Sudan University of Sciences and Technology

Collage of Graduate Studies

Evalution Of Plasma Interleukin-8 (IL-8) Levels among Neonatal Sepsis Patients in some Selected Hospitals – Khartoum State - Sudan

تقويم مستوي المادة البلازمة الخلوية 8 وسط حديثي الولادة المصابين بالنتان الدموي في بعض المستشفيات المختارة – ولاية الخرطوم - السودان

A dissertation Submitted in Partial Fulfillment of the Requirements for the MS.c Degree in Medical Laboratory Science (Microbiology)

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الآيـــــة

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صدق الله العظيم

سورة الشرح الآيات5-6

Dedication

To my beloved parents who made me what I'm today

To my husband who always support me

To my respectful brothers, sister and friends

To my little daughter Layanand my sonAbobaker

To my wonderful supervisor

To all those wonderful persons

I am trying to say thank you

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First of all, thanks to ALMIGHTY ALLAH for giving me patience and strength tocomplete this study.

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A lot of thanks to DuaaTarig and Sarrah Ahmed whom really give me their time and knowledgement.

Finally, we would like to appreciate and express our deepest feelings to all ourcolleagues, brothers, sisters, relatives and friends for encouragement and help.

مستخلص البحث

احد اهم العوامل التي تلعب دورا في امراضية الانتان الوليدي هي افراز المادة الخلوية واكثر المواد الخلوية الفعالة والذي له خاصية محفزة للايتان الوليدي هو المادة الخلوية 8.

هذه الدراسة هدفت الى قياس مستوى المادة الخلوية 8 وسط حديثي الولادة المصابين بالانتان الوليدي مع حديثي الولادة الطبيعين بولاية الخرطوم في الفترة من نوفمبر 2020 الى مايو 2021.

تم اختيار اثنين وخمسين فردا عشوائيا لهذه الدراسة وكانت اعمارهم تتروح من 1-13 يوم حيث كان متوسط الاعمار2.6 ± 3.4, 26طفل حديث ولادة مصاب بالانتان الوليدي(13 ذكر و 13 انثى) و 26 من حديثي الولادة الطبيعيين (13 ذكر و 13 انثى).

تم سحب 1 مل عينه دم وريدية من كل مشارك في انبوبة تحتوي على مانعة التجلط EDTA. تم قياس تركيز المادة الخلوية 8 عن طريق فحص الممتز المناعي المرتبط بالانزيم. حللت البيانات باستخدام الحزمة الاحصائية للمجتمع (نسخة 20).

كان الوسط الحسابي المادة الخلوية 8 26.9±221.8 ,28.7±111.1في المرضى حديثي الولادة المصابين بالانتان الوليدي و حديثي الولادة الطبيعين على التوالي. كان هنالك ارتفاع ذو دلالة احصائية في معدل المادة الخلوية في حديثي الولادة المصابين بالانتان الوليدي وحديثي الولادة الطبيعيين(كانت القيم الاحتمالية 0,005).

ليس هنالك دلالة احصائية في معدل المادة الخلوية 8 بين الانتان الوليدي المبكر والانتان الوليدي المتاخر وبين حديثي الولادة الخدج و حديثي الولادة مكتملي النمو (كانت القيم الاحتمالية 0,65 , 0,77 على التوالي).

ليس هنالك دلالة احصائية بين معدل المادة الخلوية 8 مع العمر والجنس في حديثي الولادة المصابين بالانتان الوليدي (كانت القيم الاحتمالية 0.15, 0.67 على التوالي).

لقد توصلت الدراسة الى ان تركيز المادة الخلوية 8 يمكن ان يستخدم كعلامة للتشخيص بالانتان الوليدي.

Abstract

One of the most important factors playing important role in neonatal sepsis pathogenesis is cytokine release and the most effective cytokines that with proinflammatory characteristic is interleukin-8 (IL-8). This is a case-control study was aimed to evaluate the IL-8 level in Sudanese Septic neonates cases and in control subjects in Khartoum state during the period from November (2020) to May (2021).

Fifty tow subjects were selected convincely in this study, with age varies from 1-13 days, mean of age was 3.4± 2.6. 26 subjects were septic neonates (13 males and 13 females) as case group and 26 subjects (13 males and 13 females) as healthy control group. Venous blood sample (1ml) was collected in EDTA container from each subject.

IL-8 concentration was measured using Enzyme Linked ImmunosorbentAssay(ELISA).The data was analyzed using Statistical Package Social Science Programme (Version 20).

The Means of IL-8 were 221±26.9, 111.1±28.7 in the case group and control group respectively. IL-8 level was significantly elevated in septic neonates than control group (*P. value* 0.005).In septic group there was no statistical correlation in IL-8 levels between early onset sepsis and late onset sepsis and between premature neonates and term neonates (*P. value* 0.66 and 0.77 respectively).

Also there was no statistical correlation between level of IL-8 with gender and age (*P. value* was 0.67 and 0.15 respectively).

The study concluded that IL-8 concentration may be useful as a diagnostic marker for neonatal sepsis.

