Sudan University of Science and Technology

College of Graduate Studies

Assessment of Serum Tumor Necrosis Factor-Alpha Level in Sudanese Neonates with Bacterial Sepsis at Omdurman Maternity Hospital in Khartoum State

تقييم مستوى مصل عامل النخر ألفا عند حديثي الولادة السودانيين المصابين بتعفن الدم البكتيري في مستشفى الولادة بأم درمان في ولاية الخرطوم

A Dissertation Submitted in Partial Fulfillment of the Requirements for the M.Ss. Degree in Medical Laboratory Sciences (Microbiology)

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الآية

قَالَ تَعَالَىٰ:

﴿ يَٰٓأَيُّهَا ٱلَّذِينَ ءَامَنُوٓاْ إِذَا قِيلَ لَكُمۡ تَفَسَّحُواْ فِي ٱلۡمَجَٰلِسِ فَٱفۡسَحُواْ يَفۡسَحِ ٱللَّهُ لَكُمۡۖ وَإِذَا قِيلَ ٱنشُزُواْ فَٱنشُزُواْ يَرۡفَعِ ٱللَّهُ ٱلَّذِينَ ءَامَنُواْ مِنكُمۡ وَٱلَّذِينَ أُوتُواْ ٱلۡعِلۡمَ دَرَجَٰتٖۚ وَٱللَّهُ بِمَا تَعۡمَلُونَ خَبِيرٞ ١١ ﴾

صدق الله العظيم

سورة المجادلة الآية

(11)

Dedication

To

My mother

A strong and gentle soul who taught me to trust Allah, believe in hard work and that so much could be done with little

To

My father

For earning an honest living for us and for supporting and encouraging me to believe in my self

To

My sisters

for their bearing in my resentment and nervousness, and motivating me to work

To

My fiancé

For his permanent assistance to complete this work and for his encouragement all the way and whose encouragement has made sure that I give it all it takes to finish that which I have started

To

My friends

for supporting and helping me during this work

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Abstract

Worldwide, neonatal sepsis accounts for an estimated 26% of under-five deaths, with sub-Saharan Africa (SSA) having the highest mortality rates, 49.6% of all under-five deaths.

TNF-α is the prime mediator of septic shock in neonates and wide spread tissue injury. The role of TNF-α as a marker for the prediction of neonatal sepsis has been suggested. Additionally, when used in combination with IL-6, TNF-α may achieve up to 98.5% sensitivity.

This cross-sectional, case control study was aimed to assess the serum TNF-α level in Sudanese neonates infected with bacterial sepsis and control subjects, in Omdurman Maternity Hospital in Khartoum State during the period from December (2019) to November (2020). In which 44 septic neonates and 44 of age and sex- matched healthy control neonates were enrolled.

Among the septic neonates; 27/44 (61%) had early-onset and 17 (39%) had late-onset neonatal sepsis, 25 (57%) were males and 19 (43%) were females, 20 (45%) were preterm neonates and 24 (55%) were term, while control neonates included; 23/44 (52%) males and 21(48%) females, 15 (34%) preterm neonates and 29 (66%) term neonates.

TNF-α concentration was measured using Enzyme Linked Immunosorbent Assay (ELISA).

The result showed that: there was significant difference in serum level of TNF-α between septic neonates and healthy one (*p. value* = 0.000).

Concerning onset of disease, there was no significant difference in serum levels of TNF-α in EOS and LOS (*p. value* = 0.621).

Level of serum TNF-α in preterm neonates was differ than term in both patient and control groups but the difference was statistically insignificant (*p. value* = 0.285, *p. value* = 0.124 respectively).

Regarding to gender, there was meaningless differences between males and females in both septic (*p.value* =0.467) and healthy neonates (*p.value* was 0.726).

This study concluded that; serum level of TNF-α was higher in septic neonates compared with healthy neonates. There was no significant difference in serum levels of TNF-α according to onset of disease, gestational age and gender.

TNF-α concentration was elevated in septic neonates and might be a useful marker for diagnosis of neonatal bacterial sepsis.

مستخلص البحث

في جميع أنحاء العالم ، يمثل الإنتان الوليدي حوالي 26٪ من وفيات الأطفال دون سن الخامسة ، مع وجود أعلى معدلات الوفيات في أفريقيا جنوب الصحراء الكبرى ، 49.6٪ من جميع وفيات الأطفال دون سن الخامسة.

عامل نخر الورم ألفا هو الوسيط الرئيسي للصدمة الإنتانية عند الولدان وإصابة الأنسجة المنتشرة. تم اقتراح دور عامل نخر الورم ألفا كعلامة للتنبؤ بالإنتان الوليدي. بالإضافة إلى ذلك ، عند استخدامه مع إنترلوكين 6 ، قد يحقق عامل نخر الورم ألفا حساسية تصل إلى 98.5٪.

هدفت هذه الدراسة المقطعية إلى تقييم مستوى مصل عامل نخر الورم ألفا عند حديثي الولادة السودانيين المصابين بتعفن الدم البكتيري و حديثي الولادة الطبيعيين في مستشفى الولادة بأم درمان في ولاية الخرطوم خلال الفترة من ديسمبر (2019) إلى نوفمبر (2020). تم اختيار 44 من حديثي الولادة المصابين بالإنتان البكتيري و44 من الولدان الطبيعيين المتطابقين من العمر والجنس.

من بين الولدان المصابين بالإنتان ؛ كان 44/27 (61%) منهم مصابًا بإنتان حديثي الولادة المبكر و17 (39%) مصابًا بإنتان حديثي الولادة المتأخر، 25 (57%) منهم من الذكور و 19 (43%) من الإناث وكان 20 (45%) منهم طفلا خديجاً و 24 (55%) طفلا مكتملا ، بينما شمل الولدان الطبيعيين ؛ 44/23 (52%) من الذكور و 21 (48%) من الإناث , 15 (34%) من الخدج و 29 (66%) من المكتملين.

تم قياس مستوى عامل نخر الورم ألفا في الدم باستخدام مقايسة الامتزاز المناعي المرتبط بالإنزيم.

أظهرت النتائج أن: هناك فرق كبير في مستوى مصل عامل نخر الورم ألفا بين حديثي الولادة المصابين بالإنتان البكتيري وحديثي الولادة الطبيعيين (القيمة الاحتمالية = 0.000).

فيما يتعلق بوقت ظهور المرض ، لم يكن هناك فرق كبير في مستويات المصل من عامل نخر الورم ألفا في الإنتان المبكر و الإنتان المتأخر (القيمة الاحتمالية = 0.621).

كان مستوى TNF-α في مصل الدم عند الولدان الخدج يختلف عن الناضجين في كل من مجموعات المرضى والمقارنة ولكن الفرق كان ضئيلًا من الناحية الإحصائية (كانت القيم الاحتمالية 0.285 و0.124 على التوالي).

فيما يتعلق بالجنس ، كانت هناك اختلافات لا معنى لها بين الذكور والإناث في كل من مجموعة الأطفال المرضى (القيمة الاحتمالية= 0.467) و مجموعة الأطفال الطبيعيين (القيمة الاحتمالية =0.726) .

لقد توصلت الدراسة إلى أن مستوى عامل نخر الورم ألفا مرتفع بشكل ملحوظ عند حديثي الولادة المصابين بتعفن الدم البكتيري مقارنة بحديثي الولادة الطبيعيين الغير مصابين بالمرض. ولم يكن هناك فرق كبير في مستويات عامل نخر الورم ألفا تبعاً لوقت ظهور المرض وعمر الحمل والجنس.

