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Prevalence and Quantities Assessment of Cytokines in Malaria Infection in East Nile locality-khartoum state.

معدل انتشار الملاريا والقياسات الكميه للسايتوكينات في الاصابه بمرض الملاريا

في محليه شرق النيل – ولاية الخرطوم

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الآيـــــــة

قال تعالى:

بسم الله الرحمن الرحيم

(إِنَّ اللَّهَ لاَ يَسْتَحْيِي أن يَضْرِبَ مَثَلاً مَّا بَعُوضَةً فَمَا فَوْقَهَا فَأَمَّا الَّذِينَ آمَنُواْ فَيَعْلَمُونَ أَنَّهُ الْحَقُّ مِن رَّبِّهِمْ وَأَمَّا الَّذِينَ كَفَرُواْ فَيَقُولُونَ مَاذَا أَرَادَ اللَّهُ بِهَذَا مَثَلاً يُضِلُّ بِهِ كَثِيرًا وَيَهْدِي بِهِ كَثِيرًا وَمَا يُضِلُّ بِهِ إِلاَّ الْفَاسِقِينَ)

صدق الله العظيم

(سورة البقرة - الاية 26)

Dedication

I dedicate this study with my respect to .....

my parents, sisters and brothers, teachers, colleagues and fellow members who supported me to complete this thesis.

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Above all, thanks to ALLAH for his mercy and guidance in giving me full strength to complete this work.

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Abstract

A cross sectional study was carried out from May to July 2018 in the East Nile locality which Located in the eastern part of the Khartoum state to determine the prevalence of malaria and quantities assessment of cytokines that are supposed to be involved in malaria pathogenesis.

Peripheral venous blood samples were taken from patients for making blood film as well as for serum cytokines concentration measurement .10 selected negative (control) samples and 29 positive (patients) samples were determined using enzyme-linked immunosorbent assays obtained commercially.

The prevalence of malaria among 384 randomly selected patients in the East Nile, s revealed a prevalence rate of 18.5%. *Falciparum* malaria is the most prevalent and constitutes about 13% of all infections, while *vivax* malaria has prevalence of 4.6% and the lowest prevalence rate 0.8% was for mixed infection (*P. falciparum* and *P.vivax*). The study showed that Males had higher prevalence rate 22.7% than females 15.6% rate. The prevalence based on age groups revealed that highest prevalence rate 20.1% was reported in the age group less than 10years old followed by11-49 age group with prevalence rate of 19.7% and the lowest prevalence rate 2% was reported among more than 50 year age group. the highest prevalence rate 53.8% of moderate parasitemia was reported among the less than 10 years age group, high prevalence rate 34.5% of mild parasitemia was reported among the 11-49 years age group, Lower prevalence rates of 2% and mild parasitemia 12.1% was reported among age group more than 50 years old.

In this study detailed analysis showed manifestation of the disease is significantly associated with elevated serum levels of IFN-γ in comparison with the values measured in the sera of the healthy controls. Sex-specific cytokine profiles showed that males produce high levels of IL-10, than the females, and there was a significant difference. But there was no significant different in the production of

IFN-γ, and TNF-α. levels of cytokine responses for age groups less than 10 years , 11-49 and more than 50 age group, for both children and adults, the median levels of IL-10 and IFN-γ responses were shown greater than TNF-α**.** In addition, association was found significant between IL-10 serum concentration and age groups. No significant correlation of cytokines levels and parasitaemia was found in the studied group. Study also found significant correlation of cytokines level and recurrent shown in TNF-α- at and IL – 10.

The study concludes that, the IL – 10 was significant with age group and gender as well as in recurrent infection.

المستخلص

أجريت دراسة مقطعية في الفترة من مايو إلى يوليو 2018 في محلية شرق النيل والتي تقع في الجزء الشرقي من ولاية الخرطوم لتحديد مدى إنتشار الملاريا و قياس كميات السيتوكينات المفترض ان تشارك في إمراضيه الملاريا.

تم أخذ 29 عينات إيجابية و10 سلبيات من الدم الوريدي المحيطي من المرضى لتصنيع فيلم الدم وكذلك قياس تركيز السيتوكينات المصلية تم تخزينها في -20 c حتى حين التحليل ، تم قياس تركيزات المصل من IFN- γ _ ، TNF-a IL-10 باستخدام المقايسات الممتصة المرتبطة بالإنزيم (اليزا) التي تم الحصول عليها تجاريا و تمت مقارنته مع الكنترول.

معدل إنتشار مرض الملاريا بين 384 مريضاً تم اختيارهم عشوائياً من محلية شرق النيل هو (18.5٪)، وأن معدل الاصابة بطفيل فالسبرم هو أكثر إنتشاراً ويمثل حوالي (13٪) من جميع الإصابات ، بينما معدل الإصابة بطفيل فيفاكس هو (4.6٪). وأدنى معدل إنتشار نتيجه للاصابهباكثر من نوع من طفيل فالسبرم وفيفاكس (0.8٪). واوجدت هذه الدراسة ان للذكور معدل إنتشار أعلى (22.7٪) من الإناث (15.6٪) كما بينت نسبة الإنتشار على أساس الفئات العمرية أن أعلى معدل إنتشار (20.1٪) تم الإبلاغ عنه في الفئة العمرية (أقل من10) سنه تليها الفئة العمرية (11-49) سنه بمعدل إنتشار (19.7٪) وأدنى معدل إنتشار تم الإبلاغ عن معدل (2٪) بين أكثر من 50 سنة. أعلى نسبة إنتشار (53.8٪) من العدوى الطفيلية المعتدلة كانت مرتبطة بالفئة العمريه أقل من10 واعلى معدل إنتشار (34.5٪) من العدوى الطفيلية الخفيفة سجلت بين الفئة العمرية11-49، ومعدل إنتشار أقل (20٪) عدوى طفيلية خفيفة في الدم (12.1٪) لوحظ في الفئة العمرية أكثر من 50 سنة.

أظهرالتحليل التفصيلي للسايتوكاينات ان مظاهر المرض ترتبط بشكل كبير مع ارتفاع مستويات المصل من IFN-γ بالمقارنة مع القيم المقاسة في الأمصال الصحيحة. الملامح السيتوكينية الخاصة بالجنس اوجدت أن الذكور تنتج مستويات عالية من IL-10 ، من الإناث ، وكان هناك إرتباط كبير .ولكن لم يكن هناك إختلاف كبير في إنتاج IFN-γ ، و.TNF-α , مستويات استجابات السيتوكين للفئات العمرية (أقل من 10) ، (11-49) و(أكثر من50)، لكل من الأطفال والبالغين، تم إظهار المستويات المتوسطة من IL-10 و IFN-γالاستجابات أكبر من TNF- α . بالإضافة إلى ذلك ، تم العثور على إرتباط كبير بين تركيز مصل IL-10والفئات العمرية. لم يتم العثور على أي ارتباط كبير من مستويات السيتوكينات و كثافه الطفيليات في المجموعة التي شملتها الدراسة. كما وجدت الدراسة ارتباط معنوي لمستوى السيتوكينات والإصابة المتكررة مع TNF-α- و IL – 10.

خلصت الدراسة إلى أن IL-10 كانت اعلي مع الفئة العمرية والجنس وكذلك في العدوى المتكررة .

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Abbreviations

CDC	Centers for Disease Control and prevention
CM	Cerebral Malaria
ELISA	Enzyme Linked Immunosorbent Assay
FCM	Flow cytometry
HRP-II	Histidine- rich protein II
HLA	Human leukocyte Antigen
HRP2	Histidine- Rich Protein2
IL-6	Interleukin -6
IL- 2	Interleukin 2
IFAT	Indirect Fluorescent Antibody Test
LAMP	loop- mediated isothermal amplification
I NF-γ	Interferon gamma
LDMS	Laser desorption mass spectrometry.
MIS	Malaria Indicator Survey
NCMP	National Control Malaria Program
NMCP	National Malaria Control Programme
PLDH	Plasmodium Lactate Dehydrogenase
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffer Saline
QBC	Quantities Buffy Coat
RDT	Rapid Diagnostic Test
INF-a	Tumor Necrosis Factor Alpha
TMB	Tetra Methyl Benzidine
ULVS	Ultralow-volume Spraying
WHO	World Health Organization

Chapter one
1- Introduction

1.1 Introduction

Malaria is a mosquito –borne infectious disease of human and other animals caused by eukaryotic protests of the genus *Plasmodium*. The disease result from the multiplication of *Plasmodium* parasite within red blood cells, causing symptoms that typically include fever and headache, in severe cases progressing to coma or death (Ahmed, 2011).

Five species of the parasite cause disease in humans P.falciparum, P. vivax, P. ovale, P. Malaria, and P. knowlelsi. P.falciparum is the most dangerous strain in humans and the target of most scientific research today. In 2002, scientists succeeded in sequencing the P. falciparum genome, which has allowed researchers to make great strides in better understanding ways to target it (Gardneret al., 2002).

In 2016, nearly half of the world's population was at risk of malaria. Most Malaria cases and deaths occur in sub-Saharan Africa. However, the WHO regions of South-East Asia, Eastern Mediterranean, Western Pacific, and the Americas are also at risk. In 2016, 91 countries and areas had ongoing Malaria transmission (WHO, 2000a).

Most of the country below North latitude 150 is endemic zone with relatively high transmission in Southern states, while parts of the north are exposed to epidemics following the heavy rains or floods from River Nile. Transmission of Malaria in North Sudan South to Khartoum is seasonal and depends on rainfall except in urban cities and irrigated schemes. Sudan's rainy season lasts for about three months (July to September) in the North, and up to six months (June to November) in the South. Hence, the duration of transmission varies from 3-6 months with an average of 4 months, while a longer season is noticed in the southern areas. The transmission season may last from July/August to November/December, with an earlier beginning in June in the southern areas (e.g., Kadugli, El Damazin) and

later start in August in northern areas (Wad Madani, Kosti, Kassala, El Obeid) (National Malaria Control Programme,2006).

The History of organized Malaria control efforts in Sudan goes back to the beginning of the last Century when a Balfour managed to eradicate Malaria from Khartoum in1904 (Malik *et al.,* 2006).

Immunity to Malaria develops slowly and protection against the parasite occurs later than protection against disease symptoms. Because of the different location of the parasite and the different antigens expressed at the liver and blood stages, the relevant immune responses and their specificity and regulation will not be same for the liver and blood stages of infection (Langhorne, 2005).