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LIST OF ABBREVIATIONS

Abbreviations	full name
AAP	AmericanAcademyof Pediatrics
ANC	AbsoluteNeutrophilCount
CDC	CenterforDiseaseControlandPrevention
CDS	CanadianPediatricSociety
CoNS	Coagulase-negative Staphylococci
CRP	C-reactive Protein
CSF	Cerebrospinal Fluid
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylenediaminetetraaceticacid
ELBW	Extereme Low Birth Weight
EONS	EarlyOnsetNeonatalSepsis
GBS	GroupBSterptococci
HRP	Horse Reddish Peroxidase
IF	Interferon gamma
IL-10	Interlukin-10
IL-1B	Interlukin-1 beta
IL-6	Interlukin-6
IL-8	Interlukin-8
ILS	Interlukins
KD	Kilo Dalton
LBW	Low Birth Weight
LONS	Late Onset Neonatal Sepsis

LPSLipopoly Saccharide

MALDI-TOF Matrix Assisted Laser Desorption

Ionization Time-Off light

MIFMigration Inhibitory Factor

MLMilliliters

MRSAMethicillinResistant*Staphylococcus*

aureus

NICUNeonatalIntensiveCareUnits

NmNanometer

PCRPolymeraseChainReaction

PCTProcalcitonin

PgPicogram

PMNCPolymorphNuclearCells

qPCRQuantitativepolymeraseChain

Reaction

SIRSSystemicInflammatoryResponse

Syndrome

SPSSStatisticalPackageForSocial

Sciences

SSASubSaharanAfrica

TMBTetramethylebenzidine

TNF alpha TumerNecrosisFactoralpha

VLBWVeryLowBirthWeight

VREVancomycinResistantEnterococci

CHAPTER I

INTRODUCTION

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Introduction

1.1. Introduction

The neonatal sepsis defines as blood infection that will occur in newborn at the first four weeks from birth (Paolucci*et al.,* 2012).

Neonatal sepsis is categorized into either an early-onset neonatal sepsis (EONS) or late-onset neonatal sepsis (LONS). EONS is defined by bacteremia or meningitis occurring in newborns less than 3 days old (Giannoni*etal.,* 2018). EONS could be vertically transmitted infection that usually occurs as an ascending infection from mothers cervix. Group B *streptococcus* (GBS) is leading to cause EONS followed by *Escherichiacoli* (*E. coli*) and *Listeriamonocytogenes (*Simonsen *et al.,* 2014*).* LONS is defined as sepsis in infants during 4-90 days oflife and could be caused either vertically or horizontally transmitted infection. The most important microorganisms involved in LONS include coagulase-negative *staphylococci* (CoNS), *Enterobacter*spp, *E. coli*, *Pseudomonasaeruginosa*, *Klebsiellapneumonia*, *Staphylococcusaureus* and *Candidaalbicans*(Dong and Speer, 2015;Yadav*etal.,* 2018).

Sepsis is a serious cause of morbidity and mortality among neonates, killing approximately 3 million newborns each year (Mersha*etal.,* 2019). The global burden of neonatal sepsis was measured as 2,202 per 100,000 live births(Fleischmann *etal.,* 2018). Sepsis is potentially life-threatening condition resulting from an extreme systemic immune response of the body to fight against an infection. The invasive infection frequently bacterial characterized by systemic signs ofinfection and isolation of bacteria from the blood stream. Sepsis is one of the most important reasons for hospitalization of newborns in neonatal intensive care units(NICU). Studies showed that the neonatal sepsis is

fatal and may quickly lead to septic shock and death if its untreated(Shah and Padbury, 2014).

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).

The gold standard for a definitive diagnosis is a blood culture. However, there are many problems with this test reduces its value(Dessi*etal.,* 2014). Measuring of biochemical markers have been taken as a markers such as CRP, TNF alpha and ILs such as IL-6, 8 and 10 have been evaluated as main indicators for early detection of neonatal sepsis (Prashnt*etal.,* 2013)

Cytokines are relatively small molecules with short serum half-life and play a central role in immune response in neonates with sepsis. During sepsis, cytokines levels may be observed in pictograms per milliliter of plasma or in nanograms or even micrograms(de Jong *etal.,* 2010).

Interlukin-8 is a pro-inflammatory cytokine and is predominantly produced by monocytes, macrophages and endothelial cells. IL-8 regulates the migration and activation of leukocytes, whose level evaluate promptly within 1-3 hours of infection and its half-life is less than 4 hours(Hotoura*etal.,* 2012)(Meem*etal.,* 2011).

1.2. Rationale

Severe neonatal infections are one of the most significant causes of pediatric mortality, resulting in more than 500,000 deaths each year (Black *etal*., 2010).

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 .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(Hornik*et al.*, 2012).

There are no previous studies about IL-8 in Sudanese neonatal sepsis patients.

1.3.Objectives

1.3.1. General objective

Toestimatetheplasma level of IL-8 among neonatal sepsis patients in Khartoum State.

1.3.2. Specific objectives

1-To measurethe plasma level of IL-8 in neonatal sepsis patients and in healthy using Enzyme linked immune sorbent assay.

2-To compare between the results of IL-8 in neonatal sepsis patients and normal controls.

3- To evaluate the utility of using IL-8 asdiagnostic biomarker of neonatal sepsis.

4- To associate between neonatal sepsis and risk factors including age, gender, onset of sepsis and maturity of the neonates.

CHAPTER II

LITERATURE REVIEW

CHAPTER II
LITERATURE REVIEW

2.1. Definition of neonatal sepsis

The term neonatal sepsis is used to designate a systemic condition of bacterial, viral, or fungal (yeast) origin that is associated with haemodynamic changes and other clinical manifestations and results in substantial morbidity and mortality.Traditionally, the definition of sepsis has included isolation of a pathogen from a normally sterile body fluid such as blood or cerebrospinal fluid (CSF). However, as the clinical features of sepsis can be induced by potent pro-inflammatory cytokines, the term systemic inflammatory response syndrome (SIRS) has also been used when describing neonatal sepsis. Neonatal sepsis has been classified as either early-onset or late-onset depending on the age of onset and timing of the sepsis episode. Clinical manifestations of early onset infections usually appear within the first 72 h of life (Wynn *etal.*, 2014).