إن تركيز عامل نخر الورم ألفا مرتفع لتعفن الدم البكتيري عند حديثي الولادة ويمكن أن يستخدم كعلامة مفيدة.

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LIST OF ABBREVIATIONS

ANC	Absolute neutrophil count
APPs	Acute phase Proteins
BSI	Blood stream infection
CAM	Chorioamnionitis
CD	Neutrophil cluster of differentiation
CoNS	Coagulase Negative *Staphylococci*
CRP	C-reactive protein
ELISA	Enzyme Linked Immunosorbent Assay
EOS	Early onset sepsis
GBS	Group B *Streptococcus*
GM-CSF	Granulo-macrophage colony-stimulating
HRP	Horse radish peroxidase
ICAM	Intercellular adhesion molecule
IFNγ	Interferon gamma
IL-6	Interleukin 6
IL-8	Interleukin 10
IL-8	Interleukin 8
LOS	late onset sepsis
LPS	Lipopolysaccharaide
MIF	Macrophage migration inhibitory factor
MRSA	Methicillin resistant *Staphylococcus aureus*
NICUs	Neonatal intensive care units
P. value	Probability value
PCR	Polymerase chain reaction
PCT	Procalcitonin
Pg/ml	Pico gram/milliliter
PMNs	polymorph nuclear leukocytes
PROM	Prolonged rupture of membranes
RT	Room temperature
SIRS	Systemic inflammatory response syndrome
SPSS	Statistical package for social sciences

SSA	Sub Saharan Africa
Th	T helper cells
TMB	Tetra methyl blue
TNF-α	Tumor necrosis factor alpha
VCAM	vascular cell adhesion molecule
VLBW	very low birth weight

CHAPTER I
INTRODUCTION

CHAPTER I

1. INTRODUCTION

1.1. Introduction

Neonatal sepsis is a blood stream infection associated with inflammation and life threatening organ dysfunction. It is classified as early-onset sepsis (EOS, <72 h after birth) or late-onset sepsis (LOS, >72 h) (Ng *et al*., 2018).

Bacterial sepsis remains one of the most common diseases of the neonatal period and is a global problem (Lawn *et al*., 2005).

Worldwide, neonatal sepsis accounts for an estimated 26% of under-five deaths, with sub-Saharan Africa (SSA) having the highest mortality rates, 49.6% of all under-five deaths (Adatara *et al*., 2019).

In Sudan, the prevalence of neonatal sepsis is 17.5% and the mortality is 14.5% (Abdelaziz *et al*., 2019).

Blood culture has been the gold standard for confirmation of the diagnosis but the sensitivity is low and results are usually not promptly obtained (Liu *et al*., 2014). Sepsis cannot always be excluded even when blood cultures are found to be negative (Shah and Padbury, 2014). Thus, the diagnosis of neonatal sepsis is based on a combination of clinical presentation; the use of non-specific markers, including C-reactive protein and procalcitonin and the use of molecular methods, including PCR, Cytokines, including tumor necrosis factor alpha (TNF-α) interleukin 6 (IL-6), interleukin 8 (IL-8), gamma interferon (IFNγ) (Simonsen *et al*., 2014).

Changes in the blood levels of cytokines occur rapidly in the setting of neonatal sepsis even before that of acute phase reactants (Benitz, 2010). TNF-α is the prime mediator of septic shock in neonates and widespread tissue injury, release of it may occur approximately 30 minutes after LPS injection. The role of TNF-α as a marker for the prediction of neonatal sepsis has been suggested. Additionally, when used in combination with IL-6, TNF-α may achieve up to 98.5% sensitivity (Machado *et al*., 2014). Kocabaş and colleagues demonstrated that TNF-α are the best markers in the diagnosis of neonatal Sepsis (Kocabaş *et al*., 2007). The TNF-α has appropriate accuracy for the diagnosis of neonatal sepsis and thus is a good biomarker for the diagnosis of neonatal sepsis (Lv *et al*., 2014).

1.2. Rationale

Blood culture has been the gold standard for diagnosis of neonatal sepsis (Liu *et al*., 2014). but it limited by the time needed to isolation of the infecting organism, resulting in a delay of appropriate antimicrobial therapy (Hornik *et al*., 2012). The use of biomarkers in neonatal infection has remained an important area of research in the past decades, many infection markers are components of the inflammatory cascade and reflect response to infection. Cytokines and chemokines have been demonstrated to have good diagnostic utilities as early phase inflammatory markers for diagnosis of neonatal bacterial sepsis (Lam and Ng, 2008).

There are many studies regarding biomarkers that used for early diagnosis of neonatal sepsis in Sudan; for example a study conducted by Kheir *et al* in 2013 at Soba University Hospital, they reported that C-reactive protein was a reliable diagnostic marker of neonatal sepsis, especially in developing communities with poor resources,

But the role of cytokine TNF-α as aseptic marker has not been well established yet, Therefore, this study was conducted to evaluate the potential diagnostic value of TNF-α for early diagnosis of neonatal bacterial sepsis in Sudanese neonates. The result of this study would help to knew if TNF-α was a good marker for neonatal bacterial sepsis and might help in early treatment of it and following the effectiveness of treatment. Since blood culture takes time the elevated level of TNF-α considered as rapid indicator for neonatal sepsis.

1.3. Objectives

1.3.1. General objective

To assess serum TNF-α level in Sudanese neonates infected with bacterial sepsis at Omdurman maternity hospital in Khartoum State.

1.3.2. Specific objectives

1. To estimate serum level of TNF-α by ELISA technique in septic neonates (patients) and healthy neonates (controls).
2. To compare levels of TNF-α between septic neonates and healthy one.
3. To compare serum levels of TNF-α in neonates with EOS and LOS.
4. To compare the mean of serum levels of TNF-α in preterm and term neonates among study groups.
5. To compare the mean of serum levels of TNF-α in study groups according to gender.

CHAPTER II

LITERATURE REVIEW

CHAPTER II

2. LITERATURE REVIEW

2.1. Sepsis

2.1.1. Definition of sepsis

The definition of sepsis has shifted over time. Sepsis defined as a systemic inflammatory response syndrome (SIRS) due to infection. “Severe” sepsis was defined as sepsis associated with organ dysfunction, hypoperfusion and hypotension, and “septic shock” was defined as sepsis with arterial hypotension despite adequate fluid resuscitation. Sepsis also defined as life-threatening organ dysfunction caused by a deregulated host response to infection (Sartelli *et al*., 2018).

Neonatal sepsis is a systemic infections of the newborn such as septicemia, meningitis, pneumonia, arthritis, osteomyelitis, and urinary tract infections. The term “neonatal sepsis” is reserved for blood stream infection which is seen in the first month of life of the neonate (Moges *et al*., 2017).

2.2. Neonatal bacterial sepsis

Neonatal bacterial sepsis (NBS) remains as an important cause of mortality and morbidity among infants. In most of developing countries, Gram-negative bacilli remain the major cause of neonatal bacterial sepsis (Movahedian *et al*., 2006).

It classified according to the time of onset of the disease to early onset (EOS) and late onset (LOS) (Vergnano *et al*., 2005). EOS is defined as onset of sepsis in the first 3 days of life and is usually due to transplacental, ascending, or intrapartum transmission of bacteria from mothers to infants in the perinatal period shortly before or during birth (Bhandari *et al*., 2014). LOS is defined as infection occurring after 1 week of life is attributed to the horizontal transmission of pathogens acquired postnatally and is often more insidious in onset (Bizzarro *et al*., 2005).