Cytokines seem to be involved both in protection and pathology in Malaria infection. Early and effective inflammatory response, mediated by interferon gamma (IFN-γ) interleukin-12 (IL-12) and (IL-18) dependent manner, seems to be crucial for the control of parasitaemia and resolution of Malaria infection through the mechanisms of the tumor necrosis factor-a (TNF-a) induction and enhanced release of the antiparasitic reactive nitrogen and oxygen radicals (Artavanis-Tsakonas *etal*., 2003). Severe Malaria has long been associated with high circulating levels of pro-inflammatory cytokines such as TNFa, IFN-γ, IL-1 and IL-6 (Malaguarnera and Musumeci, 2002).Their excessive production may affect the disease outcome through their direct systemic effect and by increasing cytoadherence of parasitized erythrocytes to the endothelium via up regulation of adhesion molecules in *P. falciparum* infections (Day *etal.,* 1999).

1.2 Rationale

The expression of cytokines in general as well as the balance of pro- and anti-inflammatory response are supposed to be involved in Malaria pathogenesis, but their relationship with the pattern and extent of vital organ dysfunction in Malaria infection has not been well defined yet. Severe malarial has been associated with low serum levels of IL12 and low interleukin 10 (IL-10) to TNFa serum concentrations ratio in a few studies of childhood Malaria in holoendemic areas.

In order to explore the effect of the immune response to Malaria and the development of clinical immunity, the study aimed to measured cytokines and chemokines in the plasma of patient with Malaria infection.

1.3 Objectives

1.3.1 General objective:

To determine the prevalence and quantities assessment of Malaria cytokine in East Nile locality.

1-3-2 Specific objectives:

- To detect the prevalence rate of Malaria infection in East Nile locality area.
- To compare between parasite count with cytokines, age group, and gender.
- To correlate relationship between recurrent infection and level of cytokines

Chapter Two
2-Literature review

2.1Historical background

The term Malaria originates from medieval Italian: *mala aria*—"bad air"; the disease was formerly called (*ague* or *marsh fever*) due to its association with swamps and marshland. The term first appeared in the English literature about 1829.Malaria was once common in most of Europe and North America, where it is no longer endemic, though imported cases do occur (WHO, 2006). Scientific studies on Malaria made their first significant advance in 1880, when Charles Louis Alphonse Laveran-observed parasites inside the red blood cells of infected people for the first time in Algeria, in1897 when Ross, proved the complete life-cycle of the Malaria parasite in mosquitoes. He thus proved that the mosquito was the vector for Malaria in humans by showing that certain mosquito species transmit Malaria to birds. He isolated Malaria parasites from the salivary glands of mosquitoes that had fed on infected birds (CDC, 2012).The first effective treatment for Malaria came from the bark of cinchona tree, which contains quinine, Its effectiveness against Malaria was found and the Jesuits introduced the treatment to Europe around 1640; by 1677, it was included in the London Pharmacopoeia as an antimalarial treatment (Kaufman and Rúveda, 2005).

2.2 Classification of malaria

Kingdom Protista

Subkingdom Protozoa

Phylum: Apicomplexa

Class Sporozoasida

Order Eucoccidiorida

Family *Plasmodiidae*

Genus *Plasmodium*

Species *falciparum*

malariae

ovale

vivax

Knewlsei

2.3 Transmission and life cycle *(figure 1.1)*.

The Malaria parasite exhibits a complex life cycle involving an insect vector (mosquito) and a vertebrate host (human).

2.3.1 Life cycle in the human host

The infection is initiated when sporozoites are injected with the saliva of a feeding mosquito. Sporozoites are carried by the circulatory system to the liver and invade hepatocytes. The intracellular parasite undergoes an asexual replication known as exoerythrocytic schizogony within the hepatocyte; exoerythrocytic schizogony culminates in the production of merozoites which are released into the bloodstream. A proportion of the liver-stage parasites from *P. vivax* and *P.ovale* go through a dormant period (hypnozoite) instead of immediately undergoing asexual replication, these hypnozoites will reactivate several weeks to months (or years) after the primary infection and are responsible for relapses (Wiser, 2011).

Merozoites invade erythrocytes and undergo atrophic period in which the parasite enlarges, the early trophozoite is often referred to as 'ring form' because of its morphology. Trophozoite enlargement is accompanied by an active metabolism including the ingestion of host cytoplasm and the proteolysis of hemoglobin into amino acids. The end of the trophic period is manifested by multiple rounds of nuclear division without cytokinesis resulting is a schizont, merozoites bud from the mature schizont, also called a segmented, and the merozoites are released following rupture of the infected erythrocyte. Invasion of erythrocytes reinitiates another round of the blood-stage replicative cycle, as an alternative to the asexual replicative cycle, the parasite can differentiate into sexual forms known as macro- or microgametocyte's the gametocytes are large parasites which fill up the erythrocyte, but only contain one nucleus (Wiser, 2011).

2.3.2 Life cycle in the mosquito (figure 1.1).

Ingestion of gametocytes by the mosquito vector induces gametogenesis (i.e., the production of gametes) and escape from the host erythrocyte. Microgametes formed by a process known as exoflagellation, flagellated forms which will fertilize the macrogamete**,** leading to a zygote. The zygote develops into a motile ookinete which penetrates the gut epithelial cells and develops into an oocyst. The oocyst undergoes multiple rounds of asexual replication resulting in the production of sporozoites**.** Rupture of the mature oocyst releases the sporozoites into the hemocoel (i.e., body cavity) of the mosquito and invade the salivary glands, thus completing the life cycle (wiser, 2011).

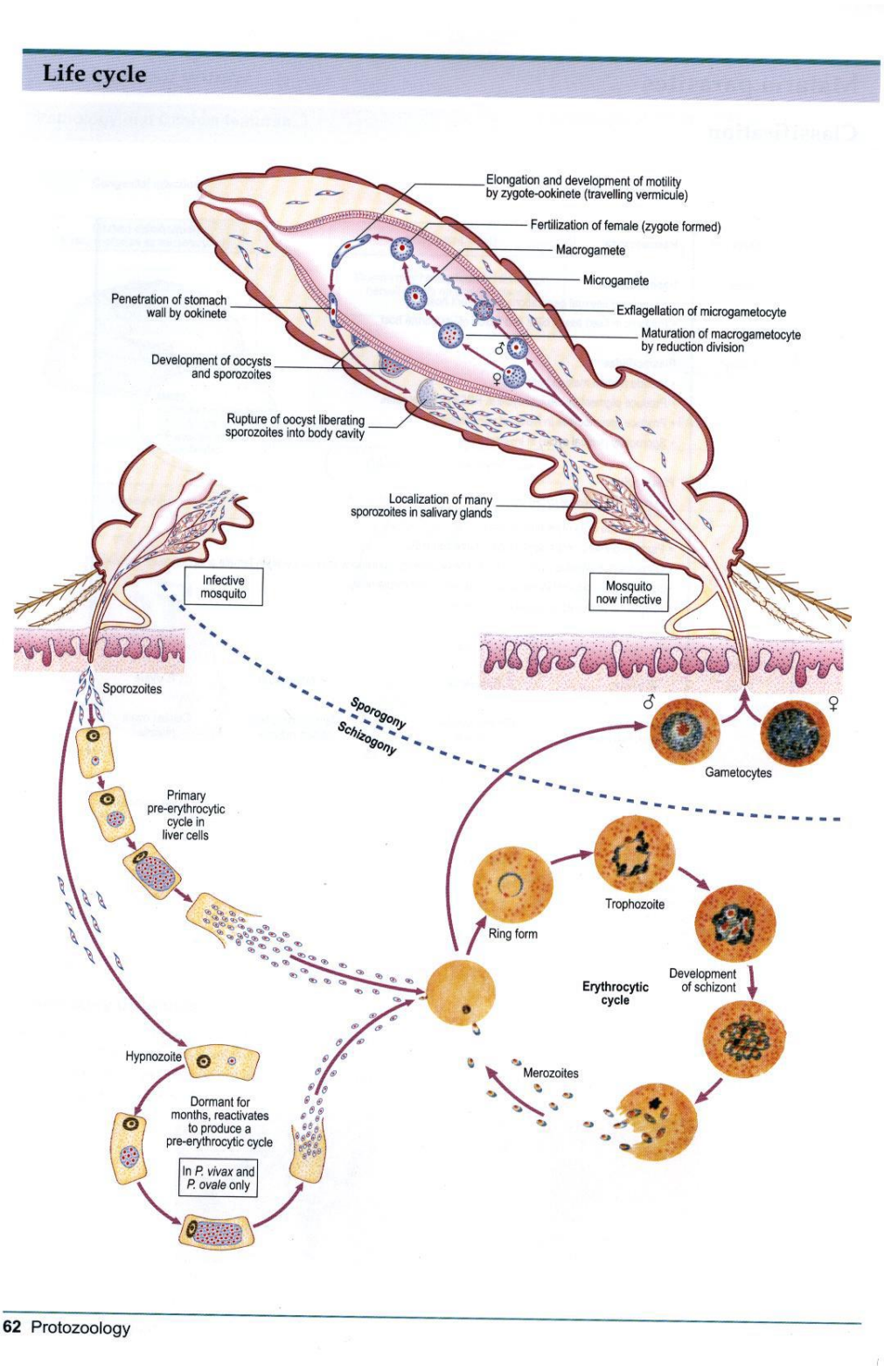

Figure (1.1)

Figure (1.1) Transmission and life cycle of malaria parasite

2.4 Pathology and pathogenesis of malaria

Most severe and fatal illness is caused by *P. falciparum***,** although *P. Vivax* and *P. malariae* infections can also cause severe immunological consequences, affecting the spleen, liver and kidneys**.** The pre-erythrocytic stage of infection produces minimal histopathological changes and absolutely no detectable symptoms or functional disturbances in the host. Infection with erythrocytic stages via blood transfusion or parenteral accidents does not involve the liver; hypnozoites don't develop and there is no risk of relapse (David and Herbert, 2002).

*P. falciparum*can cause severe Malaria because it multiples rapidly in the blood, and can thus cause severe blood loss (anemia). In addition, the infected parasites can clog small blood vessels. When this occurs in the brain, cerebral Malaria results, a complication that can be fatal. *P. malariae* causes a long-lasting, chronic infection that in some cases can last a lifetime. In some chronically infected patients *P. malariae* can cause serious complications such as the nephritic syndrome. *P.knowlesi* has a 24-hour replication cycle and so can rapidly progress from an uncomplicated to a severe infection; fatal cases have been reported (CDC, 2018).