Some clinicians define early-onset infections, especially those due to group B *Streptococcus* (GBS), as infections occurring at less than 7 days of age. Early-onset infections are acquired before or during delivery and usually represent vertical mother-to-infant transmission. Late-onset infections present after delivery, or beyond 3 to 7 days of age, and are attributed to organisms acquired from interaction with the hospital environment or the community. In extremely low gestational age and high-risk term infants, many of whom have prolonged hospital stays, the designation of late-onset sepsis might apply to any episode of sepsis from birth to hospital discharge regardless of age at the time of the episode. For GBS infections, late onset often refers to disease that occurs from 1 week to 3 months of age, with infections that develop after 3 months of age designated as very-late-onset infection (Van *etal.*, 2010).

In 2010 worldwide, 7.6 million children less than 5 years old died, predominantly due to infectious causes including sepsis; neonatal death(in the first 28 days of life), accounted for 40% of the total lives lost(Liu *etal.,* 2012).

Worldwide, neonatal sepsis occurs in about 1 to 50 out of 1,000 live births and accounts for 3 to 30% of infant and child deaths annually(Sherman, 2010; Zhou *etal.,* 2016)

2.1.1. Early-onset neonatal sepsis

Early-onset neonatal sepsis occurs inutero from either a transplacental or, more commonly, ascending bacteria entering the uterus from the vaginal environment following membrane rupture. Additionally, the newborn child might become infected when exposed to potentially pathogenic bacteria, viruses, or fungi during passage through the birth canal. The human birth canal is colonized with aerobic and anaerobic bacterial organisms that can be vertically transmitted from an ascending infection of the amniotic fluid or natal infection of the neonateduring labour or delivery(Rampersaud*etal.*, 2012)

Chorioamnionitis, often referred to as intra-amniotic infection, is an acute inflammation of the fetal membranes, presumably due to bacterial infection. Chorioamnionitis results from microbial invasion of amniotic fluid, often as a result of prolonged rupture of the chorioamniotic membrane. The clinical syndrome of chorioamnionitis might include maternal signs and symptoms (fever, leucocytosis, cloudy or odorous discharge, and lower abdominal tenderness) and fetal signs (tachycardia is most common). Chorioamnionitis might also present asymptomatically with laboratory or pathological abnormalities supporting the syndrome. The rate of histological chorioamnionitis is inversely related to gestational age at birth and directly related to duration of membrane rupture(Worthame*tal.*, 2016; Goldenberg *etal.*, 2011). *Ureaplasmaparvum*and *Ureaplasmaurealyticum*, both genital

mycoplasmas, are the most common bacteria isolated from placentas with histological chorioamnionitis and from amniotic fluid.
*Ureaplasma*sppcolonisation of the respiratory tract of preterm infants has been associated with bronchopulmonary dysplasia. The understanding of the association between maternal chorioamnionitis and neonatal outcomes is an area of active investigation by maternal and neonatal research teams(Higgins *etal.*, 2016).

2.1.2. Late-onset or acquired neonatal sepsis

During the first 3 months of life, the innate immune system, including phagocytes, natural-killer cells, antigen presenting cells, and the complement system, provide a defence against pathogens. Decreased function of neutrophils and low concentrations of immunoglobulins increase the susceptibility of preterm infants to invasive infection. As infants age, they are exposed to environmental organisms that might be pathogenic to those with an immature immune system. Contact with hospital personnel, family members, nutritional sources, and contaminated equipment all represent opportunities for pathogen exposure. Hand contamination is the most common source of postnatal infections in infants admitted to hospital, underscoring the importance of hand hygiene (Bizzarro*etal.*, 2011).

Late-onset bloodstream infections occur more frequently in neonates with central venous access than in infants without central venous access who are usually older, and these infections are more likely to be attributed to Gram-positive organisms, including coagulase-negative *staphylococci* and *streptococci*. Most cases of meningitis are late-onset infections resulting from haematogenous spread via the choroid plexus into the CNS; less often, late-onset meningitis results from contiguous spread as a result of contamination of open neural tube defects, congenital sinus tracts, ventricular devices, or penetrating wounds from fetal scalp monitors. Abscess formation, ventriculitis,

septic infarcts, hydrocephalus, and subdural effusions are complications of meningitis that occur more often in neonates(Bizzarro*etal.*, 2011).

2.2. Etiology of neonatal bacterial sepsis

The organisms and pathogens that are most associated with neonatal sepsis differ depending upon country involved. Pathogens range from gram positive and negative bacteria to viruses and fungi, with bacteria being the most frequentlyIdentified(Shane*etal.*,2017).

2.2.1 Organisms associated with early-onsetneonatal sepsis (EONS)

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 pneumonie), *Staphylococcus aureus*, *Enterococcus*, *H.influenzae* (excluding group B) and *Listeriamonocytogenes* (Klobučar, 2017).Gram-negative organisms such as *Klebsiella*, *Pseudomonas* and *Salmonella* are also more common (Shane*etal.*, 2017).