2.3. Etiology of neonatal bacterial sepsis

2.3.1. Organisms associated with early-onset sepsis (EOS)

The two most common pathogens making up 70% of all the early neonatal infections are *Streptococcus* group B (GBS) and *E.coli.* Other less common pathogens making up the final 30% are other *streptococci* (Viridians and pneumonia), *Staphylococcus aureus*, *Enterococcus*, *H.influenzae* (excluding group B) and *Listeria monocytogenes* (Klobučar, 2017).Gram-negative organisms such as *Klebsiella*, *Pseudomonas* and *Salmonella* are also more common (Zaidi *et al*., 2009).

2.3.2. Organisms associated with late-onset sepsis (LOS)

Coagulase Negative *Staphylococci* (CoNS) have emerged as the single most commonly isolated pathogen among VLBW infants with LOS. And *Staphylococcus aureus* is associated with 4–8% of LOS (Camacho-Gonzalez *et al*., 2013). Methicillin resistant *Staphylococcus aureus* (MRSA) has been isolated in 28% of staphylococcal infections in preterm neonates (Shane *et al*., 2012). Gram-negative bacilli responsible for neonatal LOS mainly include *Escherichia coli*, *Klebsiella spp*., *Enterobacter spp*. and *Pseudomonas spp* (Dong and Speer, 2015). A study by Abdelaziz *et al* done at Maternity Hospital in Sudan reported Gram-negative organisms as the commonest pathogen isolated. *Klebsiella pneumoniae* (21.2%) was the most frequent gram-negative organism and Methicillin resistant *Staphylococcus aureus* (MRSA) (33.7%) was the most common isolated bacteria (Abdelaziz *et al*., 2019). Similar finding to Abdelaziz was reported by Babiker *et al* in a study done at Soba University Hospital in Sudan which reported MRSA and *K. pneumoniae* are the most common isolated organisms followed by *P. aeruginosa, S. aureus, E. fecalis and E. coli* (Babiker *et al*., 2018).

2.4. Epidemiology of neonatal bacterial sepsis

Eighty-five percent of newborns with early-onset infection present within 24 hours and onset is most rapid in premature neonates (Khamis *et al*., 2015). With improved survival of preterm infants, LOS has become an important cause of morbidity and mortality among low birth weight infants (Gladstone *et al*., 1990).

The incidence of neonatal sepsis is related to the gestational age of the newborn and Preterm neonates have higher incidence of sepsis than term neonates. However, the incidence of sepsis is significantly higher in neonates with very low birth weight (VLBW) than neonates with normal weight this due to the deficiencies in the immune mechanism in Preterm neonates than in full term babies (Anderson *et al*., 2009).

The incidence of neonatal sepsis is affected by socioeconomic status such as gender (males are four times more affected than females), income, as long as the standard of neonatal care received. The global incidence of neonatal sepsis is estimated at 5-6/1000 live births, 6-21/1000 live births in sub-Saharan Africa, 1.8-12/1000 live births in the Middle East and North Africa (Khamis *et al*., 2015). The rates of blood stream infection (BSI) in neonates are 3-20 times higher in developing countries, and in some countries, approximately half of the patients in neonatal intensive care units (NICUs) acquire infection. (Sorsa, 2019). It is estimated that 3 million newborns and 1.2

million children suffer from sepsis globally every year. Three out of every ten deaths due to neonatal sepsis are thought to be caused by resistant pathogens (WHO, 2018).

Neonatal sepsis that caused by Gram negative rods bacteria is a significant cause of morbidity and mortality among neonates it was responsible for 18%-78% of all neonatal sepsis (Babiker *et al*., 2018).

2.5. Mode of transmission and pathogenesis of neonatal bacterial sepsis

Throughout pregnancy, the fetus is relatively protected from the microbial flora of the mother by the chorioamniotic membrane, the placenta and poorly understood antibacterial factor in amniotic fluid (Klein, 2001). One of the maternal risk factors that have been heavily studied is prolonged rupture of membranes (PROM). It is defined as the rupture of the membranes longer than 24 hours before delivery and carries the increased risk of ascending infection, which can put both the mother and child at risk (Caughey *et al*., 2008).

Initial colonization of the neonate usually takes place after rupture of the chorioamniotic membranes. Prolonged rupture of the chorioamniotic membranes (>18 hours) often results in chorioamnionitis, which is a bacterial infection that results in the inflammation of the fetal membranes and is considered a strong risk factor for the development of neonatal sepsis (Klobučar, 2017).

Fetal infection can result from aspiration of infected amniotic fluid, which may result in congenital pneumonia, with or without bacteraemia. Infected amniotic fluid can also be ingested leading to gastrointestinal colonization with invasion of the blood stream and systemic infection. Vigorous resuscitation at birth particularly if it involves endotracheal intubation or insertion of umbilical vessel catheter are associated with an increased risk of bacterial infection (Chama, 2013).

After birth, neonates are exposed to infectious agents in the nursery or in the community and postnatal infection may be transmitted by direct contact with hospital personnel, mother or other family members. The most common source of postnatal infections in hospitalized newborns is hand contamination of health care personnel. Other sources include sophisticated equipment for respiratory and nutritional support, arterial and venous umbilical catheters, central venous catheters, peripheral arterial and venous canulation. Also urinary indwelling catheters provide enormous opportunities for relatively non-virulent pathogens to establish infection and invade the host (Stoll, 2007).

2.6. Host susceptibility

The body produces several different types of pro inflammatory cytokines in attempt to protect itself. The process begins by the bacterial agent entering the circulatory system and interacting with the complement cascade causing the release of several pro inflammatory mediators such as C3 and C5a. These mediators cause vessel vasodilation and a release of proinflammatory cytokines such as interleukin-1, 6 and 8 (IL-1-6-8) (Dessi *et al*., 2014). These cytokines facilitate the adhesion of leukocytes to the endothelial wall, releasing nitric oxide and reactive oxygen species that then cause injury to the endothelial cell wall (Silveira *et al*., 2010).

The bacterial antigen can facilitate the formation of microthrombi in the microcirculation, further compromising tissue perfusion and the immaturity of the neonate's immune system fails to produce adequate anti-inflammatory cytokines (Dessi *et al*., 2014). Due to this immaturity of the immune system compared to adults, they have an increased rate of infections (Yost *et al*., 2009). Neonates are also not able to proficiently combat the effects of the poly microbial flora that they are exposed to during and after birth. It is therefore during this period their major source of defense against any pathogen is their innate immunity and the passive protection that is acquired in utero from their mothers (Yoon, 2010).

2.7. Clinical manifestations of neonatal sepsis

Neonates with bacterial sepsis may have either non-specific signs and symptoms or focal signs of infection (Anderson *et al*., 2009). These include temperature instability, hypotension, poor perfusion with pallor and mottled skin, metabolic acidosis, tachycardia or bradycardia, apnoea, respiratory distress, grunting, cyanosis, irritability, lethargy, seizures, feeding intolerance, abdominal distension, jaundice, petechiae, purpura and bleeding (Gonzalez *et al*., 2003).

2.8. Diagnosis

One of the major difficulties in the management of neonatal sepsis is getting an accurate diagnosis. Unlike older patients, newborns have very subtle presentations, and multiple conditions resemble neonatal sepsis. Auxiliary tests have limited value and are difficult to interpret due to low sensitivity and changing normal ranges during the neonatal period. Blood cultures also lack sensitivity due to specific characteristics of the neonatal population. As a result, a combination of findings is necessary to provide a correct diagnosis of neonatal sepsis (Camacho-Gonzalez *et al*., 2013).