Pathological processes in Malaria are the result of the erythrocytic cycle. After developing in hepatocytes for 7 to 10 days, schizonts rupture, releasing merozoites which invade erythrocytes, where they develop through ring forms to trophozoites and finally to multi segmented schizonts. In the case of *P. falciparum,*this process results in the following changes to the infected erythrocyte: altered membrane transport mechanisms, decreased deformability and other mechanical and rheological changes, development (in some strains) of electron-dense protuberances or knobs beneath the surface membrane, expression of (strains specific) variant surface neoantigens, development of cytoadherent and resetting properties resulting in sequestration of erythrocytes containing later trophozoites and schizonts in deep vascular beds and digestion of hemoglobin to pigment. The secondary effects of these changes are related to the host's immunological

response to parasite antigensand altered red cell surface membranes: stimulation of the reticuloendothelial system, changes in regional blood flow and vascular endothelium, systemic complications of altered biochemistry, anemia, tissue and organ hypoxia and a marked systemic inflammatory response characterized by release of cytokines such as TNF-a and interleukins (Day *etal*., 1999).

Infection with *P. falciparum* can cause disease patterns of various intensities, including mild, almost asymptomatic disease, an acute but self-limiting febrile illness with constitutional symptoms such as malign, nausea‹ vomiting and diarrhea, and severe life-threatening illness. Severe Malaria is defined by the discovery of asexual blood-stage infection with *P. falciparum,*in association with a number of different clinical and laboratory abnormalities known to carry a bad prognosis, these include severe anemia, respiratory distress, cerebral malaria, jaundice, renal failure, shock, acidosis, metabolic and haemostatic abnormalities (WHO, 2000).

2.4.1 Malaria caused by *P. falciparum*

Malaria caused by *P. falciparum* is referred to as *falciparum* malaria, formerly known as sub tertian (ST) or malignant tertian (MT) malaria. It is the most wide spread, accounting for up to 80% of Malaria cause world wide .*P. falciparum* is the most pathogenic of the human Malaria species with untreated infections causingsevere disease and death, particularly in young children, pregnant women and non-immune adults. The pathogenicity of *P. falciparum* is mainly due tothecytoadherence of *falciparum* parasitized red cells causing the cells to adhere to one another and to the walls of capillaries in the brain, muscle, kidneys and elsewhere and in pregnant women, in the placenta. Sequestration of parasitized cells in the micro circulation causes congestion, hypoxia, blockage and rupturing of small blood vessels. High levels of parasitaemia resulting in the activation of cytokines and the destruction of many red cells. *Falciparum* malaria parasitaemia

can exceed more than 250000 parasites/ul of blood. Up to30–40% of red cells may become parasitized (Cheesbrough, 2009).

2.4.1.1 Cerebral malaria

Cerebral Malaria is restricted to *falciparum* malaria, adhesion to cerebral endothelial cells may have some effect on blood-brain barrier permeability, and evidences accumulating that Intraparenchymal inflammatory and immune response may play a role in translating the effects of sequestration into local neuronal dysfunction (Taylor *et al*.**,** 1998)**.**

2.4.1.2 Anemia

Anemia is an inevitable consequence of erythrocyte parasitization as all parasitized red blood cells. (PRBCs) are destroyed at merogony. However, other processes, such as dyserythropoiesis، enhanced splenic clearance and even blood loss, contributes to Malaria anemia. The survival of non-parasitize erythrocytes was found to be reduced for several weeks after clearance of parasitaemia in patients with falciparum and *vivax* malarias (WHO, 2000b).

2.4.1.3 Hyper-reactive Malaria splenomegaly

Hyper-reactive malarial splenomegaly syndrome (HMSS) is a massive enlargement of the spleen due to an exaggerated immune response to repeated attacks of malaria;it is seen more commonly among residents of endemic areas of Malaria (McGillivray, 2000). It's characterized with lassitude fever, weight loss, hypergammaglobulin (especially IgM), anemia and cryoglobulinemia, a clinical response to prolonged antimalarial prophylaxis is diagnostic, pathogenesis is unclear. In some patients, the condition will progress to splenic lymphoma with villous lymphocytes (Mohamedani *et al*., 1999).

2.4.1.4 Malaria haemoglobinuria

In severe Malaria and approximately one in ten adult patients develop significant intravascular haemolysis of both infected and uninfected erythrocytes leading to haemoglobinuria (black water fever), causing anemia and contributing to renal failure. Glucose-6 phosphate dehydrogenase deficiency is a predisposing factor (Tran *et al.,* 1996).

2.4.1.5 Malaria in pregnancy

Normal immune responses are reduced during pregnancy. In areas of stable Malaria transmission, a pregnant woman will have acquired partial immunity to malaria. This will protected against serious clinical *falciparum* Malaria but not prevent heavy parasitic infection of the placenta and anemia (often severe) which can result in a low birth weight baby which may not survive, first pregnancies are at greatest risk. In areas of unstable Malaria transmission, pregnant women lack protective immunity and are at serious risk of developing severe life-threatening *falciparum* malaria, particularly in the last few months of pregnancy and for several weeks after delivery. Untreated infections can result in abortion, still-birth, premature labor or low birth weight; cerebral malaria, pulmonary edema, and hypoglycemia frequently occur (Cheesbrough, 2009).

2.4.1.6 Hypoglycemia

This is a common finding particularly in children and pregnant women with severe *falciparum* malaria. Hypoglycemia is an increasingly recognized complication of *falciparum* malaria. The cinchona alkaloids quinine and guanidine, release insulin from pancreatic islet cells. This reduces hepatic and increases peripheral glucose uptake by tissues, resulting in hypoglycemia. In this situation inappropriately high plasma insulin concentrations will be associated with increased lactate and alanine; glucose consumption may be increased, in patients with Malaria as a result of and

low ketene concentrations fever, infection and anaerobic glycolysis in the host tissues and by the parasite burden (David and Herbert, 2002).

2.5. Immunology of malaria

In areas of stable endemicity repeated exposure to the parasite leads to the acquisition of specific immunity, which restricts serious problems to young children; Malaria in older subjects causes a relatively mild febrile illness. However, even in people exposed to Malaria for the first time, there is a range of possible outcomes, from death at one extreme to the occasional subject who appears resistant to infection at the other, in this case, any resistances non-specific; it does not depend on prior exposure to Malaria and may be either acquired or innate (David and Herbert, 2002).

2.5.1 Innate resistance to malaria

The Malaria parasite faces a succession of challenges within the host. It has to attach to enter and thrive in, first, hepatocytes and then erythrocytes. Having overcome these hurdles, it has to leave the host to carry on the next part of its cycle in the mosquito. Along its way, the parasite is susceptible to a whole range of potential interruptions, including simple physical barriers, non-specific protective responses, alterations in the supply of essential nutrients and the operation of specific immune mechanisms (David and Herbert, 2002).

2.5.1.1 Red cell polymorphisms

Interest is focusing on host molecules thought to be involved in the pathogenesis of severe malaria, on the grounds that polymorphisms altering the function of these molecules would make them subject to either positive or negative selection, depending on the effect of the functional change on disease severity. Different polymorphisms affecting the promoter region of the gene coding for TNF-a, which is believed to play an important role in severe malaria, have been shown to be associated with both protection and increased susceptibility (Knight *et al.,* 1999).

Similarly, a polymorphism in intracellular adhesion molecule -1 (ICAM-1), one of the endothelial receptors for infected cell cytoadherence are associated with an increased risk of cerebral Malaria (Fernandez-Reyes *et al*., 1997).

2.5.2 Acquired immunity to malaria

Acquired immunity may be either active or passive. Active (acquired) immunity is an enhancement of the defense mechanism of the host as a result of a previous encounter with the pathogen or parts. Passive (acquired) immunity is conferred by the prenatal or postnatal transfer of protective substances from mother to child or by the injection of such substances. In humans, various types of acquired or adaptive immunity against *Plasmodium* have been defined:

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

(ii) Anti-parasite immunity, conferring protection against parasitemia, which Affects the density of parasites.

(ii) Premonition, providing protection against new infections by maintaining a low-grade and generally asymptomatic parasitemia (Denise *et al*., 2009).

2.5.3 Humoral immunity to malaria

The humeral arm of the immune system is believed to play a key role in naturally acquired partial immunity to Malaria (Bull and Marsh, 2002).

Early studies showed that transfer of serum from partially immune individuals to non-immune individuals conferred some protection from severe outcomes related to Malaria (Sabchareon *et al.,* 1991). Immuno-epidemiological studies have also shown associations between high levels of some Malaria antibodies with protection (Roussilhon *et al.,* 2007).

2.5.4 Cell mediated immune response to malaria

Cell-mediated immune responses induced by Malaria infection may protect against both pre-erythrocytic and erythrocytic parasite stages. CD4 T cells are essential for immune protection against asexual blood stages in both murine and human malaria. However, the role of CD8 T cells, which have important effecter functions in pre-erythrocytic immunity and which contribute to protection against severe malaria, is less clear. It has been proposed that CD8 T cells may regulate immunosuppressant in acute Malaria and down-modulate inflammatory responses. As human erythrocytes do not express MHC antigens, lysis of infected erythrocytes by CD8 cytotoxic T lymphocytes has no role in the defense against blood-stage parasites (Perlmann, 2002).

2.5.5 Cytokines in the immunopathology of malaria

Cytokines are chemical messengers of the immune system. They are produced by many different cell types and signal via specific cell surface receptor complexes. They divided into pro- and anti-inflammatory groups. Pro-inflammatory cytokines can induce cellular apoptosis, stimulate expression of adhesion molecules, chemokines and other pro-inflammatory cytokines, modulate the architecture of local tissue microenvironments and stimulate the production of microbicidal products, such as reactive nitrogen and oxygen intermediates. Many anti-inflammatory cytokines can counter the activities of pro-inflammatory cytokines, and are often produced during inflammatory immune responses as part of homeostatic mechanisms to prevent tissue damage anti-inflammatory cytokines have also been found to play key roles in the initiation of cellular immunity (Stager *et al*., 2003 and Carvalho *et al*. 2002).

Cerebral Malaria (CM) is associated with relatively high levels of pro-inflammatory cytokines in the circulation (Clark *et al*., 1991).

2.6 Diagnosis of malaria

Laboratory diagnosis of Malaria requires the identification of the parasite or its antigens/ products in the patient's blood. The requirements of a diagnostic test are specificity, sensitivity, ease of performance and a reasonable cost. However, current available techniques can be separated in three categories microscopy, immunological techniques and molecular techniques (Malaria - West Nile Virus, 2016).