2.2.2 Organisms associated with late-onset neonatal sepsis (LONS)

Coagulase Negative *Staphylococci* (CoNS) have emerged as the single most commonly isolated pathogen among VLBW infants with LONS. 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 LONS mainly include *Escherichia coli*, *Klebsiella spp*., *Enterobacter spp*. and *Pseudomonas spp* (Dong and Speer, 2015). A study by Abdelazi*zet al* done at Maternity Hospital in Sudan reported Gram-negative organisms as the commonest pathogen isolated. *Klebsiellapneumoniae* (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).

2.2.3 Other organisms

Other organisms rather than bacteria can cause neonatal sepsis such as Viruses which include echovirus, enterovirus, parechovirus, coxsackie virus, adenovirus,
parainfluenza virus, rhinovirus, herpes simplex virus, respiratory syncytial virus, and coronavirus (Shane *etal.,* 2017). *Candidaalbicans* and other Candida species are the most common fungi associated with neonatal sepsis (Turhan*etal.,* 2015).

2.3. Risk factors

Risk factors include central venous catheter use and other invasive medical devices as well as prolonged hospitalization (Heo*etal.,* 2015) Other risk factors include preterm rupture of membranes, amnionitis, meconium aspiration, LBW, VLBW, ELBW, preterm birth, greater than three vaginal examinations during labor, fever in the mother during labor, or any other infection in the mother during labor (Cortese*etal.,* 2016). In full-term infants, males have a greater incidence of sepsis compared to female infants, an association not found in preterm infants (Shane *etal.,* 2017).

2.4. Pathophysiology

The immature immune system is the major contributing factor for increased neonatal susceptibility to sepsis. The immature function of polymorphonuclear neutrophils, macrophages and T lymphocytes makes these cells incapable of

carrying out a complete inflammatory responses in neonates. Furthermore neonates have a limited number of immunoglobulinsat birth and cannot generate a quantitative and/or qualitative adequate mounting response against infectious agents. The insufficient time that premature has in the uterus decrease the transfer of immuneglobulins to the fetus. This deficiency makes premature infants at much higher risk for sepsis when compared to term infants(Raymond *etal.*, 2017).

2.5. Clinical symptoms

Clinical signs and symptoms of sepsis in newborns vary by gestational age and severity of infection. Rarely will infants present with fever unless they are born to a febrile mother and have immediately after delivery. It is much more common foe a septic infant to be hypothermic upon presentation (Lim *etal.*, 2012).

General symptoms include; lethargy, hypothermia and poor feeding and non specific signs may include anuria and acidosis. As pneumonia is often the presenting infection respiratory symptoms are common and may include; apena, tachypnea and nasal flaring. Cardic symptoms may include; cyanosis, desaturation, brachycardia, poor perfusion, reduced capillary refill and hypotension. It is important to realize that suitable changes in respiratory states of newborns temperature instability or feeding problems can be the first signs of life threatening infections(Lim *etal.*, 2012).

2.5.1 Complications

Neonatal sepsis remains a significant contributor to morbidity and mortality in neonates. Prematurity and delayedtreatment are commonly associated with adverse outcome. VLBW infants have been found to have a higher risk of chronic lung disease and ELBW infants are at a greater risk of neurodevelopmental risks such as; hearing and visual deficits, cerebral palsy and impaired psychomotor and mental development. On the other hand, the

unnecessary overuse of antibiotics can increase the chances of sever candiadiasis and multi-drug resistant organisms (Wynn and Wong, 2010).

2.6. Diagnostic Testing

The newborn's prior probability of sepsis obtained from maternal risk factors such as chorioamnionitis and premature rupture of membranes is combined with findings based on the clinical examination, creating a scoring system that can determine the need for antibiotics and level of monitoring required. This scoring system has been shown to reduce the proportion of newborns undergoing extensive laboratory evaluation and administration of antibiotics without any adverse effects(Kuzniewicz*etal.*, 2017).

2.6.1 Complete blood cells count (CBC)

Complete blood cells count should be performed to assess for total and differential white blood cell count (WBC), absolute and immature neutrophil count, and the ratio of immature to total neutrophil count. Although an absolute leukocytosis has low sensitivity for neonatal sepsis, they may aid in clinical decision-making in cases where a low-to-moderate clinical suspicion for sepsis is present. Interestingly, a low WBC count, low absolute neutrophil count (ANC), and an immature to total neutrophil ratio (I/T) of 0.2 or greater have been shown to be highly predictive of infection (Newman *etal.*, 2014).

2.6.2 Blood culture

Blood culture remains the gold standard for confirmation of sepsis but is limited by low sensitivity and duration of time before a culture is determined to be positive (often around 24 to 72 hours). Fastidious organisms, maternal antibiotics, and small sample collection limit the sensitivity of blood cultures. False positives may occur due to inadequate skin antisepsis prior to sample collection. At least 0.5 ml of blood should be collected to improve the diagnostic yield. Samples should be collected from two different sites to reduce false positive results. If a central venous catheter is present, blood culture

should be taken from both the line and a separate peripheral source, to assess for the differential time to positivity. This helps in distinguishing catheter-associated infections from other sources of infection, which has implications in clinical management. Swab cultures from surface sites such as the eyes, ears, umbilicus, groin, throat, pharynx, and rectum may provide information about colonizing organisms. They, however, do not contribute to the decision on starting antibiotics, especially if the neonate appears well on clinical examination. Placental cultures may indicate the possible pathogen the fetus was exposed to but does not indicate infection(Shane *etal.*, 2017). Placental culture results should not, therefore, be used as a reason for antibiotic therapy. Urinary tract infections are uncommon in the first 72 hours of life. Urine cultures are therefore only performed in the evaluation of LONS(Ruangkit*etal.*, 2016).