2.8.1. Blood culture

The isolation of an organism confers many advantages, including the optimal choice and duration of antibiotic treatment. Blood cultures are still the gold standard in the diagnosis of neonatal sepsis. However, obtaining cultures from neonates can be difficult as sample volumes are small and a substantial number of cultures turn out to be contaminated or negative. The minimum volume required for a reliable culture result has been estimated as 1.0 ml (Resch *et al*., 2012).

Negative culture include insufficient blood sample volumes, intermittent or low density bacteriaemia and prior use of antibiotics and difficulty in pathogen isolation, positive blood culture ranged from 23%-70% in neonatal sepsis (Ambe *et al*., 2007).

2.8.2. Complete Blood Cell Count

Complete blood cell count is difficult to interpret in the neonatal period because it varies significantly with day of life and gestational age. Low values of white blood cells, low values of absolute neutrophil counts and high immature/total ratio are associated with early-onset sepsis. High or low white blood cells counts, high absolute neutrophil counts, high immature/total ratio and low platelet counts are associated with late-onset sepsis, all of these findings have low sensitivities (Zea-Vera and Ochoa, 2015).

Components of the white cell count, including absolute neutrophil count (ANC) and immature to total neutrophil ratio (I:T) have also been shown to be more useful for excluding infants without infection rather than identifying newborns who are infected (Polin *et al*., 2012).

2.8.3. Molecular techniques for early detection of neonatal sepsis

A meta-analysis of 23 studies on molecular diagnosis of neonatal sepsis found that real time PCR assays performed the best, with 96% sensitivity and 96% specificity. Ribosomal RNA unique to bacteria are detected by 16 s RNA. It has a high sensitivity, but has a high frequency of contamination, and it cannot determine bacterial antibiotic sensitivities (Liu *et al*., 2014).

2.8.4. Biomarkers of bacterial neonatal sepsis

The diagnosis of neonatal sepsis is complicated by non-specific clinical symptoms, a high-false negative rate, and a delay in obtaining blood culture results. An ideal biomarker needs to have a high degree of accuracy in recognizing the presence or absence of definite infection at an early stage, to guide the initiation and duration of antibiotic therapy. The diagnostic utility of the following biomarkers seems to be

most practical in the early (IL-6, IL-8, TNF- α, neutrophil CD_{64}), mid (procalcitonin) and late (C-reactive protein) phases of neonatal sepsis (Bhandari, 2014).

2.8.4.1. Acute phase proteins (APPs)

They are an evolutionarily conserved family of proteins produced mainly in the liver in response to infection and inflammation (Janciauskiene *et al*., 2011).

2.8.4.1.1. C Reactive Protein (CRP)

C Reactive protein is one of the most extensively studied, most available, and most frequently used laboratory tests for the diagnosis of neonatal sepsis, It is an acute phase reactant synthesized by the liver, has a half- life of 24–48 hrs and takes 10–12 hrs for CRP to change significantly after onset of infection. Serial determination of CRP 24–48 hrs after the onset of symptoms increases its sensitivity (Mishra *et al.*,2006).

Serial CRP measurements may also be helpful in monitoring the response to treatment in infected neonates and thus may help clinicians guide the duration of antibiotic therapy. The specificity and positive predictive value of CRP ranges from 93–100%. Thus, CRP can be considered as a "specific" but "late" marker of neonatal infection. If the CRP levels remain persistently normal, it correlates strongly with the absence of infection thereby guiding safe discontinuation of antibiotic therapy (Shah and Padbury, 2014).

2.8.4.1.2. Procalcitonin (PCT)

Procalcitonin is an acute phase marker and has been intensively investigated for its diagnostic role in neonatal sepsis since the mid 1990s. It has been reported that serum concentrations of PCT begin to rise four hours after exposure to the bacterial endotoxin, peak at six to eight hours, and remain elevated for at least 24 hours (Dandona *et al*., 1994).

PCT is synthesized in the liver and induced by similar means with acute phase reactants such as CRP. In fact, it has been shown that a great amount of PCT is produced in human liver cells after TNF-α and IL-6 stimulation (van Rossum *et al*., 2004).

In many studies, PCT sensitivity in the early diagnosis of neonatal sepsis was found to be 83-100% while the specificity was 70-100% (Simon *et al*., 2004).

2.8.4.2. Cell surface markers

With the advances in flow cytometric technology, cell surface antigens can be detected in blood cells. Neutrophil cluster of differentiation (CD) $CD_{11\beta}$ and CD_{64}

have been found to be reliable markers for detecting early and late-onset neonatal sepsis respectively with a high sensitivity and specificity (Ng, 2004).

2.8.4.2.1. $CD_{11\beta}$

It is an α subunit of the β2 integrin adhesion molecule. It is a cell-surface antigen of neutrophil and is normally expressed at a very low level on the surface of non-activated cells. Its expression on neutrophil cell surface, however, increases substantially within a few minutes after the cell comes into contact with bacteria or endotoxins. This unique property enables $CD_{11\beta}$ to be used as a potential early warning marker for detection of bacterial infection. Similarly, the high affinity FcγRI (Ng *etal*., 2002). Its expression is influenced by IL-8, a strong neutrophil activating agent. The sensitivity and specificity are as high as 96–100% (Nupponen *et al*., 2001). And it has better diagnostic accuracy for early than late-onset neonatal sepsis (Ng, 2004).

2.8.4.2.2.CD_{64}

It a high affinity receptor that binds monomeric IgG, is normally expressed by monocytes and weakly on resting neutrophils. The expression of neutrophil CD_{64} (nCD_{64}) is considered to be a very early phase of the host's immune response to bacterial infection, increasing about one hour after invasion. It is stimulated by inflammatory cytokines, then increases in a graded manner. nCD_{64} expression remains stable for more than 24 hrs. The development of flow cytometric technology has made it possible to measure nCD_{64} quickly and precisely with minimal blood volumes, the sensitivity of nCD_{64} ranges from 57 to 89%, and the specificity ranges from 62 to 100%, indicating that nCD_{64} is a reliable marker in the diagnosis of neonatal sepsis (Dai *et al*., 2017).

2.8.4.3. Cytokines profile

Multiple cytokines have been studied for diagnosis of neonatal sepsis including Il-6, Il-8, Il-10 and TNF -α. IL-6 and IL-8 increase very rapidly with bacterial invasion but they promptly normalize in serum levels (within the first 24 hours) (Ng *et al*., 2003). cytokine analysis may be useful in predicting late-onset infection (Shah and Padbury, 2014).

Many new chemokines, antimicrobial peptides, acute phase reactants and cell surface antigens are being investigated. Some of them include interferonY inducible protein (IP-10), monokine induced by interferon Y (MIG), monocyte chemoattractant protein-1 (MCP-1), growth related oncogenes α (GRO-α). The technologies include use of

genomics and proteomics for identification of host response biomarkers and quantitative flow cytometry also under developed (Singh and Devi, 2013).

2.9. Cytokines

Cytokines are small secreted proteins released by cells have a specific effect on the interactions and communications between cells. Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). It is common for different cell types to secrete the same cytokine or for a single cytokine to act on several different cell types (pleiotropy) (Zhang and An, 2007).

Cytokines are redundant in their activity, meaning similar functions can be stimulated by different cytokines. They are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines, Cytokines can also act synergistically or antagonistically (Xie *et al*., 2006).