2.6.1 Microscopy method

2.6.1.1 Thick and thin blood films

A thick blood film is the most suitable for the rapid detection of Malaria parasites, particularly when they are few. In a thick film the blood is not fixed. The red cells are lyzed during staining, allowing parasites and white cells to be seen in a much larger volume of blood (Cheesbrough, 2009). Thin blood film is required to confirm the *Plasmodium* pieces if this is not clear from the thick film. The blood cells are fixed in a thin film, enabling the parasites to be seen in the red cells. Examination of a thin film greatly assists in the identification of mixed infections. By counting the percentage of parasitized red cells before and after treatment, thin films are also of value in assessing whether a patient with *falciparum* Malaria is responding to treatment in areas where drug resistance is suspected. Examination of a thin film also gives the opportunity to investigate anemia and white cell abnormalities (Cheesbrough, 2009).

2.6.1.2 Diagnosisof Malaria in blood film

The young trophozoite appears incomplete rings or spots of blue cytoplasm with detached red chromatin dot. In the late trophozoites of *P.vivax*, the cytoplasm may be fragmented and Schiiffner's stippling may be less obvious; the band forms of *P.malariae* are less characteristic. However the schizonts and gametocytes of these species retain their usual appearance, as do the crescents of *P.falciparum* (David and Herbert, 2002).The advantages of this method is an inexpensive method, it gives the examiner the opportunity to quantify parasites and differentiate Malaria species. The diagnostic accuracy depends on quality of blood smear and equipment, abilities of the microscopic, parasite density and the time spent on reading the smear. All these may result in therapeutic delays. Disadvantages not suitable for large- scale epidemiological studies, false positive, defective blood film preparation may lead to artifacts that can be incorrectly regarded as Malaria parasites. Sometimes, platelets also confound diagnosis false negative. It is associated with low parasite density or low number of fields examined by the microscopic (David and Herbert, 2002).

2.6.2 Quantities Buffy Coat (QBC) test

The QBC technique was designed to enhance microscopic detection of parasites and simplify Malaria diagnosis. This method involves staining parasite deoxyribonucleic acid (DNA) in micro-hematocrit tubes with fluorescent dyes, e.g. acridine orange, and its subsequent detection by epi-fluorescent microscopy (Chotivanich *etal.*, 2006).

2.6.3 Immunological techniques

2.6.3.1 Antigen-based techniques

2.6.3.1.1 Rapid Diagnostic Test (RDT)

Rapid diagnostic tests are immunochromatographic tests that detect specific parasite antigens in blood, RDTs currently available are described in more detail as follows; Histidine- Rich Protein2 (HRP2) is a water-soluble protein produced by trophozoites and young gametocytes of *P.falciparum*. Tests based on HRP2 detect only *P. falciparum* malaria. HRP2 has been shown to persist and may be detectable for more than two weeks after clinical symptoms of Malaria have disappeared and parasites are apparently cleared from the host, *Plasmodium* lactic acid (Lactate) dehydrogenase (pLDH) is produced by both trophozoites and gametocytes of Malaria parasites. The pLDH antigen is present in and released from parasite-infected erythrocytes and gametocytes. Currently available pLDH RDTs usually detect pLDH specific to *P. falciparum*; some are also pan- specific to all *Plasmodium* species that infect humans.*Plasmodium* aldolase is an enzyme produced by all species of human *Plasmodium* parasites (pan-specific).Tests to detect aldolase appear to be less sensitive than tests that detect the other parasite products,RDTs are simple to use and can be carried out by non-laboratory health staff after formal training and when supported by regular supervisory follow up (National Malaria Control Programme, 2018)

2.6.3.2 Antibody-based techniques

2.6.3.2.1 Enzyme- linked immunosorbent assay (ELISA)

ELISA using the avidin-biotin amplification system for detection of antibodies to sonicated parasite extracts. The use of a perceptible substrate allows the test to be read (as an antibody titer) with the naked eye (Londner *et al*., 1987).Cytokine sandwich ELISA is sensitive enzyme immunoassays that can specifically detect and quantities the concentration of soluble cytokine and chemokine proteins are

exquisitely specific because antibodies directed against two or more distinct epitopes are required. Therefore, sandwich ELISA can discriminate between cytokines that can have overlapping biological functions which are not resolvable in a bioassay. Although cytokine sandwich ELISA is very useful for cytokine detection and measurement, several limitations for the interpretation of ELISA data must be mentioned (Carter and swain, 1997).

2.6.3.2.2 Indirect fluorescent antibody test (IFAT)

The antigen consists of infected blood bound to a 12-spot microscope slide. When the slides are dried, they are examined by fluorescence microscopy. Antibody in the test serum reacts with antigen of parasites and the anti-immunoglobulin reaction with the antibody is demonstrated by the fluorescence of the parasites, the disadvantages of this method are the requirement of a fluorescence microscope and the need for high. Technical skill (Malaria - West Nile virus, 2016).

2.6.4 Molecular techniques

2.6.4.1 PCR technique

PCR-based techniques are development in the molecular diagnosis of malaria, and have proven to be one of the most specific and sensitive diagnostic methods, particularly for Malaria cases with low parasitemia or mixed infection (Morassin *etal*., 2002).The PCR technique continues to be used extensively to confirm Malaria infection, follow-up therapeutic response, and identify drug resistance (Chotivanich *etal*., 2006).

The Advantage of PCR technique its can detect as few as1-5 parasites/µl of blood (≤ 0.0001% of infected red blood cells) compared with around 50-100 parasites/µl of blood by microscopy or RDT, can help detect drug-resistant parasites, mixed infections, and may be automated to process large numbers of samples(Hawkes and Kain, 2007). Although PCR appears to have overcome the two major problems of Malaria diagnosis-sensitivity and specificity, the utility of PCR is limited by

complex methodologies, high cost, and the need for specially trained technicians. PCR, therefore, is not routinely implemented in developing countries because of the complexity of the testing and the lack of resources to perform these tests adequately and routinely (Mens *etal*., 2006).

2.6.4.2 Loop- mediated isothermal amplification (LAMP)

The LAMP technique is claimed to be a simple and inexpensive molecular malaria-diagnostic test that detects the conserved 18S ribosome RNA gene of *P. falciparum* (Poon *et al*.,2006).Other studies have shown high sensitivity and specificity, not only for *P. falciparum*, but also *P. vivax*, *P. ovale* and *P. malariae* (Han *et al*., 2007).

The LAMP is more reliable and useful for routine screening for Malaria parasites region where vector-borne diseases, such as malaria, are endemic. LAMP appears to be easy, sensitive, quick and lower in cost than PCR (Erdman and Kain, 2008).

2.6.4.3 Microarrays

Play an important role in the future diagnosis of infectious diseases. Theprinciple of the microarrays technique parallels traditional southern hybridization. This technique would be miniaturized and automated for point of care diagnostics. A pan-microbial oligonucleotide microarray has been developed for infectious disease diagnosis and has identified *P. falciparum* accurately in clinical specimens. This diagnostic technique, however, is still in the early stages of development (Palacios *et al*., 2007).

2.7 Prevention and control

The goal of Malaria vector control is to eliminate the *Anopheline* population there are three main methods;

2.7.1 Biological control

Several methods of biological control currently exist. One involves the introduction of *Bacillus thuringiens* especially *BT israelensis* (BTI) a mosquito bacterial pathogen, into a targeted mosquito population (McNeil, and Donald, 2005).

2.7.2 Elimination of breeding sites

This can be made unsuitable for mosquito larvae through a variety of methods. They include increasing water flow or ditching, removing protective aquatic vegetation, or other actions that completely destroy breeding areas (filling or draining) (McNeil and Donald, 2005).

2.7.3 Chemical Control of Larvae

Larvicides target larvae in the breeding habitat before they can mature into adult mosquitoes and disperse. Larvicide treatment of breeding habitats helps reduce the adult mosquito population in nearby areas (Mulla and Su, 1999).

2.7.4 Chemical Control of Adult Mosquitoes

The treatment of choice to control adult mosquitoes is ultralow-volume spraying (ULV). ULV spraying provides adequate protection for limited periods of time. To provide continuous protection in large areas with many breeding sites, ULV insecticides must be applied on a repetitive schedule, typically twice daily, daily, or every other day (Navy, 2000).

2.8 Treatment

Malaria is treated with antimalarial medications; the ones used depend on the type and severity of the disease. While medications against fever are commonly used, their effects on outcomes are not clear (Meremikwu *et al.*, 2012).Simple or uncomplicated Malaria may be treated with oral medications. The most effective

treatment for *P. falciparum* infection is the use of artemisinins in combination with other antimalarials (known as artemisinin-combination therapy (ACT), which decreases resistance to any single drug component (Kokwaro, 2009).The additional antimalarialsinclude: amodiaquine, lumefantrine, mefloquine or sulfadoxine /pyrimethamine, another recommended combination is dihydroartemisinin and piperaquine (Keating,2012).

To treat Malaria during pregnancy, the WHO recommends the use of quinine plus clindamycin early in the pregnancy (1st trimester), and ACT in later stages (2nd and 3rd trimesters) (Manyando *et al*., 2011).

Infection with *P. vivax, P. ovale* or *P. malariae* usually does not require hospitalization. Treatment of *P. vivax* requires both treatment of blood stages (with chloroquine or ACT) and clearance of liver forms with primaquine (Waters and Edstein, 2012).Severe and complicated Malaria are almost always caused by infection with *P. falciparum*. The other species usually cause only febrile disease (Kochar*et al*., 2005). Cerebral Malaria is the form of severe and complicated Malaria with the worst neurological symptoms. Recommended treatment for severe Malaria is the intravenous use of antimalarial drugs. For severe malaria, parenteral artesunate was superior to quinine in both children and adults (Sinclair *et al*., 2012).In another systematic review, artemisinin derivatives (artemether and arteether) were as efficacious as quinine in the treatment of cerebral Malaria in children (Kyu *et al*.,2009).

