but statistically significant genetic
differentiation (Soulama et al., 2010). In the contrast, however, Binks et al (2001) in Sudan and Heidari et al (2009) in Iran didn’t find such genetic differentiation.

Regarding the effect of the distribution of this dimorphism on the outcome of the disease, we compared their distribution to the phenotypes of the disease which was categorized into three groups: Asymptomatic, Mild, and Severe malaria. There was no significant differences in the distribution of FCR3 and CAMP among asymptomatic, mild and severe clinical forms of malaria among whole populations from the three study areas p= 0.2503. When the distribution of genotypes among different malaria cases was examined in different study areas separately, a significant distribution of genotypes among mild and severe malaria cases was detected in Sennar area only, where the C allele was associated with severe cases (p = 0.03564). This result was in agreement to some extend with a study among Ghanaian children; where results showed that the C-segment is associated with fatal outcome in children with severe malaria, suggesting that it may contribute to the virulence of the parasite (Cramer et al., 2004). Our finding is in contrast to Heidari et al 2009 as they concluded that there was no significant correlation between clinical outcomes and the EBA-175 fragments types in their study area in Iran.

In order to detect the possible effects of sickle hemoglobin (HbS) trait on the differential selection of F and C alleles, eleven sickler cases infected with Plasmodium falciparum were genotyped for F and C fragments, the C allele found to be more frequent than F allele, 7: 4 C to F respectively. This finding contradicts
with Cramer's et al (Cramer et al, 2004) and suggests an uncoupling of the C allele and virulence. The Clinically protected human hosts, including sicklers, are known to get immunity faster than others due to modulated immune response. Sicklers were found to have antiparasitic as well as anti inflammatory cytokine responses (Hassan et al., 2009). The higher frequency of the C allele among sicklers may suggest a survival advantage of these parasites in these clinically protected individuals. This survival advantage may render sicklers into safe reservoirs for the C allele parasites. If this is the case, sicklers would help in the wide distribution of the C allele parasites in the societies among whom the sickle gene exist and that is probably the case in Um-Salala. These speculations might be augmented by our finding that the C allele population showed higher average parasitaemia (40615-/μl) than the F allele population (33042-/μl).

Furthermore, aiming to detect the relation of this dimorphism to the severity of the disease, F and C distribution was correlated to the parasitemia level on the infected individuals, hence the C allele found to have the highest average of parasitemia than the F one, 40615.8-/μl: 33042.4-/μl C to F respectively. Giving the possibility that C allele might be related to the severe cases more than F allele. However, Heidari et al (2009) they didn’t find significant correlation between parasitemia, gender, age of subjects and clinical outcome with the two fragments of EBA-175 gene.
Conclusion and Recommendations

In conclusion:

- This study showed that significant differential distribution of F and C fragments in different geographical areas endemic with Malaria which could explain the different pattern of malaria in Um-Salala village and villages in central Sudan.

- No significant association was observed between F and C alleles and different Malaria phenotypes, this association was found in Sennar area but the samples size was too small so this results needs to be verified.

Recommendations:

- More studies on larger populations are needed to understand the distributional pattern of EBA-175 alleles according to clinical outcomes of the disease.
References


A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses, Nature America Inc 6:6, 689-692


MARA/ARMA (Mapping Malaria Risk in Africa / Atlas du Risque de la Malaria en Afrique.


Appendix 1: Questionnaires including information about the family and clinical pictures.

University of Khartoum
Institute of Endemic diseases

Enrollment form

ID/No……………………………………………………………………………………………

Date……………………………………………………………………………………………

Gender…………………………………………………………………………………………

Age……………………………………………………………………………………………

Place of Birth…………………………………………………………………………………

Tribe…………………………………………………………………………………………

**General appearance:**

- Well
- Ill
- Severely ill

Temperature……………………………………………………………………………………

**Symptoms and clinical examination:**

- Fever
- Backache
- Vomiting
- Anemia
- Cough
- Spleenomegally
- Diarrhea
- Joint pain
- Abdominal pain
- Nausea
- Convulsions
- Headache

**Samples**

- Filter paper
- Blood
- Buccal
- Buccal swab

**Laboratory results:**

- HB……………………………………….g/dl.
- Random blood glucose…………………………………………………………….mg/dl.
- ICT for Malaria: Negative Positive
- Thin blood film: Negative positive
- Parasitemia ………………………………..(parasites/200 Leukocytes).
Appendix 2: The criteria of the severe malaria according to the definition of the WHO. (1999-2000)