2.6.3 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 and Presepsin) phases of neonatal sepsis (Bhandari., 2014).

2.6.3.1 Acute phase reactants

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

2.6.3.1.1 C-reactive protein (CRP)

C-reactive protein may not be elevated in early stages of infection, due to the time taken for its synthesis in the liver and eventual appearance in the blood.

Serial measurements of CRP combined with other acute phase reactants such as procalcitonin, IL-6, and IL-8 may improve its diagnostic accuracy(Hofer *etal.*, 2012).

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).

2.6.3.1.2 Procalcitonin (PCT)

Procalcitonin is the precursor of calcitonin hormone produced in very low concentration by the C cells of the thyroid gland under normal condition. It is produced by macrophages and monocytes of various organs during severe bacterial infection in response to bacterial lipopolysaccharide (LPS), which is apotent inducer of PCT into the circulation(Rashwan*etal.*, 2019).

Procalcitoninis more specific than CRP for bacterial infections and rises more rapidly in response to infection than CRP. In normal birth weight infants, a PCT level greater than 0.5ng/mL is associated with a nosocomial infection, whereas a level of greater than 2.4ng/mL in VLBW infants should prompt antibiotic therapy (Auriti*etal.*, 2012). It has been shown that procalcitonin-guided decision making is superior to standard care in reducing antibiotic therapy in neonates with suspected sepsis(Stocher*etal.*, 2017).

2.6.3.1.3 Presepsin

Presepsinhas been found to have a high level of diagnostic accuracy and has been recommended as a valuable marker in neonatal sepsis, albeit not as a single diagnostic test(Parri*etal.*, 2019).

2.6.3.2 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). Cytokine analysis may be useful in predicting late-onset infection (Shah and Padbury, 2014).

2.6.4 Newer Diagnostic Techniques

Automated blood culture systems monitor continuously for positive signals, which improves time to detection of pathogens. Matrix-assisted laser desorption ionization time-offlight (MALDI-TOF) mass spectroscopy can identify organisms in blood cultures much earlier, allowing antibiotic therapy specific to the organism(s) involved (Singhal*etal.*, 2015).

Multiplex polymerase chain reaction (PCR) can detect the identity of the bacteria or fungi, as well as the presence of antimicrobial resistance genes within hours of identification of the pathogen(Liesnfeld*etal.*, 2014). PCR can be performed on blood and other body fluids directly without the need to first culture causative organisms.Quantitative real-time amplification systems, known as qPCR, can be used to rapidly rule out the presence of organisms in body fluids, considering its high negative predictive value and a short time to results. The technique is based on 16S ribosomal deoxyribonucleic acid (DNA) amplification. qPCR utilizes a small sample volume and can be used for other bodily fluids such as pleural or peritoneal fluid. Disadvantages include the inability to perform antibiotic susceptibility testing, difficulty in differentiating a recent infection from an active infection, and the presence of contaminants that can give false positive results. Hence, clinical correlation should be made in the interpretation of these results (Herk*etal.*, 2016).

2.7. Treatment and Management

Management varies depending on a number of factors including age, site of infection, suspected causative organism, microbial resistance patterns, and available resources. Consensus among authors exists that antibiotic therapy should be initiated as soon as neonatal sepsis is suspected, but there is no consensus regarding duration of treatment (Herk*etal.,* 2016).

2.7.1 EONS Empiric Antibiotic Therapy

Recommendations from the Canadian Pediatric Society (CPS) and the AAP recommend initiating antibiotic therapy if clinical symptoms are present, with the AAP also recommending antibiotics in the presence of abnormal laboratory values or more than one risk factorThe presence of maternal chorioamnionitis with no neonatal clinical signs warrants antibiotic initiation as per the AAP and only if present with laboratory abnormalities per the CPS. The US Center for Disease Control and Prevention (CDC) recommends empiric antibiotic therapy for all newborns with a maternal diagnosis of chorioamnionitis, regardless of the infant's clinical condition. Reevaluation at 48 hours and discontinuation of antibiotics if infection was unlikely was universally recommended (Herk*etal*., 2016).

Antibiotic therapy should include intravenous ampicillin for GBS, and coverage for *Escherichiacoli* and other gram negative bacteria implicated in neonatal sepsis, such as gentamicin, with local antibiotic resistance patterns considered ((Puopolo*etal.,* 2018). The routine empirical use of broad-spectrum antibiotic agents should only be considered among term newborn infants who are critically ill until culture results are available. Elective genetic testing prior to aminoglycoside use is increasingly being considered to decrease the incidence of permanent hearing loss(Russell and Sharland, 2012).