Cytokines are made by many cell populations (neutrophils, monocytes, macrophages, B-cells, and T-cells), but the predominant producers are helper T cells (Th) and macrophages (Xie *et al*., 2006).

Cytokines act as mediators and modulators within highly localized environments and regulate immunological responses, hematopoietic development, and cell-to-cell communication as well as host responses to infectious agents and inflammatory stimuli (Lefkowitz and Lefkowitz, 2001).

Elevated concentrations of cytokines indicate activation of cytokine pathways associated with inflammation or disease progression. For this reason, cytokine measurements are important as these proteins are widely used as biomarkers to understand and predict disease progression and monitor the effects of treatment. Since cytokines are biomarkers of inflammatory-based diseases, nearly every type of disease has involvement of cytokines as potential biomarkers (Stenken and Poschenrieder, 2015).

2.9.1. The role of cytokines in neonatal sepsis

Cytokines play a central role in immune response in neonates with sepsis. During sepsis, cytokine levels may be observed in picograms per milliliter of plasma or in nanograms or even micrograms per milliliter. In the 1990s, sepsis was believed to be associated with an exacerbated release of mainly pro inflammatory cytokines, such as tumor necrosis factor (TNF-α), interleukin-1 (IL-1, IL-6, and IL-12), interferon- γ (IFN- γ), and macrophage migration inhibitory factor (MIF) (Machado *et al*., 2014).

TNF, IL-1β and IL-6 are the cytokines that mediate the initial response of the innate immune system to injury or infection. TNF and IL-1β both activate endothelial cells, attracting circulating polymorph nuclear leukocytes (PMNs) to the site. They also enter the circulation, causing fever and other systemic symptoms. IL-6 enhances the liver's production of the acute phase reactants, including CRP, and stimulates a shift in the production of cells in the bone marrow so that more PMNs are produced. Therefore, these three cytokines are essentially responsible for the features of systemic inflammatory response syndrome SIRS and could be potentially useful as biomarkers of sepsis (Faix, 2013).

IL-6 is a cytokine involved in the early host response to infection preceding the increase in CRP. It is synthesized by endothelial cells, mononuclear phagocytes, fibroblasts, amnion, and trophoblastic cells shortly after stimulation by microbial products (Tasci *et al*., 2006).

IL-6 circulating concentration can drop precipitously following antimicrobial treatment, suggesting that IL-6 can be used as a therapeutic monitoring parameter. However, in some cases, such as chorioamnionitis, the passive transfer of IL-6 can result in an elevated IL-6 level after birth, which may lead to a false-positive result. Thus, it is necessary to assess maternal risk factors (Ye *et al*., 2017).

IL-8 is a pro inflammatory cytokine predominantly produced by monocytes, macrophages and endothelial cells with similar kinetic properties with IL-6. IL-8 is considered to be one of the accurate marker having sensitivities ranging from 80-91% and specificities from 76-100%, which the diagnostic accuracy is enhanced by concomitant measurement of CRP (Tripathi and Malik, 2010). IL-8 levels increase about 90 minutes after infection and peak at about 120 minutes in septic neonates (van Deventer *et al*., 1993).

IL-10 and TGF- β are Anti-inflammatory cytokines, important inflammatory mediators, since they play a major role in preventing excess pro inflammatory response during sepsis, This cytokines suppresses the production of pro inflammatory mediators including TNF-α, IL-1, IL-6, TNF-α, and GM-CSF in cells of the immune system (Zhang and An, 2007).

IL-10 has been associated with septic shock in both adults and children. A high IL-10/TNF ratio has also been associated with severe late-onset neonatal sepsis. (Machado *et al*., 2014).

Because IL-6 and IL-10 are two indicators associated with sepsis, the IL-6/IL-10 ratio has higher specificity in the differential diagnosis of neonatal sepsis. In addition, the appropriate response of IL-10 may have a protective effect on systemic inflammatory response syndrome, and a high IL-6/IL-10 ratio has been observed in patients with a worse prognosis (Ng and Lam, 2006).

2.9.2. The role of Tumor Necrosis Factor Alpha (TNF-α) in neonatal sepsis

Tumor necrosis factor (TNF) is a pleiotropic cytokine have major roles in physiology and pathology, which causing necrotic and apoptotic cell death, cellular regulation, differentiation, inflammation, regulation of immune cells and tumorigenesis (Locksley *et al*., 2001)

TNF-α acts via specific trans membrane receptors, TNF receptor TNFR1, and TNFR2 (Lewis *et al*., 1991). TNF- α is the prime mediator of septic shock in neonates and widespread tissue injury and it regulates the secretion of IL-1β (Silveira *and* Procianoy, 1999).

High levels of TNF- α appear to be related to the severity of the disease and Systemic release of TNF- α can cause vasodilation and increased vascular permeability leading to systemic edema, with decreased blood volume and hypoproteinemia that can progress to shock. There was stimulus for leukocyte and platelet adhesion, with clots formation in small vessels and consumption of coagulation proteins that may lead to disseminated intravascular coagulation. This condition may also progress to multiple-organ failure and death in early onset neonatal (Simonsen *et al*., 2014).

TNF-α once released, act on different target cells, such as macrophages, endothelial cells, and neutrophils. leads to an enhanced production of macrophages from progenitor cells. All these effects enhance pro inflammatory responses in sepsis. Because of its unique ability to orchestrate downstream cytokine cascade, TNF-α is considered to be a “master regulator” of inflammatory cytokine production (Schulte *et al*., 2013).

2.10. Previous studies

In a study done by Roman and other participants in 1993 in Spain, TNF-α was measured by immunoradiometric in septic and healthy newborns. In which serum TNF-α level was significantly higher in the group with sepsis than in the healthy neonates.

Another study also done in Spain by Reyes in 2003, who investigated serum levels of TNF-α in neonates with positive blood cultures, neonates with negative blood cultures and healthy neonates. They reported that serum level of TNF-α are significantly higher in infected neonates than in the healthy neonates.

In 2013, Prashant *et al*. in London measured serum TNF-α (ng/ml) in infected neonates with positive blood culture and non-infected as control group. TNF-α showed a significant increase in the infected neonates when compared to the control neonates.

There were meta-analysis was performed by Lv and co–authors in 2014, in China to investigate the diagnostic value of TNF- α in neonatal sepsis (NS). Accordingly, 347 studies were collected totally, in which 15 articles and 23 trials were selected to study the NS in their meta-analysis. The TNF- α test showed moderate accuracy of the diagnosis of NS, both in early-onset neonatal sepsis (sensitivity = 0.66, specificity = 0.76, Q* = 0.74) and in late onset neonatal sepsis (sensitivity = 0.68, specificity = 0.89, Q* = 0.87). They reported that the TNF-α has appropriate accuracy for the diagnosis of NS and thus is a good biomarker for the diagnosis of NS.

In Turkey in 2007, Kurt and his colleagues measured serum level of TNF-α in neonatal sepsis by ELISA at the time of diagnosis and after therapy, their study was performed on newborns who were hospitalized for neonatal sepsis, who were classified as culture-proven sepsis, as culture-negative sepsis and as healthy newborns, they reported that at time of diagnosis serum TNF-α levels of culture-proven sepsis and culture-negative sepsis were significantly higher than those of the control groups and levels at seventh day after antibiotic treatment.

Another study done by Fattah *et al*. in 2017 in Riyadh, KSA who measured TNF-α levels in healthy control neonates, clinical sepsis group with negative blood culture and sepsis group with positive blood culture. The study observed significant elevated in plasma levels of TNF-α in patients group when compared with control group.