2.9 Vaccination

There are a number of reasons for the failure to develop an effective vaccine include; multistage life cycle with stage-specific expression of proteins, large genome: 25-30 mega-bases, 5000-6000 genes, 14 chromosomes, allelic/antigenic variation, complex, genetically variable, human immune response, parasite adaptations to avoid immune response (Baird *et al*, 1998).A number of clinical

trials with pre-erythrocytic stage *P. falciparum* vaccines have already been completed and numerous others are planned or in progress. The Walter Reed Army Institute of Research (WRAIR) and Glaxo Smith Kline Biological (GSK) has done extensive work on a CSP recombinant vaccine, RTS.S/AS02 (Stoute *et al.*, 1998).Vaccine candidates that target antigens on gametes, zygotes, or ookinetes in the mosquito midgut aim to block the transmission of malaria. These transmission-blocking vaccines induce antibodies in the human blood; when a mosquito takes a blood meal from a protected individual, these antibodies prevent the parasite from completing its development in the mosquito (Crompto*n et al*., 2010). Other vaccine candidates, targeting the blood-stage of the parasite's life cycle, have been inadequate on their own. For example, SPf66was tested extensively in areas where the disease is common in the 1990s, but trials showed it to be insufficiently effective (Graves and Gelb and, 2006).

Chapter three
3. Materials and methods

3.1 Study design

It is a cross sectional based lab study.

3.2 Study area and duration

This study was carried out in the East Nile locality which is located in the eastern part of the Khartoum state, it constitutes about 25% of the region of the state; this study was conducted in different pre-urban area in the northeast during the period from May to July 2018.

3.3 Study population

The study population includes all ages and gender of population admitted to hospital and health centers with Malaria infection. The age groups were categorized as follows: less than 10 years, 11-49 years, and more than 50 years old.

3.4 Sample size

Three hundred eighty four (384) samples selected randomly from hospital and health centers admitted with Malaria infection. The sample size was calculated on the following simple formula (Daniel, 1999).

$$N = \frac{Z2\ p\ (1-p)}{d2}$$

Where N = sample size

Z = statistic for a level of confidence =1.96.

P = prevalence study in same area (50%)

d = precision 5%, = 0.0

3.5 Sample collection

Five ml venous blood samples was collected in plain container from each patient, thick and thin films were prepared again after getting positive result of malaria, after centrifugation the serum were separated and stored in another labeled plain container. Then thick and thin blood films were stained with Giemsa stain 10% concentration, thin were fixed by methanol. Slides were placed on a drying rack and allowed the methanol-fixed thin smear to dry completely in air. Slides were placed for staining blood films face down on staining rack. The timer was set to 10 min. At the end of the staining time, each slide was removed individually. The stain were flushed from the slides by adding drops of buffered water until all the stain has been washed away. The slides were placed in the drying rack.

3.6 Microscopic examinations

After the film dry were examined microscopically and determined stage, spices and parasite count considered as

+ 1-10 parasites per 100 thick film fields
+ + 11-100 parasites per 100 thick film fields
+ + + 1-10 parasites per one thick film field
+ + + + more than 10 parasites per one thick film field.

3.7 ELISA

The stored serum were binged to lab and were allowed to thawed, then serum concentrations of IFN-y, IL-10, TNF-a were determined using enzyme-linked immunosorbent assays obtained commercially.

Procedure

100 µL diluted capture antibody solution were added to each well and sealed plate were incubated overnight between2-8°c, the plates were washed four times then blocked by adding 200 µL assay diluents (A) to each well, then were sealed and

incubated for 1 hour with shacked on palate shaker 500 rpm with a 0.3cm circular orbit. The plates were washed four times then 100 ul diluted standers and samples added to each wells, the plate were sealed and incubated at room temperature for 2 hour with shaking. The plates were washed four times, then 100 µL diluted detection antibody solution were added to each well, sealed and were incubated at room temperature for 1 hour with shaking. The plates were washed four times then 100ul diluted avidin – HRP solution were added to each well the plate sealed and were incubated at room temperature for 30 minutes with shaking. The plates were washed five times then were soaked for 30 second to 1 minutes per wash and 100 µL freshly TMB substrate solution were added to each well and incubated in the dark for 20 minutes. Finally, 100 µL of stop solution were added to each well, the absorbance was read with (SPECTROstar Nano S/N 601-0682) at 540 nm and 570 nm within 15 minutes.

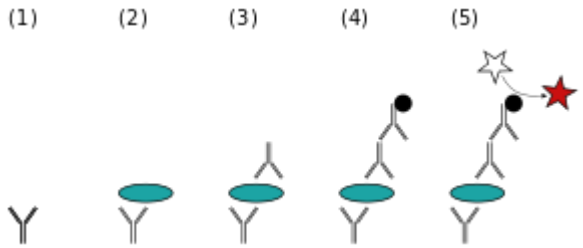

Figure (2.1) a sandwich ELISA

(1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form (Schmidt et al ., 2012).

3.8 Data collection

The primary data were collected with questionnaire which designed contain simple investigative questions or indicators which include demographic, health data and history of Malaria infection.

3.9 Statistical analysis

Data were analyzed using statistical package for social sciences (SPSS) (version-20). Chi square test statistical analysis was performed and the *P*.valueof less than 0.05 was considered statically significant

3.10 Ethical consideration

Ethical clearance for this study was obtained from committee of college of medical laboratory science – Sudan University of Science and Technology. An informed consent was obtained from all subjects included in this study**.**

Chapter four
4. Result

Prevalence of Malaria in the study area using blood film

Out of 384 blood samples collected from different pre-urban area in east Nile locality, during the period from May to July 2018 (pre- Malaria season) 71 (18.5%) were found to be positive and 313 (81.5 %) were negative when using blood films (Table 4.1).

Table (4.1): Overall prevalence of Malaria in study area using blood film

Blood film	Frequency	percentage%
(Positive) +ve	71	18.5
(Negative) –ve	313	81.5
Total	384	100.0

Overall prevalence of Malaria according to gender using blood films

The overall prevalence rates of Malaria according to gender as shown in (Table 4.2) males had higher prevalence rate (22.7%) while the lowest prevalence rates was reported among females (15.6%).

Table (4.2): Overall prevalence of Malaria according to gender using blood films

Gender	No examined	Number positive (%)
Male	154	35 (22.7%)
Female	230	36 (15.6%)
Total	384	71 (18.5%)

Prevalence of Malaria according to age in the study area

The prevalence based on age groups revealed that highest prevalence rate (20.1%) was reported in the age group (less than 10) followed by 11-49 age group with prevalence rate (19.7%) and the lowest prevalence rate (2%) was reported among more than 50 year age group (Table 4.3).

Table (4.3): Prevalence of Malaria according to age in the study area

Age group	No examined	Number positive (%)
Less than 10	134	27 (20.1%)
11 - 49	198	39 (19.7%)
More than 50	52	5 (2%)
Total	384	71 (18.5)

Distribution of *Plasmodium* species in the study area

The prevalence according to *Plasmodium* species revealed that those with infection due to *P. falciparum* had the highest prevalence rate (13%) followed by P.*vivx* (4.6%) And the lowest prevalence rate (0.8%) with mixed infection (*P. falciparum* and *P.vivax*) while there was no any positive result for *P. malariae* and *P. ovale* (Table 4.4).

Table (4.4): Distribution of Plasmodium species in the study area

Species	Frequency (%)
P.falciparum	51 (13%)
P.vivax	17 (4.6%)
P.ovale	0 (0%)
P. malariae	0 (0%)
(Mix infection*P.f and P.v)*	3(0.8%)
Total	71 (18.5%)

Overall prevalence of Malaria according to density (parasitemia) using blood films

The overall prevalence rates of Malaria according to parasitemia showed that, the highest prevalence rate (58%) was reported among parasite count (+) (mild) parasitemia, the lowest prevalence rate (13%) was reported among parasite count (++) (moderate) parasitemia, while there was no sever parasitemia (parasite count (+++) and (++++) (Table 4.5).

Table (4.5): Overall prevalence of Malaria according to parasite count

Parasite count	Frequency (%)
One cross (+)	58 (81.7%)
Two crosses (++)	13 (18.3%)
Three crosses(+++)	0 (0%)
Four crosses (++++)	0 (0%)
Total	71(18.5)

Correlation between parasitemia and age

Table 4.6 shows the correlation between parasitemia and age group, it showed that, the highest prevalence rate of parasitemia (53.4%) was reported in parasite count (+) among 11-49 age group, followed by the rate of (34.5%) which was related to less than 10 age group and the lowest rate was reported in the age group more than 50 (12.1%) , the highest prevalence rate (53.8%) was related in parasite count (++) among less than 10 age group followed by prevalence rate of (46.2%) among the 11-49 age group, The difference in rates in patasitemia among age group was found to be statistically insignificant at *P*.value=0.108).

Table (4.6): Correlation between parasitemia and age

Density	Age group			Total	p.value
	Less than 10	11-49	More than 50		
One cross (+)	20(34.5%)	31(53.4%)	7(12.1%)	58	0.108
Two crosses (++)	7(53.8%)	6(46.2%)	0	13	
Three crosses (+++)	0	0	0	0	
Four crosses (++++)	0	0	0	0	

Cytokine profile in patient and controls

A significant difference was observed between its mean serum concentrations in patients compared to the controls. Mean serum level of IFN-γ was found to be significantly higher in patients than controls (p.value =0.026) TNF-α serum level not shown higher in patients compared to the controls (p.value= 0. 646). Mean serum level of IL-10 showed insignificant differences between patients and control at p.value =0.071) (table 4.7).

Table (4.7): Mean and Std.Deviationin of cytokine profile in patient and controls

Sample	NO	Cytokines profile		
		IFN-γ(Ng/ml)	TNF-α(Ng/ml)	IL-10(Ng/ml)
Patients Mean SD	29	 61.98 ± 71.93	 5.91 ± 5.67	 48.87 ± 52.99
Control Mean SD	10	 11.05 ± 11.05	 5.36 ± 3.78	 12.48 ± 8.08
p.value		0.026	0.646	0.071

Correlation between gender and cytokines profile

Table 4.8 shows sex-specific cytokine profiles, males produce high levels of IL-10, than the females, and there was a significant difference at P.value = 0.015). But there was no significant different in the production of IFN-γ, (p.value =0.533) and TNF-α, (p.value =0.281).

Table (4.8): Correlation between gender and cytokines profile

Gender	No	Mean	P.value
IFN-γ Male Female	 16 23	 70.75 55.89	 .533
TNF-α Male Female	 16 23	 7.09 5.09	 .281
IL-10 Male Female	 16 23	 73.05 32.05	 .015

Correlation between age and cytokines profile

Table 4.9 shows the correlation and levels of cytokine responses for age groups less than 10 , 11-49 and more than 50 age group, for both children and adults, the median levels of IL-10 and IFN-γ responses were shown greater than TNF-α. In addition, association was found significant between IL-10 serum concentration and age group at (p.value = 0.029).