2.7.2 Treatment of LONS

Early diagnosis, appropriate antibiotic administration, and timely supportive management are the keys to successful treatment(Huang *etal.,* 2015). Most cases are attributable to Staphylococcus species and GBS, but about one-third are caused by gram negative organisms. Most empiric antibiotic regimens include ampicillin, a third-generation cephalosporin, or meropenem, plus an aminoglycoside or vancomycin. In preterm infants, the most common isolates are CONS(Pont- Thibodeau*etal.,* 2014). Vancomycin and teicoplanin are the antibiotics of choice for a proven and significant CONS infection, but their excessive use has been associated with the development of vancomycin-resistant enterococcus (VRE) infections and gram-negative infections. Their use as first-line antibiotics for nosocomial infection should be avoided. A combination of flucloxacillin and gentamicin can be used to treat the majority of cases caused by other organisms(Russell and Sharland., 2012). Clindamycin or metronidazole are sometimes added to cover anaerobic organisms in cases of necrotizing enterocolitis. Cefotaxime is commonly reserved for the treatment of infants with meningitis(Huang *etal.,* 2015). Infants with risk factors for candidal sepsis should receive fungal empiric therapy(Wynn, 2016). Treatment with a beta-lactam or beta-lactamase inhibitor combined with an aminoglycoside for *Enterobacter*, *Serratia*, or *Pseudomonas* sepsis is recommended by many experts(Huang *etal.*, 2015).. Meropenem is recommended for preterm infants with systemic extended-spectrum beta-lactamase infections. In one study, prolonged intravenous infusion of meropenem (over 4 hours every 8 hours) in neonates with gram-negative LONS was associated with better clinical outcome compared to the conventional strategy (over 30 min every 8 h)(Shabaan*etal.*, 2017).

2.8.Cytokines

Cytokines are peptides that have a fundamental role in communication within the immune system and in allowing the immune system and host tissue cells to exchange information.

Cytokines act via binding to receptor that in turn sends a signal to the recipient cell leading to a change in function or phenotype. Such signal cascades are complex and integrate a variety of environmental factors (Mclnnes, 2017). Cytokines activity can be blocked by antagonist molecules which bind cytokines or their receptors(Schreiber and Walter, 2010).

2.8.1 Function of cytokines

Cytokines can stimulate the production of blood cells, aid in the development, maintenance and repair of tissues. Also cytokines regulate the immune system, drive inflammation through interferons, interlukins and tumer necrosis factor alpha (Ferreira, 2019).

While cytokines is an umbrella term that includes many types messenger, more specific names are given to cytokines based on either the type of cell that makes them or action they have in the body, according to this cytokines classified into four types(Tisoncik et al., 2012).

2.8.1.1 Lymphokines

Lymphokines madeby lymphocytes which attract immune cells such macrophages (Tisoncik et al., 2012).

2.8.1.2 Monokines

Monokines made by monocytes which attract neutrophils (Tisoncik et al., 2012).

2.8.1.3 Chemokines

Chemokines are associated with chemotactic actions (Tisoncik et al., 2012).

2.8.1.4 Intrelukins

Interlukins are made by one leukocyte but act on other leukocytes mediating communication between cells. Specific interlukins can have a major impact on cell-cell communication(Tisoncik et al., 2012).

2.8.2 Cytokines signaling mechanisms

2.8.2.1 Paracrine signaling

Paracrine signaling occurs between neighboring cells where the signals elicit quick responses and last only a short while due to the degradation of paracrine ligands(Drabsch and Dijke,. 2013).

2.8.2.2 Autocrine signaling

As the name suggest, in autocrine signaling, a cell signals itself through a moiety that it synthesis, ultimately leading to a biological response within the same cell. Autocrine signaling can either occur within the cytoplasm of the cell or by secreted growth factor/cytokine interacting with receptors on the surface of the same cells (Drabsch and Dijke,. 2013).

2.8.2.3 Endocrine signaling

In endocrine signaling growth factor/cytokine moieties are secreted inti the blood and carried by blood and tissue fluids to the target cells wherebysubsequent responses are triggered(Drabsch and Dijke,. 2013)

2.8.3 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), interleukins (IL-1, IL-6, and IL-8), 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 (Faix, 2013).

2.8.4 Interlukin-8(IL-8)

The IL-8 chemokine is an important mediator of the innate immunity with well defined immune modulatory effects on T-cell function and inflammatory response (Pollicino*et al.,* 2013). IL-8 is an 8.4 KD a non glycosylated protein produced by processing of a precursor protein of 99 amino acids belonging to the CXC subfamily of chemokines which is characterized by two essential cysteine residues, separated by a third intervening amino acid (Qazi*et al.,* 2011). IL-8 is a proinflammatory chemokine produced by various cell types to recruit leukocytes to sites of infection or tissue injury. Acquisition of IL-8 and/or its receptors CXCR1 and CXCR2 are known to be a relatively common occurrence during tumor progression (David *et al.,* 2016). IL-8 was originally identified for its role in chemoattraction of neutrophils, for which it was named neutrophil chemotactic factor (NCF) and neutrophil activating protein (Turner *et al.,* 2014).

2.8.4.1 Pro-inflammatory effects of IL-8

IL-8 is an oxidative stress-responsive proinflammatory chemokine, released fromepithelial cells following particle-induced oxidative stress leading to neutrophil influx and inflammation. Proinflammatory stimuli are considered to be a major regulator of IL-8 levels in response to injury. IL-8 is involved in many of the wound healing processes. It not only serves as a chemotactic factor for leukocytesand fibroblasts but also stimulates fibroblast differentiation into myofibroblasts andpromotes angiogenesis (Qazi*et al.,* 2011).

2.8.5 Neonatal sepsis and IL-8

IL-8 is released in the serum immediately after activation of humoral and cellular immune system which mediated the host response to bacterial infection(Barug*etal.,* 2014) (Hotoura*etal.*,2012).
Many studies have showed IL-8 is an early-phase biomarker for diagnosis of neonatal sepsis and IL-8 test may be a valid non-invasive, effective and rapid method for diagnosis (Meem*etal.*,2011). It increases early in the course of neonatal sepsis (Barug*etal.*,2014).

Further investigations suggest that the initial levels of IL-8 were the most predictive factors for death in patients with sepsis(Mera*etal.*,2011).