In 2010, in Egypt, Shouman and Badr measured serum TNF-α in septic and healthy neonates and reported that; serum TNF-α was significantly higher in the septic group compared with the non-septic group.

CHAPTER Ш
MATERIALS AND METHODS

CHAPTER III

3. MATERIALS AND METHODS

3.1. Study design

This is descriptive, cross-sectional, case-control and hospital based study.

3.2. Study area and duration

Study was conducted in Omdurman Maternity Hospital (the hospital in metropolitan Khartoum, in Omdurman city, is one of the largest maternity hospital in Africa and is the largest maternity hospital in Sudan. It was founded in 1957) in Khartoum State during the period from December 2019 to November 2020.

3.3. Study population

Study population consisted of Sudanese neonates of age between 1- 15 days, divided into 2 groups as follows: clinical sepsis group (patients group) consist of neonates with positive blood culture and who were hospitalized for clinical suspicion of neonatal sepsis in neonatal intensive care units (NICU) and healthy control subjects (control group) consist of healthy neonates who were born normally at Omdurman Maternity Hospital without any abnormal signs or symptoms of infection.

3.3.1. Inclusion criteria

Preterm and term neonates, confirmed bacterial sepsis, had signs of EOS, or LOS and hospitalized in the neonatal intensive care units (patients group), while healthy preterm and term neonates without any signs of infections (control group) were included in this study.

3.3.2. Exclusion criteria

All neonates with situations that affect cytokine level including congenital malformations, congenital infections associated with TORCH complex, neonates under antibiotics therapy, severe perinatal asphyxia and trauma (surgical or birth), anoxic delivery, fetal distress, diabetic mother, mother with systemic lupus erythematosus and other immune disease were excluded from this study.

3.4. Ethical considerations

Permission to carry out this study was obtained from Scientific Research Committee, College of Medical Laboratory Science in Sudan University of Science and Technology and from hospital. Verbal consent was taken from neonate's parents.

3.5. Sample size

Eighty-eight (n= 88) neonates were enrolled in this study. Forty-four were septic neonates and forty-four were healthy neonates.

3.6. Method of data collection

Data was collected through review of collected data in file of each neonate through check list which includes demographic, clinical and laboratory data.

3.7. Sampling technique

Non-probability, convenience sampling technique.

3.8. Laboratory processing

3.8.1. Collection of specimen

One ml of venous blood was collected from patients and controls in plain containers and allowed to clot at room temperature. Then the specimens were centrifuged at 3000 rpm for 10 minutes. Sera was separated in plain containers and stored at -20 ℃ until processing.

3.8.2. ELISA (Enzyme linked Immunosrorbent Assay)

Serum levels of TNF-α were measured using sandwich ELISA technique. 100 µL of diluted capture antibody solution was added to all wells of a 96-well plate; sealed and incubated overnight between 2℃ and 8°C. Plate was washed 4 times with diluted wash buffer. To block non-specific binding and reduce background, 200 µL 1X Assay diluent A was added per well, plate sealed and incubated at RT for 1 hour with shaking at 200 rpm on a plate shaker and washed 4 times with wash buffer. 100 µL of diluted standards were added to the standards wells and 100 µL of serum samples were added to the samples wells. Plate sealed and incubated at room temperature for 2 hours with shaking and washed 4 times with wash buffer. Then100 µL of diluted detection antibody solution was added to each well, plate sealed and incubated at RT for 1 hour with shaking and washed 4 times with wash buffer. 100 µL of diluted Avidin-HRP solution was added to each well, plate sealed and incubated at RT for 30 minutes with shaking. Plate washed 5 times with wash buffer and For the final wash, wells were soaked in wash buffer for 30 seconds to 1 minute for each wash. To minimize background, 100 µL of freshly mixed TMB substrate solution was added to each well and incubated in the dark for 15 minutes. Reaction was stopped by

addition of 100 µL of stop solution to each well and absorbance was read at 450 nm within 15 minutes.

3.8.3. Interpretation of results

For results calculation the standard curve was plotted on log-log axis graph paper with analyte concentration on the x-axis and absorbance on the y-axis. A best fit line was drawn through the standard points. To determine the unknown analyte concentrations in the samples, the absorbance value of the unknown on the y-axis was found and a horizontal line was drawn to the standard curve. At the point of intersection, vertical line was drawn to the x-axis and the corresponding analyte concentration was read.

3.9. Statistical analysis

Data were analyzed by using statistical package for social sciences (SPSS) program (version 20) by using independent T-test. The probability value ≤ 0.05 was considered significant.

CHAPTER IV
RESULTS

CHAPTER IV

4. RESULTS

Eighty-eight (n=88) neonates of age 1-15 days were enrolled in this study and they were two groups: septic group (patient group) and healthy neonates (control group).

In which there were 44 septic neonates with mean age 4± 2S.D, there were 25 (57%) males and 19 (43%) were females, 20 (45%) were preterm neonates and 24 (55%) were term, 27 (61%) had EOS and 17 (39%) with LOS as shown in table 4-1.

While 44 neonates were healthy (control group) with mean age 4±2 S.D, in which there 23 (52%) were males and 21 (48%) were females, 15 (34%) were preterm neonates and 29 (66%) were term (table 4-1).

There was significant difference in serum level of TNF-α between septic neonates and healthy one (*p. value* = 0.000) as presented in table 4-2.

Concerning onset of disease, there was no significant difference in serum levels of TNF-α in EOS and LOS (*p. value* = 0.621) as illustrated in table 4-3.

In this study level of serum TNF-α in preterm neonates was differ than term in both patient and control groups but the difference was statistically insignificant, (*p. value* = 0.285, *p. value* = 0.124 respectively) as shown in table 4-4.

Regarding to gender, there was meaningless differences between males and females in both septic(*p. value* =0.467) and healthy neonates (*p. value* was 0.726) (table 4-5).

Table 4-1: Distribution of demographic data among study population

Variable	Patient group (n=44) Frequency (%)	Control group (n=44) Frequency (%)
Gender		
Male	25 (57%)	23 (52%)
Female	19 (43%)	21 (48%)
Gestational age		
Term	24 (55%)	29 (66%)
Preterm	20 (45%)	15 (34%)
Onset of disease		
EOS	27(61%)	-
LOS	17(39%)	

Table 4-2 : Comparison of TNF-α level between patient and control neonates

Subject	patient group (n=44)	Control group (n=44)	*P. value*
Mean of TNF-α	13.90 ±9.57 S.D	4.43 ±2.58 S.D	0.000

Table 4-3: Comparison of TNF-α levels according to onset of disease in septic neonates

Onset of disease	**EOS** (n=27)	**LOS** (n=17)	*P. value*
Mean ±SD of TNF-α	14.48 ±8.67	12.99 ±11.06	0.621

Table 4-4: Comparison of TNF-α level between term and preterm neonates in patients and controls

Subject	Term	Preterm	*P. value*
Mean ± SD of TNF-α in patients	12.48 ± 11.05	15.61 ± 7.33	0.285

Subject	Term	Preterm	*P. value*
Mean ± SD of TNF-α in controls	4.86 ± 2.81	3.60 ± 1.87	0.124

Table 4-5: Comparison of TNF-α level according to gender in patient and control neonates

Subject	Male	Female	*P. value*
Mean ± SD of TNF-α in patients	14.83 ± 8.96	12.68± 10.43	0.467

Subject	Male	Female	*P. value*
Mean ± SD of TNF-α in controls	4.56 ± 2.74	4.29 ± 2.45	0.726

CHAPTER V

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

CHAPTER V

5. DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1. Discussion

In this study the level of serum TNF-α was significantly higher in septic neonates compared with healthy one. This result was similar to that reported by Ucar *et al*. (2008) in Turkey, Caldas *et al*. (2008) in Spain Prashant *et al*. (2013) in London and Sugitharini *et al*. (2013) in India who were demonstrated that; there was significantly elevated serum TNF-α level in the septic neonates versus normal subjects.