Table (4.9): Correlation between age and cytokines profile

Age group	**Cytokines profile**			**p.value**
	Less than 10 N0=8	11-49 No=24	More than 50 No=7	
IFN-γ(Ng/ml) Mean SD	61.98 ± 71.93	61.98 ± 71.93	61.98 ± 71.93	0.830
TNF-α(Ng/ml) Mean SD	5.91 ± 5.67	5.91 ± 5.67	5.91 ± 5.67	0.709
IL-10**(**Ng/ml) Mean SD	48.87 ± 52.99	48.87 ± 52.99	48.87 ± 52.99	0.029

Correlation between parasitemia and cytokines profile

Table 4.10 shows the correlation and levels of cytokine responses for density of parasite, no significant correlation of cytokines levels and parasitaemia was found in the studied group IFN-γ, (p.value =0.922) TNF-α (p.value =0.525) and IL-10 (p.value =0.380).

Table (4.10): Correlation between parasitemia and cytokines profile

Density	No	Mean		
		IFN-γ Ng/ml)	**TNF-α(**Ng/ml)	**IL-10** (Ng/ml)
Cross +	25	79.49	5.80	60.50
Two crosses ++	4	79.94	7.98	67.17
p.value		0.922	0.525	0.380

Correlation between recurrent infectioninfection and cytokines profile

Table 4.11 shows the correlation and levels of cytokine responses for recurrent infection among patient significant correlation of cytokines level and recurrent infection shown in TNF-α- at (p.value =0.011) and IL - 10 at (p.value =0.007), statistically insignificant correlation of IFN-γ levels and recurrent infection was found in the studied group at (p.value =0.151).

Table (4.11): Correlation between recurrent and cytokines profile

Recurrent	No	Mean	SD	p.value
IFN-γ				
Yes	15	83.06	70.61	0.151
No	24	48.82	71.02	
TNF-α				
Yes	15	8.76	8.10	0.011
No	24	4.13	2.03	
IL-10				
Yes	15	77.21	65.55	
No	24	31.16	34.26	0.007

Chapter five

Discussion:

This study determined the prevalence of malaria among 384 randomly selected patients in the East Nile locality which is located in the eastern part of the Khartoum state. Findings revealed a prevalence rate of 18.5%. This rate finding was greater than the rate reported in khartoum by EL Mekki *et al*, (2012) who reported the prevalence of malaria in Dar Al salam camp was 5% and 11% in Jabal Awlia camp. El Sayed *et al*, (2000) who reported that, Khartoum which was formerly malaria free and it was considered as hypoendemic or mesoendemic area in which malaria is unstable and epidemic outbreaks are common we agree with history finding.

Falciparum malaria is the most prevalent and constitutes about 13% of all infections, while benign tertian *vivax* malaria has prevalence of 4.6% and the lowest prevalence rate 0.8% for mixed infection (*P. falciparum* and *P.vivax*) .However, there was no prevalence for *P. malariae* and *P. ovale*. Males had higher prevalence rate 22.7% than females 15.6% rate. Our study finding agreed with a study in Khartoum by Abdulla *et al*, (2007) who reported that the overall prevalence of malaria was 28, 2 % and was higher in males than in females.

The intensity of the highest prevalence rate 53.8% of moderate parasitemia was reported among the less than 10 years age group. Although children are more susceptible to malaria infection due to slow developing immune system, high prevalence rate 34.5% of mild parasitemia reported among the 11-49 years age group. Lower prevalence rates of 2% and mild parasitemia 12.1% was reported among age group more than 50 years old, this finding was closer to the finding of Igwe *et al*, (2014) in Nigeria who reported that the highest prevalence of asymptomatic malaria parasitemia 87.5% was found in the parturient who were ≤19 years while the lowest prevalence of 68.2% occurred in those who were 40‑49 years old . In the present study observed that, the patasitemia among age group was

found to be statistically insignificant and we agreed with study done by El Khalifa *et al*,(2008) who found no significant age difference among 5 years and above during 2003 and 2004.

In this study, serum levels IFN-γ, TNF-a and IL-10 were measured in healthy and patients with *P. falciparum* and *P. vivax* within studied group. IFN-γ was found to be significantly higher in patients than controls; this finding was in line with a study in Poland by Wroczynska *et al*, (2005) who reported that the mean serum level of IFN- γ was found to be significantly higher in severe as well as uncomplicated malaria group compared to the controls. Also another study done by Favre *et al,* (1997), who reported available data are consistent with a requirement for an early production of in particular interferon (IFN-γ) to mount resistance against infection. Interestingly, in this study significant correlation between IL-10 with genders specific as well as age also an associations was found between initial IL-10 levels and parasite densities, that near to the finding of Hugosson *et al*, (2004) who reported during treatment, indicating that the IL-10 levels may play a role in the clearance of parasites during treatment., also suggesting age-related differences in immunity and the development of partial clinical tolerance.

No difference was seen between cytokines regarding to the parasites density and IFN-γ, TNF-α and IL-10 this was in line with study done by Janine *et al*, (2001). Serum IL-10 levels had no clear or statistically significant association with level of parasitemia. Also agreed with a study by Nnaemaka *et al*, (2009) who found no significance correlation between IL-10, IL-12 and IFN-γ in the asymptomatic individuals with parasitaemia, similar relations regarding mentioned by Wroczynska *et al*, (2005). In this study, the significant high production of IL-10 was remarkable in patients with recurrent malaria, as well as TNF-α, our finding was agreed with study done by Edward *et al,* (2008) who reported that the high levels of IL-10 observed during malarial episodes may be beneficial by reducing the inflammatory response, but may be detrimental by decreasing antiparasitic

cellular immune responses, this is clearly shown by our data with significantly finding of TNF- α level in patients with the recurrent malaria which in line with Medzhitov *et al*, (2012).who reported that the observations are consistent with the idea that repeated malaria may drive the host towards a disease tolerance state in order to reduce the negative impacts of infection-related pathology. In subjects who are routinely exposed to malaria infection, the pro-inflammatory response may be diminished or quickly controlled by regulatory mechanisms. This effect may be particularly exaggerated in this study area, where transmission is especially intense.

Chapter six
Conclusion and recommendations

6.1 Conclusion

Findings of this study revealed that, Malaria still remains a public health problem, in East Nile locality. *P. falciparum* was the dominant parasite species followed by *P. vivax* but a few cases of infection (mostly mixed infections with *P. falciparum* parasites) were found among the study area. Male had high prevalence rate than female. Prevalence rates of Malaria according to parasitemia was high among mild parasitemia, low among moderate parasitemia, while moderate parasitemia was reported with high prevalence rate among age group less than10 years old, while adult had mild parasitemia. Concentrations of serum cytokines was higher in patients, except IFN-γ which was found to be significantly higher in patients than controls. No correlation of parasitemia to changes in cytokine levels, highly significant correlation between gender, age and IL-10. Patients with the history of recurrent Malaria infections had significant high serum TNF- α and IL-10.

6.2 Recommendations

- This study demonstrated the need to focus on awareness programs to prevent Malaria and to use existing knowledge in practice to control the Malaria burden in East Nile locality.
- Agricultural projects must be outside the villages and not in the middle because they are considered a permanent source of mosquito breeding.
- However, the results of this study should be considered as preliminary; therefore further studies are needed on larger group of patients to determine the cytokines involvement in particular clinical manifestations of the disease.

References

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Appendix

Appendix 1

Preparation of stain

Giemsa stain

Preparation of 500 mL

•Giemsa powder (Azure B type): 3.8 g

•Glycerol, pure: 250 mL

•Methyl alcohol (certified pure): 250 mL

The stain is prepared best by mixing alcohol and glycerol and then gradually adding small quantities of powder in a porcelain mortar and grinding until most of the powder is dissolved. Some residue may remain and, by leaving the mixture for about a week without filtering, the maximum amount of the stain will be absorbed. The prepared stock solution can then be filtered and should be kept in a glass bottle away from the sunlight.

Stock solutions of Giemsa stain must always be diluted by mixing an appropriate amount of the stain with distilled neutral or slightly alkaline water

Buffer

•Potassium dihydrogen phosphate KH2P 0 4): 0.7 g

•Disodium hydrogen phosphate (Na2H P 0 4): 1.0 g

• Distilled water: 1 L

Appendix 2

Enzyme Linked Immunosorbent Assay (ELISA) protocol

Human IFN-γ- BioLegend's ELISA MAX™ Deluxe Sets

Materials

- Uncoated Micro well plates: 96-well Nunc MaxiSorp™
- A microplate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 2 µL to 1 mL
- Deionized (DI) water
- Coating Buffer: 8.4 g $NaHCO_3$, 3.56 g Na_2CO_3, add DI H_2O to 1.0 L, pH to 9.5 (BioLegend Cat. No. 421701 is recommended.)Assay Diluent: 10% Fetal Bovine Serum or 1% BSA in Phosphate-Buffered Saline (PBS) (BioLegend's ELISA MAX™ Deluxe Sets Cat. No 430104. Is recommended.)
- PBS: 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add DI water to 1.0 L, pH to 7.4
- Wash Buffer: Phosphate-Buffered Saline (PBS) + 0.05% Tween-20 (BioLegend Cat. No. 421601 is recommended.)
- Wash bottle or automated micro plate washer
- TMB Substrate Solution -BioLegend's ELISA MAX™ DeluxeSets lot. No.B231228 is recommended.
- Stop Solution (2 N H_2SO_4)
- Tubes to prepare standard dilutions
- Timer
- Absorbent paper

Appendix 3
Human IFN-γ- BioLegend's ELISA MAX™ Deluxe Sets

Human IFN-γ ELISA MAX™ Deluxe Set

Certificate of Analysis

Product Name: Human IFN-γ ELISA MAX™ Deluxe Set
Product Cat. No: 430104 (5 plates) / 430105 (10 plates) / 430106 (20 plates)
Lot No: B207130
Expiration Date: 30-SEP-2017

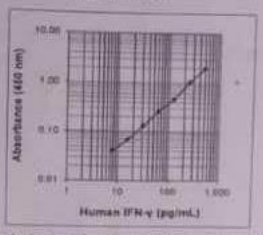
Contents Description	Quantity (5 plates)	Volume (per bottle)	Part No.	Lot No.
Human IFN-γ ELISA MAX™ Capture Antibody (200X)	1 vial	300 µL	79001	B196549
Human IFN-γ ELISA MAX™ Detection Antibody (200X)	1 vial	300 µL	79940	B196550
Human IFN-γ Standard	2 vials	14 ng	79103	B207132
Avidin-HRP (1,000X)	1 vial	60 µL	79004	B206881
Substrate Solution A	1 bottle	30 mL	78570	B204109
Substrate Solution B	1 bottle	30 mL	78571	B204108
Coating Buffer A (5X)	1 bottle	30 mL	79008	B198919
Assay Diluent A (5X)	1 bottle	60 mL	78888	B202070
Nunc™ MaxiSorp™ ELISA Plates, Uncoated	5 plates	-	423501	-

Storage Conditions

1. Unopened set: Store set components between 2°C and 8°C. Do not use this set beyond its expiration date.
2. Opened or reconstituted components:
   2.1. Reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze/thaw cycles.
   2.2. Other components: Store opened reagents between 2°C and 8°C and use within one month.