2.9. Previous studies

In Iran in 2010, a case control study conducted on 80 neonates , control grouop including 42 infants and 38 infants as case group. Study aimed to estimate serum IL-8 and CRP levels. Serum IL-8 was significantly higher in infants with confirmed sepsis than healthy infants. The results showed that IL-8 may be a valid and early predictive marker of neonatal infection. Also IL-8 is associated with severity of infection(Bskabadi*etal.,* 2010).

In 2018 in Egypt a study conducted on 85 neonates which divided into 3 groups: control(10 cases), suspected(45 cases) and infected(30 cases). Study aimed to estimate serum IL-6, 8 and CRP levels. The results showed that IL-6 and IL-8 are more sensitive than CPR. In such study the result means the level of IL-8 is significantly higher in suspected, infected groups than control group with P value 0.001(Elfaragy*etal.*,2018).

Wu and others in2016 were carried out a prospective study. 75 newborns whodeveloped septicemia and 50 healthy newborns as control were selected to evaluate the levels of serum IL-6 and IL-8. Results demonstrated that levels of IL-6 and IL-8 of septicemia group were higher than those of the control group(Wu *etal.*,2016).

In 2013 in Iran a total of 84 infants aged 72 hrs or less were enrolled in a prospective case control trial. The case group (41) and control group (43). IL-6, 8 and 10 were measured for all infants. Result showed that there are statistically differences between control and case groups for serum median level of IL-6, 8 and 10 (P-value 0.001)(Boskabadi*etal.,* 2013).

In 2015 Zhou and other conducted a systemic review and meta-analysis to investigate the diagnostic value of the IL-8 in neonatal sepsis. Eight studies (548) neonates were included in this study. Meta-analysis showed IL-8 had a moderate accuracy for the diagnosis of neonatal sepsis. IL-8 is a helpful biomarker for early diagnosis of neonatal sepsis (Zhou *etal.,* 2015).

In 2017 in Egypt a study aimed to evaluate the value of IL-8 as early diagnostic biomarkers for EONS was conducted. 40 neonates with prenatal risk for neonatal sepsis with their mother were taken as case group. 10 healthy neonates and their healthy mothers were taken as control group. IL-8 were evaluated in cord blood of neonates and in sera of mothers. IL-8 were significantly higher in the study group than the control group (EL-Mashad*etal.,* 2017).

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1. Study design

This was descriptive case control analytical study.

3.2. Study area and duration

Study was conducted in Soba Teatching Hospital and Ibrahim Malik hospital in Khartoum state during the period from November 2020 to May 2021.

3.3. Study population

Neonates of age between 1- 21 dayswereenrolled, divided into towgroups 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 ofhealthy neonates who were born normally at Ibrahim Malik Hospital without any abnormal signs or symptoms of infection.

3.4. Inclusion criteria

Neonateswithconfirmed bacterial sepsis and have sings of early onset sepsis or late onset sepsis and hospitalized while healthy neonate without any signs of infections will included.

3.5. Exclusion criteria

All neonates with situation thataffectedcytokineslevelsincluding congenital malformations, congenital infections associated with TORCH complex, 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.

3.6. Ethical consideration

Permission to carry out the study was obtained from Scientific research Committee, College of Medical Laboratory Science, Sudan University of

Science and Technology. Also taken from laboratory manger. Every sample was collected after verbal consent was taken from parents of neonates.

3.7. Sample size

Total Eighty-eight (n= 88) blood samples from neonates were enrolled in this study. Forty-four will septic neonates (case group) and forty-four will healthy neonates (control group).

3.8. Sampling technique

Conviencesampling technique.

3.9. Data collection

Questionnaire was used to collect demographic, clinical and laboratory data.

3.10. Laboratory processing

3.10.1. Specimen collection

One ml of venous blood were collected from patients and control in EDTA container. Then the samples were centrifuged and plasma separated in cryovial tube and stored at -20ºC until analysis. Plasma levels of IL-8 was measured using ELISA (Biolegend's ELISA MAXTM).

3.10.2. Estimation of IL-8 levels by Enzyme Linked Immune Sorbent Assay

3.10.2.1. ELISA Procedure

Indayoneplatewascoatedby added 100µL of diluted capture antibody solution to each well, sealed the plate and incubated overnight between 2˚C and 8˚C. Indaytow plate washed 4 times with at least 300µL of wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean absorbent paper. The plate blocked by added200 µL 1X Assay Diluents A to each well, sealed the plate and incubated at room temperature for 1 hour with shaking at approximately 500 rpm (with a 0.3 cm circular orbit). All

subsequent incubation with shaking was performed similarly. Plate was washed 4 times; 100 µL of diluted standareds and samples was added to the appropriate wells. Sealed the plate and incubated at room temperature for 2 hours with shaking. Then plate was washed 4 times; 100µL of diluted detection antibody solution was added to each well, sealed the plate and incubated at room temperature for 1 hour with shaking. plate was washed 4 times; 100 µL diluted Avidin-HRP solution was added to each well, sealed the plate and incubated at room temperature for 30 minutes with shaking. Plate washed 5 times; soaking for 30 seconds to 1 minute per wash. 100µL of freshly mixed TMB substrate solution was added to each well and incubated in the dark for 30 minutes. 100µL of stop solution was added to each well. Absorbance was road at 450 nm and 570 nm within 15 minutes. The absorbance 570 nmm can be substracted from the absorbance at 450 nm (www.biolegend.com).