This study was different from Santana *et al*. (2001) in Spain who recorded that there was no statistically significant differences between septic and healthy neonates in level of TNF-α. This differences could be due to the short half-life of TNF-*α* and its interaction with soluble receptor, so the detection becomes difficult.

The present study demonstrated that; there was no statistically significant difference in serum TNF-α level between EOS and LOS. This finding was matched to findings of Kocabaş *et al*. (2007) in Turkey and Fattah *et al*. in Riyadh, KSA (2017) who were showed there was no significant difference in serum TNF-α level between EOS and LOS.

In the current study, serum level of TNF-α in preterm was differ than term neonates in the both patient and control groups but the difference was statistically insignificant, Shouman and and Badr (2010) in Egypt recorded similar to this finding, they reported that there was no statistical differences in serum TNF-α level between two groups.

This study found that; males had higher levels of TNF-α than females in both septic and healthy neonates but the differences were statistically insignificant. There was no similar study concerning gender.

Finally the level of TNF-α was higher in septic patients than normal neonates and no differences between EOS and LOS, gestational age and different sex.

5.2. Conclusions

This study conclude that; serum level of TNF-α was higher in septic neonates compared with healthy neonates. There was no significant difference in serum levels of TNF-α according to onset of disease, gestational age and gender.

TNF-α concentration was elevated in septic neonates and might be a useful marker for diagnosis of neonatal bacterial sepsis.

5.3. Recommendations

Large sample size should be used to get proven results.

Combination of two biomarkers at least should be used to detect the presence of bacterial sepsis, for example combination of CRP and TNF-α should be tested.

Other bacterial sepsis biomarkers should be tested to evaluate their diagnostic value for early diagnosis of neonatal bacterial sepsis.

Regular measurement of serum TNF-α level as indicator for evaluation of prognosis in neonates with sepsis infection**.**

Serum TNF-α level could be measured in suspected septic neonates prior blood culture result.

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Appendix (1)

Check list

Sudan University of Sciences and Technology

Assessment of Serum Tumor Necrosis Factor-Alpha (TNF-α) Level in Sudanese Neonates with Bacterial Sepsis at Omdurman Maternity Hospital in Khartoum State

-Date: …….

-ID Number: ……..

-Postnatal age: ……..…….day(s)

-Gender: Male{ } Female { }

Medical history

-Gestational age: Preterm{ } term{ }

-The time of infection:

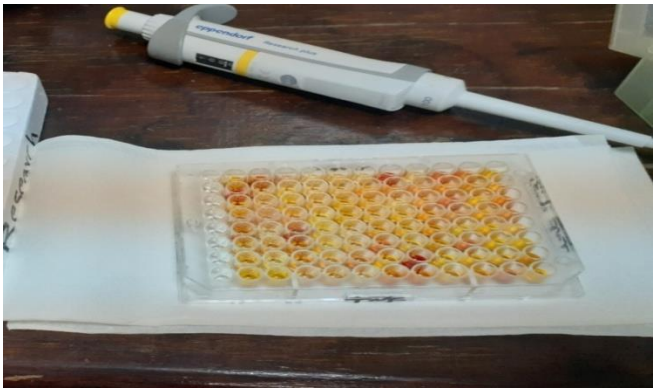
With in 72 hrs{ } After 72 hrs { }

Investigation results

Level of Serum TNF-α: normal { } decreased { } increase { }

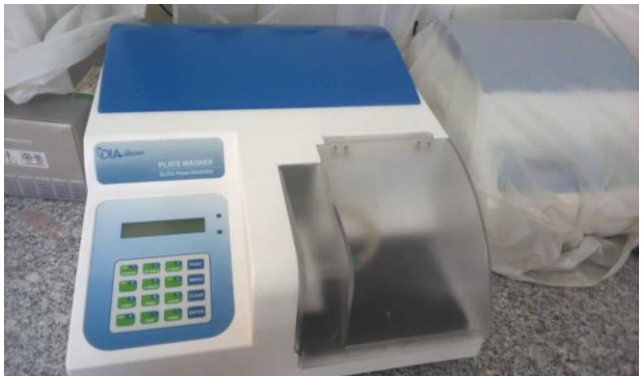
Appendix (2)

2.1. ELISA Kits of TNF-α



2.2. ELISA micro plate

2.3. ELISA Reader



2.4. ELISA Washer

Appendix (3)

BioLegend®
Enabling Legendary Discovery™

Human TNF-α

ELISA MAX™ Standard Set

Cat. No. 430201

BioLegend's ELISA MAX™ Standard Set contains the capture and detection antibodies, recombinant protein standard, and Avidin-HRP required for the accurate quantification of natural and recombinant human TNF-α. These BioLegend's ELISA MAX™ Standard sets are cost-effective and designed for experienced ELISA users. Optimization of reagent concentrations and assay conditions may be required.

It is highly recommended that the instruction sheet be read in its entirety before using this product. Use the recommended assay protocol, microwell plates, buffers, diluent, and substrate solution to obtain desired assay results. Do not use this set beyond the expiration date.

Materials Provided

1. Human TNF-α ELISA MAX™ Capture Antibody (200X)
2. Human TNF-α ELISA MAX™ Detection Antibody (200X)
3. Human TNF-α Standard
4. Avidin-HRP (1000X)
5. Lot-Specific Certificate of Analysis/**ELISA MAX™ Standard Set Protocol**

Introduction

Human TNF-α (Tumor Necrosis Factor-Alpha), also termed Cachectin, Cytotoxic Factor (CF), CTX, Hemorrhagic Factor, Macrophage-Derived Cytotoxic Factor, and Macrophage Cytotoxic Factor (MCF), is a potent multifunctional cytokine which can exert regulatory, inflammatory and cytotoxic effects on a wide range of normal lymphoid and non-lymphoid cells and tumor cells.

Principle of the Test

BioLegend's ELISA MAX™ Standard Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). A human TNF-α specific mouse monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and TNF-α binds to the immobilized capture antibody. Next, a biotinylated mouse monoclonal anti-human TNF-α detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB Substrate Solution, producing a blue color in proportion to the concentration of TNF-α present in the sample. Finally, the Stop Solution changes the reaction color from blue to yellow, and the microwell absorbance is read at 450 nm with a microplate reader.

For research purposes only. Not for use in diagnostic or therapeutic procedures.

Materials to be Provided by the End-User

- Microwell plates: BioLegend Cat. No. 423501 is recommended
- Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20
- 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, pH 7.4, 0.2 µM filtered. (BioLegend Cat. No. 421203 is recommended)
- Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, e.g. 2N H_2SO_4
- Plate Sealers: BioLegend Cat. No. 423601 is recommended
- PBS (Phosphate-Buffered Saline): 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 L; pH to 7.4, 0.2 µm filtered
- Deionized (DI) water
- A microplate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 2 µL to 1 mL
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer

Storage Information

- Store kit components between 2°C and 8°C.
- After reconstitution of the lyophilized standard with Assay Diluent, aliquot into polypropylene vials and store at -70°C. Do not repeatedly freeze/thaw the recombinant protein standard as loss of activity may occur.
- Prior to use, bring all components to room temperature (18°C-25°C). Upon assay completion return all components to appropriate storage conditions.