Note: Precipitation of Assay Diluent A (5X) may be observed when stored long term between 2°C and 8°C. The precipitation does not alter the performance of the assay. If heavy precipitation is observed, it can be filtered to clarify the solution.

Lot #: B207130



This standard curve is for demonstrative purposes only.
A standard curve must be run with each assay.

This is to certify that the product was manufactured under stringent process controls to ensure lot to lot consistency and complete lot traceability. The product has been tested and meets quality control specifications.

Signature: _____ (Quality Control) Date: 9/30/2015

BioLegend is ISO 9001:2008 and ISO 13485:2003 Certified

FOR RESEARCH USE ONLY

BioLegend | 9727 Pacific Heights Blvd | San Diego, CA 92121 U.S.A.
Phone: (858)-768-5800 | Fax: (877)-455-9587 | **biolegend.com**

ELISA MAX™ Deluxe Set Protocol

Materials to be Provided by the End-User

1. Phosphate-Buffered Saline (PBS): 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1.0 L, pH to 7.4, 0.2 µm filtered.
2. Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20.
3. Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, e.g. 2N H_2SO_4.
4. Plate Sealers: BioLegend Cat. No. 423601 is recommended.

Reagent Preparation

Reagents Description	Dilute with	Dilution for 1 plate
Coating Buffer A (5X)	Deionized Water	2.4 mL in 9.6 mL DI H_2O
Capture Antibody (200X)	1X Coating Buffer A	60 µL in 12 mL Buffer
Assay Diluent A (5X)	PBS	12 mL in 48 mL PBS
Detection Antibody (200X)	1X Assay Diluent A	60 µL in 12 mL Buffer
Avidin-HRP (1,000X)	1X Assay Diluent A	12 µL in 12 mL Buffer

Standard reconstitution: Reconstitute the lyophilized Human IFN-γ Standard by adding 0.2 mL of 1X Assay Diluent A to make the 70 ng/mL standard stock solution. Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.

Prepare 1,000 µL of the top standard at 500 pg/mL by adding 7.1 µL of reconstituted standard stock solution to 992.9 µL 1X Assay Diluent A. Perform six two-fold serial dilutions of the 500 pg/mL top standard with 1X Assay Diluent A in separate tubes. 1X Assay Diluent A serves as the zero standard (0 pg/mL).

Samples: For cell culture supernatant samples, the end user may need to determine the dilution factors in a preliminary experiment. Serum or plasma samples should be tested initially without any dilution. If dilution is required, samples should be diluted in 1X Assay Diluent A before adding to the wells.

TMB Substrate Solution Preparation: TMB Substrate Solution is a mixture of equal volumes of Substrate Solution A and Substrate Solution B. Mix the two components immediately prior to use. For one plate, mix 5.5 mL Substrate Solution A with 5.5 mL of Substrate Solution B in a clean container (solution should be clear and colorless).

ELISA Procedure Summary

Day 1

Add 100 µL diluted Capture Antibody solution to each well, seal the plate and incubate overnight between 2°C and 8°C.

Day 2

1. Wash plate 4 times*, block the plate by adding 200 µL 1X Assay Diluent A to each well, seal plate and incubate at room temperature for 1 hour with shaking on a plate shaker (e.g. 500 rpm with a 0.3 cm circular orbit). All subsequent incubations with shaking should be performed similarly.
2. Wash plate 4 times*, add 100 µL diluted standards and samples to the appropriate wells.
3. Seal the plate and incubate at room temperature for 2 hours with shaking.
4. Wash plate 4 times*, add 100 µL diluted Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.
5. Wash plate 4 times*, add 100 µL diluted Avidin-HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes with shaking.
6. Wash plate 5 times*, soaking for 30 seconds to 1 minute per wash. Add 100 µL of freshly mixed TMB Substrate Solution to each well and incubate in the dark for 20 minutes.
7. Add 100 µL Stop Solution to each well. Read absorbance at 450 nm and 570 nm within 15 minutes. The absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

***Plate Washing:** Wash step is crucial to assay precision. Wash the plate with at least 300 µL of Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean absorbent paper.

For more detailed set information, please refer to the online manual at:
www.biolegend.com/media_assets/pro_detail/datasheets/430104.pdf

BioLegend®

Appendix 4

TNFα-BioLegend's ELISA MAX™ Deluxe Sets

Materials

- Uncoated Microwell plates: 96-well Nunc MaxiSorp™
- A micro plate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 2 µL to 1 mL
- Deionized (DI) water
- Coating Buffer: 8.4 g $NaHCO_3$, 3.56 g Na_2CO_3, add DI H_2O to 1.0 L, pH to 9.5 (BioLegend Cat. No. 421701 is recommended.)
- Assay Diluent: 10% Fetal Bovine Serum or 1% BSA in Phosphate-Buffered Saline (PBS) (BioLegend's ELISA MAX™ Deluxe Sets Cat. No 421203. is recommended.)
- PBS: 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add DI water to 1.0 L, pH to 7.4
- Wash Buffer: Phosphate-Buffered Saline (PBS) + 0.05% Tween-20 (BioLegend Cat. No. 421601 is recommended.)
- Wash bottle or automated microplate washer
- TMB Substrate Solution -BioLegend's ELISA MAX™ Deluxe Sets cat. No.421101 is recommended.
- Stop Solution (2 N H_2SO_4)BioLegend's ELISA MAX™ Deluxe Sets cat. No.421101 is recommended.
- Tubes to prepare standard dilutions
- Timer
- Absorbent paper

Appendix 5
TNFα-BioLegend's ELISA MAX™ Deluxe Sets

Human TNF-α ELISA MAX™ Standard Set

ficate of Analysis

Name: Human TNF-α ELISA MAX™ Standard Set

Cat. No: 430201 (5 plates) / 430202 (10 plates) / 430203 (20 plates)

8192721

ion Date: 31-DEC-2016

nts Description	Quantity (5 plates)	Volume (per bottle)	Part No.	Lot No.
n TNF-α ELISA MAX™ e Antibody (200X)	1 vial	300 µL	79017	B192520
n TNF-α ELISA MAX™ ion Antibody (200X)	1 vial	300 µL	79018	B192521
n TNF-α Standard	2 vials	8 ng	79019	B192720
-HRP (1,000X)	1 vial	60 µL	79004	B189506

ge Conditions

Unopened set: Store set components between 2°C and 8°C. Do not use this set beyond its expiration date.

Opened or reconstituted components:

2.1 Reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze/thaw cycles.

2.2 Other components: Store opened reagents between 2°C and 8°C and use within one month.

erials to be Provided by the End-User

Microwell plates: BioLegend Cat. No. 423501 is recommended.

Plate Sealers: BioLegend Cat. No. 423601 is recommended.

Phosphate Buffered Saline (PBS): 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1.0 L, pH to 7.4, 0.2 µm filtered.

Coating Buffer: 8.4 g $NaHCO_3$, 3.56 g Na_2CO_3, add deionized water to 1.0 L, pH to 9.5, 0.2 µm filtered. (BioLegend Cat. No. 421701 is recommended).

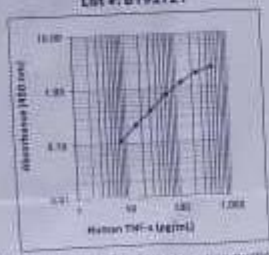
Assay Diluent: 10% Fetal Bovine Serum or 1% BSA in PBS, 0.2 µm filtered (BioLegend Cat. No. 421203 is recommended).

Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20.

TMB Substrate Solution: BioLegend Cat. No. 421101 is recommended.

Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, e.g. 2N H_2SO_4.

Lot #: B192721



This standard curve is for demonstrative purposes only. A standard curve must be run with each assay.

This is to certify that the product was manufactured under stringent process controls to ensure lot to lot consistency and complete lot traceability. The product has been tested and meets quality control specifications.

Signature: _____ (Quality Control) Date: 1/7/15

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ELISA MAX™ Standard Set Protocol

Note: Bring all reagents to room temperature before beginning assay. Do not mix Avidin-HRP or antibodies from different sets, lots, and/or manufacturers. All reagents should be diluted immediately prior to use. Human TNF-α standard from different manufacturers should not be used with this set.

Antibody and Avidin-HRP Preparation

Reagents Description	Dilute with	Dilution for 1 plate
Capture Antibody (200X)	Coating Buffer	60 µL in 12 mL Buffer
Detection Antibody (200X)	Assay Diluent	60 µL in 12 mL Buffer
Avidin-HRP (1,000X)	Assay Diluent	12 µL in 12 mL Buffer

Standard Reconstitution and Preparation

Standard reconstitution: Reconstitute the lyophilized Human TNF-α standard by adding 0.2 mL of Assay Diluent to make the 40 ng/mL standard stock solution. Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.

Prepare 1,000 µL of the top standard at 500 pg/mL by adding 12.5 µL of reconstituted standard stock solution to 987.5 µL Assay Diluent. Perform six two-fold serial dilutions of the 500 pg/mL top standard with Assay Diluent in separate tubes. Assay Diluent serves as the zero standard (0 pg/mL).

Sample Preparation

Human TNF-α ELISA MAX™ Standard Set is specifically engineered for the accurate quantification of natural and recombinant Human TNF-α in cell culture supernatants. For cell culture supernatant samples, the end user may need to determine the dilution factors in a preliminary experiment. If dilutions are necessary, samples should be diluted in the corresponding cell culture medium.

For other sample types, such as serum and plasma, optimization of reagent concentrations and assay conditions may be required.

ELISA Procedure Summary

Day 1

Add 100 µL diluted Capture Antibody solution to each well, seal the plate and incubate overnight between 2°C and 8°C.