<table>
<thead>
<tr>
<th>Defining Criteria</th>
<th>Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>cerebral malaria \ (unrousable coma)</td>
<td>Unrousable coma not attributable to any other cause in a patient with falciparum malaria. Coma should persist at least 30 minutes after a generalized convulsion to make the distinction from transient post-ictal coma.</td>
</tr>
<tr>
<td>severe normocytic \ anemia</td>
<td>Normocytic anemia with hematocrit $&lt; 15%$ or hemoglobin $&lt; 5\text{ g/dL}$ in the presence of parasitemia $&gt; 10,000$ parasites per $\mu\text{L}$. If microcytic indices seen, need to consider iron deficiency anemia, thalassemia and hemoglobinopathy.</td>
</tr>
<tr>
<td>renal failure</td>
<td>Urine output $&lt; 400\text{ mL}$ in 24 hours in adults, or $12\text{ mL}$ per kg in children, failing to improve after rehydration, and with serum creatinine $&gt; 265\text{ µmol/L}$ (3 mg/dL)</td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td>Whole blood glucose $&lt; 2.2\text{ mmol/L}$ ($&lt; 40\text{ mg/dL}$)</td>
</tr>
<tr>
<td>circulatory collapse, shock</td>
<td>Hypotension (systolic blood pressure $&lt; 50\text{ mm Hg}$ in children 1-5 years old; $&lt; 70\text{ mm Hg}$ in adults) with cold, clammy skin or a core-to-skin temperature difference $&gt; 10\text{ °C}$</td>
</tr>
<tr>
<td>Spontaneous bleeding. DIC</td>
<td>spontaneous bleeding from gums, nose, GI tract or other sites, with laboratory evidence of DIC</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>repeated generalized seizures</td>
<td>more than 2 observed seizures (&gt;=3) within 24 hours despite cooling</td>
</tr>
<tr>
<td>Acidemia or acidosis</td>
<td>arterial pH &lt; 7.25, plasma bicarbonate &lt; 15 mmol/L</td>
</tr>
<tr>
<td>Malarial hemoglobinuria</td>
<td>need to exclude hemoglobinuria due to antimalarial medications and to G6PD deficiency</td>
</tr>
<tr>
<td><strong>Additional Criteria</strong></td>
<td><strong>Finding</strong></td>
</tr>
<tr>
<td>impaired consciousness but rousable</td>
<td>impaired consciousness less marked than unrousable coma, can localize a painful stimulus</td>
</tr>
<tr>
<td>prostration and extreme weakness</td>
<td>patient unable to sit or walk, with no other obvious neurological explanation</td>
</tr>
<tr>
<td>Hyperparasitemia</td>
<td>very high parasite densities are associated with increased risk of severe disease but is affected by the immune status (more than 5% parasitemia in non-immune is serious, but may be well tolerated in semi-immune children); &gt; 500,000 per µL</td>
</tr>
<tr>
<td>Jaundice</td>
<td>total bilirubin &gt; 50 µmol/L (&gt; 3 mg/dL)</td>
</tr>
<tr>
<td>Hyperpyrexia</td>
<td>rectal temperature &gt; 40 °C</td>
</tr>
</tbody>
</table>
Appendix 3: Laboratory work requirements:

A. Equipment:

Autoclave

Incubator (iso-temp)

Horizontal Gel electrophoresis apparatus

Micropipette

Power supply

Thermo-cycler

Vortex

Freezer

Glass ware

Gel documentation system

B. Reagent:

10XTBE buffer

Tris Base.................................108g

Boric Acid..............................55g

EDTA.................................9.5g

H2O.................................750ml

2% Agrose gel electrophoresis:

Agrose ......................1.5g

d H2O..............................68ml

10XTBE.........................8ml
**PBS Buffer** (5x in 500 ml):

- NaCl ..................................................20.45 g
- KCl .....................................................0.465 g
- Na2HPO4*7 H2O ....................................10.142 g
- KH2PO4..............................................0.545 g
- pH 7.2

**6X Loading dye:**

- Glycerol.............................................30% (v/v)
- Bromophinol Blue .........................25% (w/v)
- Xylin cyanole ...................................25% (w/v)
- 1mM EDTEA........................................25%