Health Hazard Warnings

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details (www.biolegend.com/msds).
2. TMB substrate solution is harmful if ingested. Additionally, avoid skin, eye or clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum and/or plasma in accordance with NCCLS regulations.

Specimen Collection and Handling

Cell Culture Supernatant: If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at < -20°C. Avoid repeated freeze/thaw cycles.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 X *g*. Remove serum layer and assay immediately or store serum samples at < -20°C.
Avoid repeated freeze/thaw cycles. Serum specimens should be clear and non-hemolyzed.

Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1,000 X *g* within 30 minutes of collection. Assay immediately or store plasma samples at < -20°C. Avoid repeated freeze/thaw cycles. Plasma specimens should be clear and non-hemolyzed.

Reagent and Sample Preparation

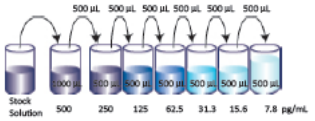
Do not mix reagents from different sets or lots. Reagents and/or antibodies from different manufacturers should not be used with this set. All reagents should be diluted immediately prior to use.

Preparation of 1X Reagent for 1 Plate

Material	Dilute with
60 µL of Capture Antibody (200X)	12 mL of Coating Buffer
60 µL of Detection Antibody (200X)	12 mL of Assay Diluent
12 µL of Avidin-HRP (1,000X)	12 mL of Assay Diluent

Standard: Lyophilized vials are under vacuum pressure. **Refer to Lot-Specific Certificate of Analysis** for Standard Reconstitution. Allow the reconstituted standard to sit fo 15 minutes at room temperature, then mix gently prior to making dilutions.

Prior to use, refer to **Lot-Specific Certificate of Analysis** for preparation of 1,000 µL of the top standard at a concentration of 500 pg/mL from stock solution in Assay Diluent. Perform six two-fold serial dilutions of the 500 pg/mL top standard with Assay Diluent in separate tubes. After diluting, the human TNF-α standard concentrations are 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, and 7.8 pg/mL, respectively. Assay Diluent 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. 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.

Assay Procedure

Do not use sodium azide in any solutions as it inhibits the activity of the horseradish-peroxidase enzyme.

1. One day prior to running the ELISA, dilute Capture Antibody in Coating Buffer. Add 100 µL of this Capture Antibody solution to all wells of a 96-well plate. Seal plate and incubate overnight between 2°C and 8°C.
2. Bring all reagents to room temperature (RT) prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.
3. Wash plate 4 times with at least 300 µL Wash Buffer per well and blot residual buffer by firmly tapping plate upside down on absorbent paper. **All subsequent washes should be performed similarly.**
4. To block non-specific binding and reduce background, add 200 µL Assay Diluent per well.
5. Seal plate and incubate at RT for 1 hour with shaking on a plate shaker(e.g. 300 rpm with a 0.3 cm circular orbit). All subsequent incubation with shaking should be performed similarly.
6. While plate is being blocked, prepare standard dilutions and appropri-

ate sample dilutions (if necessary).

7. Wash plate 4 times with Wash Buffer.
8. Add 100 µL/well of standard dilutions and samples to the appropriate wells. If needed, samples can be further diluted with Assay Diluent before adding 100 µL/well diluted samples.
9. Seal plate and incubate at RT for 2 hours with shaking.
10. Wash plate 4 times with Wash Buffer.
11. Add 100 µL of diluted Detection Antibody solution to each well, seal plate and incubate at RT for 1 hour with shaking.
12. Wash plate 4 times with Wash Buffer.
13. Add 100 µL of diluted Avidin-HRP solution to each well, seal plate and incubate at RT for 30 minutes with shaking.
14. Wash plate 5 times with Wash Buffer. For this final wash, soak wells in Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.
15. Add 100 µL of TMB Substrate Solution and incubate **in the dark for 15-30 minutes or until the desired color develops*.** Positive wells should turn blue in color. It is not necessary to seal the plate during this step.
16. Stop reaction by adding 100 µL of Stop Solution to each well. Positive wells should turn from blue to yellow.
17. Read absorbance at 450 nm within 30 minutes. If the reader can read at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

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

Assay Procedure Summary

Day 1

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

Day 2

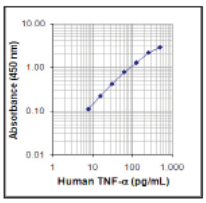
1. Wash plate 4 times
2. Add 200 µL Assay Diluent to block, incubate at room temperature for 1 hour with shaking
3. Wash plate 4 times
4. Add diluted standards and samples to the appropriate wells, incubate at room temperature for 2 hours with shaking
5. Wash plate 4 times
6. Add 100 µL diluted Detection Antibody solution to each well, incubate at room temperature for 1 hour with shaking
7. Wash plate 4 times
8. Add 100 µL diluted Avidin-HRP solution to each well, incubate at room temperature for 30 minutes with shaking
9. Wash plate 5 times, soaking for 30 seconds to 1 minute per wash
10. Add 100 µL of TMB Substrate Solution to each well, incubate in the dark for 15-30 minutes or until the desired color develops
11. Add 100 µL Stop Solution to each well
12. Read absorbance at 450 nm and 570 nm

Calculation of Results

Plot the standard curve on log-log axis graph paper with cytokine concentration on the x-axis and absorbance on the y-axis. Draw a best fit line through the standard points. To determine the unknown cytokine concentrations in the samples, find the absorbance value of the unknown on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the corresponding cytokine concentration. If the samples were diluted, multiply by the appropriate dilution factor. The data is best calculated with computer-based curve-fitting software using a 3- or 4-parameter logistics curve-fitting algorithm. If a test sample's absorbance value falls outside the standard curve ranges, that test sample needs to be reanalyzed at a higher or lower dilution as appropriate.

Typical Data

Standard Curve: This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Performance Characteristics

Specificity: No cross reactivity was observed when this kit was used to analyze multiple human, mouse and rat recombinant proteins.

For more information about BioLegend ELISA MAX™ Sets and LEGEND MAX™ ELISA Kits with precoated plates, visit **www.biolegend.com.**

Troubleshooting

High Background:

- Background wells were contaminated.
- Matrix used had endogenous analyte.
- Plate was insufficiently washed.
- TMB Substrate Solution was contaminated.

No signal:

- Incorrect or no antibodies were added.
- Avidin-HRP was not added.
- Substrate solution was not added.
- Wash buffer contained sodium azide.

Low or poor signal for the standard curve:

- Standard was incompletely reconstituted or was stored improperly.
- Reagents were added to wells with incorrect concentrations.
- Plate was incubated with inappropriate temperature, timing or agitation.

Signal too high, standard curves saturated:

- Standard was reconstituted with less volume than required.
- One or more reagent incubation steps were too long.
- Plate was incubated with inappropriate temperature, timing, or agitation.

Sample readings out of range:

- Samples contain no or below detectable levels of analyte.
- Samples contain analyte concentrations greater than highest standard point.

High variations in samples and/or standards:

- Pipetting errors may have occurred.
- Plate washing was inadequate or nonuniform.
- Samples were not homogenous.
- Samples or standard wells were contaminated.

References Using This Set

1. Davis, AM., et al. 2008. J Immunol. 180:6923.
2. Kulkarni, O., et al. 2009. J. Pharmacol Exp Ther. 328:371.

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For other technical resources, please visit: **www.biolegend.com/support** or email: **techserv@biolegend.com**