Day 2

1. Wash plate 4 times*. Block the plate by adding 300 µL Assay Diluent to each well, seal plate and incubate at room temperature for 1 hour with shaking on a plate shaker (e.g. 500 rpm with a 0.3 cm circular orbit). All subsequent incubations with shaking should be performed similarly.
2. Wash plate 4 times, add 100 µL diluted standards and samples to the appropriate wells, seal the plate and incubate at room temperature for 2 hours with shaking.
3. Wash plate 4 times, add 100 µL diluted Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.
4. Wash plate 4 times, add 100 µL diluted Avidin-HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes with shaking.
5. Wash plate 5 times, soaking for 30 seconds to 1 minute per wash, add 100 µL of TMB Substrate Solution to each well, incubate in the dark for 15-30 minutes** or until the desired color develops.
6. Add 100 µL Stop Solution to each well. Read absorbance at 450 nm and 570 nm within 12 minutes. The absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

***Plate Washing:** Wash step is crucial to assay precision. Washing is typically repeated 4-5 times between each step to remove unbound material. Wash the plate with at least 300 µL of Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean absorbent paper. All subsequent washes should be performed similarly.

****Optimal substrate incubation time depends on laboratory conditions and the optical linear ranges of ELISA plate reader.**

For more detailed set information, please refer to the online manual at: www.biolegend.com/media_assets/pro_detail/datasheets/430201.pdf

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Appendix 6
IL-10 - BioLegend's ELISA MAX™ Deluxe Sets

Materials

- Uncoated Micro well plates: 96-well Nunc MaxiSorp™
- A micro plate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 2 µL to 1 mL
- Deionized (DI) water
- Coating Buffer: 8.4 g $NaHCO_3$, 3.56 g Na_2CO_3, add DI H_2O to 1.0 L, pH to 9.5 (BioLegend Cat. No. 421701 is recommended.)Assay Diluent: 10% Fetal Bovine Serum or 1% BSA in Phosphate-Buffered Saline (PBS) (BioLegend'sELISA MAX™ Deluxe Sets Cat. No 421203. is recommended.)
- PBS: 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add DI water to 1.0 L, pH to 7.4
- Wash Buffer: Phosphate-Buffered Saline (PBS) + 0.05% Tween-20 (BioLegend Cat. No. 421601 is recommended.)
- Wash bottle or automated micro plate washer
- TMB Substrate Solution -BioLegend's ELISA MAX™ Deluxe Sets cat. No.421101 is recommended.
- Stop Solution (2 N H_2SO_4)BioLegend's ELISA MAX™ Deluxe Sets cat. No.423001 is recommended.
- Tubes to prepare standard dilutions
- Timer
- Absorbent papers

Reagent Preparation

- Dilute (5X) Coating Buffer to 1X with deionized water.
  For one plate, dilute 2.4 mL (5X) Coating Buffer in 9.6 mL deionized water
- . 2. Dilute pre-titrated Capture Antibody 1:200 in 1X Coating Buffer. For one plate, dilute 60 µL Capture Antibody in 12 mL 1X Coating Buffer.
- Dilute (5X) Assay Diluent A to 1X with PBS (pH 7.4). For one plate, dilute 12 mL 5X Assay Diluent A in 48 mL PBS.
- Dilute the pre-titrated Biotinylated Detection Antibody 1:200 in 1X Assay Diluent. For one plate, dilute 60 µL Detection Antibody in 12 mL 1X Assay Diluent A**.**
- . Dilute Avidin-HRP 1:1000 in 1X Assay Diluent A. For one plate, dilute 12 µLAvidin-HRP in 12 mL 1X Assay Diluent A.
- TMB Substrate Solution is a mixture of equal volumes of Substrate Solution A with Substrate Solution B. Mix the two components immediately prior to use. For one plate mix 6 mL Substrate Solution A with 6 mL of Substrate Solution B in a clean container (solution should be clear and colorless)**.**

Appendix 7
IL-10 - BioLegend's ELISA MAX™ Deluxe Sets

Human IL-10 ELISA MAX™ Deluxe Set

ertificate of Analysis

oduct Name: Human IL-10 ELISA MAX™ Deluxe Set
oduct Cat. No: 430604 (5 plates) / 430605 (10 plates) / 430606 (20 plates)
t No: B232253
piration Date: 28-Feb-2019

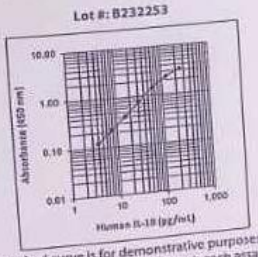
Contents Description	Quantity (5 plates)	Volume (per bottle)	Part No.	Lot No.
Human IL-10 ELISA MAX™ Capture Antibody (200X)	1 vial	300 µL	79029	B229580
Human IL-10 ELISA MAX™ Detection Antibody (200X)	1 vial	300 µL	79030	B229581
Human IL-10 Standard	2 vials	30 ng	79031	B232246
Avidin-HRP (1,000X)	1 vial	60 µL	79004	B231254
Substrate Solution A	1 bottle	30 mL	78570	B231227
Substrate Solution B	1 bottle	30 mL	78571	B231228
Coating Buffer A (5X)	1 bottle	30 mL	79008	B232549
Assay Diluent A (5X)	1 bottle	60 mL	78888	B230792
Nunc™ MaxiSorp™ ELISA Plates, Uncoated	5 plates	-	423501	-

Storage Conditions

1. Unopened set: Store set components between 2°C and 8°C. Do not use this set beyond its expiration date.
2. Opened or reconstituted components:
   2.1. Reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze/thaw cycles.
   2.2 Other components: Store opened reagents between 2°C and 8°C and use within one month.

Note: Precipitation of Assay Diluent A (5X) may be observed when stored long term between 2°C and 8°C. The precipitation does not alter the performance of the assay. If heavy precipitation is observed, it can be filtered to clarify the solution.

Lot #: B232253



This standard curve is for demonstrative purposes only. A standard curve must be run with each assay.

This is to certify that the product was manufactured under stringent process controls to ensure lot to lot consistency and complete lot traceability. The product has been tested and meets quality control specifications.

Signature: _____ (Quality Control) Date: 3/2/17

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ELISA MAX™ Deluxe Set Protocol

Materials to be Provided by the End-User

1. Phosphate-Buffered Saline (PBS): 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1.0 L, pH to 7.4, 0.2 µm filtered.
2. Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20.
3. Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, e.g. 2N H_2SO_4.
4. Plate Sealers: BioLegend Cat. No. 423601 is recommended.

Reagent Preparation

Reagents Description	Dilute with	Dilution for 1 plate
Coating Buffer A (5X)	Deionized Water	2.4 mL in 9.6 mL DI H_2O
Capture Antibody (200X)	1X Coating Buffer A	60 µL in 12 mL Buffer
Assay Diluent A (5X)	PBS	12 mL in 48 mL PBS
Detection Antibody (200X)	1X Assay Diluent A	60 µL in 12 mL Buffer
Avidin-HRP (1,000X)	1X Assay Diluent A	12 µL in 12 mL Buffer

Standard reconstitution: Reconstitute the lyophilized Human IL-10 Standard by adding 0.2 mL of 1X Assay Diluent A to make the 150 ng/mL standard stock solution. Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.

To prepare 250 pg/mL top standard, perform an initial 1:10 dilution by adding 10 µL standard stock solution to 90 µL of 1X Assay Diluent A. Then add 16.7 µL to 983.3 µL of 1X Assay Diluent A. Perform six two-fold serial dilutions of the 250 pg/mL top standard with 1X Assay Diluent A in separate tubes. 1X Assay Diluent A serves as the zero standard (0 pg/mL).

Samples: For cell culture supernatant samples, the end user may need to determine the dilution factors in a preliminary experiment. Serum or plasma samples should be tested initially without any dilution. If dilution is required, samples should be diluted in 1X Assay Diluent A before adding to the wells.

TMB Substrate Solution is a mixture of equal volumes of Substrate Solution A and Substrate Solution B. Mix the two components immediately prior to use. For one plate, mix 5.5 mL Substrate Solution A with 5.5 mL of Substrate Solution B in a clean container (solution should be clear and colorless).

ELISA Procedure Summary

Day 1

Add 100 µL diluted Capture Antibody solution to each well, seal the plate and incubate overnight between 2°C and 8°C.

Day 2

1. Wash plate 4 times*, block the plate by adding 200 µL 1X Assay Diluent A to each well, seal the plate and incubate at room temperature for 1 hour with shaking at approximately 500 rpm (with a 0.3 cm circular orbit). All subsequent incubation with shaking should be performed similarly.
2. Wash plate 4 times*, add 100 µL diluted standards and samples to the appropriate wells.
3. Seal the plate and incubate at room temperature for 2 hours with shaking.
4. Wash plate 4 times*, add 100 µL diluted Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.
5. Wash plate 4 times*, add 100 µL diluted Avidin-HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes with shaking.
6. Wash plate 5 times*, soaking for 30 seconds to 1 minute per wash. Add 100 µL of freshly mixed TMB Substrate Solution to each well and incubate in the dark for 30 minutes.
7. Add 100 µL Stop Solution to each well. Read absorbance at 450 nm and 570 nm within 15 minutes. The absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

***Plate Washing:** Wash step is crucial to assay precision. Wash the plate with at least 300 µL of Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean absorbent paper.

For more detailed set information, please refer to the online manual at: www.biolegend.com/media_assets/pro_detail/datasheets/430604.pdf

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Appendix 8

Sudan University of science and technology

Faculty of Medical Laboratory

M.SC in Parasitology and medical entomology

Questionnaire

Questionnaire about:

Prevalence and Quantities Assessment of Cytokines in Malaria Infection in East Nile locality.

Demographic Data:

1- **Age:**.....…… years

2- **Sex**: (a) - Male ☐ (b) Female ☐

3- ***Residence*****…….....**

4- **Occupation**

(a) private sector ☐ (b) House wife ☐ (c) Government employee ☐

Health Data:

Do you suffering from chronic disease?

BP ☐ Renal disease Heart disease ☐ ☐

DM ☐ other.....

Last time you had malaria?

.....

How many times did you have Malaria in this year?

……..……

Any sensitive to drugs? Yes ☐ No ☐

If yes? What type of drugs?

…….....

Witch drug did you take for treatment?

Quinine ☐

Quartum ☐

Artesunate ☐

Preventive measures:

Changing stored water: Yes ☐ No ☐

Do you use bed nets? Yes ☐ No ☐

Spraying insecticides Yes ☐ No ☐

Lab result:

Blood film ☐

P.fP.vmix infection ☐ ☐

Stage Density ☐ ☐

Rapid test (ICT)

-ve ☐ **+ve** ☐

Type of positive

p.f ☐ **P.v** ☐ **Pan** ☐

Profiling serum cytokine result by ELISA