3.10.2.2. ELISA Washer process

First the wash solution is pump from the wash bottle, the solution is dispense to the cuvette by short pins, and then the wash liquid isaspirate from the cuvette by long pins, at the end the waste liquid was pumped into the waste bottle by the vacuum pupp(www.diasource.be 2020).

3.10.2.3. ELISA reader principle

White light produced by the lamps is focused into a beam by the lens and passes through the sample. Part of the light is absorbed by the sample and the remaining light is transmitted. It is filtered by interference filters and focused onto the photodiodes. The photodiode converts the received light into an electrical signal which is transformed into a digital form, from which the microprocessor calculates the absorbance, taking in account of the blank and dichromatic selection (www.diasource.be 2020).

3.11. Statistical analysis

Data will analyze 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

RESULTS

4-Results

Fifty tow neonates with varies age from 1-13days were enrolled in this study, 26 neonates were case, 13/26(50%) of them were males and 13/26(50%) of them were females with mean of age 5.0± 2.7. Other 26 neonates were apparently healthy control, 13/26(50%) of them were males and 13/26(50%) of them were females with mean age1.7± 0.8 as shown in table(4.1). .

Mean level of IL-8 in case group(221.8±182.8pg/ml), in control group(111.1± 28.7pg/ml) with statistically significant differences between case and control groups(*P-value* was 0.005) as in table (4.2).

The mean level of IL-8 in males and females of case group were 246.9±222.2 and 196.6±137.3 respectively, *P-value* was 0.49. The mean level of IL-8 was 102.4±28.3 in males and 119.8± 26.9 in females of control group *P.value* was 0.12. There was no statistical association between level of IL-8 and gender in case and control group.

Mean level of IL-8 in EONS and LONS patients was 229.5±196.2 and 196.2±140.7 respectively.Concerning onset of disease, there was no significant difference in level of IL-8 between EONS and LONS, P-value was 0.65 as shown in table (4.3).

Mean level of IL-8 in term and preterm neonates in study group were 232.3±233.6 and 211.3±121.7 respectively. There was no statistical significant differences between level of IL-8 in term and preterm neonates,*P-value* was 0.77 as shown in table (4.4).

Table (4.1): Distribution of demographic data of study population

Variable	Patient group (n=26) Frequency (%)	Control group (n=26) Frequency (%)
Gender		
Male	13 (50%)	13 (50%)
Female	13 (50%)	13(50%)
Gestational age		
Term	13 (50%)	26(100%)
Preterm	13(50%)	
Onset of disease		
EONS	20(76%)	-
LONS	6(24%)	

Table(4-2): Association of IL-8 level between patients and control neonates

Subject	patient group (n=26)	Control group (n=26)	*P. value*
Mean of IL-8	221.8 ±182.8 S.D	111.1 ± 28.7 S.D	0.005

Table(4-3): Association of IL-8 levels and onset of disease in septic patients

Onset of disease	**EONS** (n=20)	**LONS** (n=6)	*P. value*
Mean ± SD of IL-8	229.5±196.2	196.2±140.7	0.65

Table(4-4): Association of IL-8 level and maturity of the neonates in case group

Subject	Term	Preterm	*P. value*
Mean ± SD of IL-8 in case group	232.3±233.6	211.3±121.7	0.77

Chapter V
Discussion, Conclusions and Recommendations

Chapter V

Discussion, Conclusions and Recommendations

5.1. Discussion

Interleukin-8 (IL-8) is an important mediator of inflammation and the immune response in human disease. IL-8 plays a key role in the body's defense mechanism
by regulating neutrophil activity, but prolonged presence of inflammation induced
IL-8 in circulation may cause variable degrees of tissue damage (Dong and Zheng, 2015).

In the present study plasma level of IL-8 was measured in 26 septic neonates and in 26 apparently health control. The result relieve that the mean level of IL-8in case group was higher than control and the difference was significant (*P.value*
0.005). This result was supported by Elfaragy*etal* (2018), In such study the result means the level of IL-8 is significantly higher in suspected, infected groups than control group with *P.value* 0.001. Boskabadi et *al*(2010) in their study showed that serum concentration of IL-8 in neonates with confirmed sepsis is significantly higher than in healthy neonates.

In the present study there is no correlation between the mean level of IL-8 and gender in case group *P.value*was 0.67. This resultagree with Boskabadi*etal* (2010)*P.value*> 0.05.

In the present study there is no correlation between the mean level of IL-8 and age in case group *P.value*was 0.15. This result agree with Nasked (2017) who reported that there was no association between age subgroup.

In the present study there is no significant statistical differences of IL-8 between term and preterm neonates *P.value* was 0.77 and between EONS and

LONS P.value was 0.65. There was no similar studies concerning onset of the disease and maturity of neonates (term and preterm neonates).

Finally the level of IL-8 was higher in septic patients than normal neonates and no differences between EONS and LONS, maturity of neonates and gender.

5.2 Conclusion

This study conducted thatplasma level of IL-8 was highly significant in septic neonates group than healthy neonates group.

There was no association between IL-8 levels with gender and age.

There was no association between IL-8 levels with the onset of the disease and maturity of neonates.

Plasma IL-8 can be considered useful as diagnostic marker for bacterial neonatal sepsis.

5-3 Recommendations

Further studies may be conducted considering increase of sample size, routinmeasurement of IL-8 level in neonates with bacterial sepsis.

Combination of two biomarkers at least should be used to detect the presence of bacterial sepsis, for example combination of CRP and IL-8 should be tested.

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

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Appendices

Appendix (1)

Sudan university of Science and Technology

Collage of Post Graduates

Evaluation of interleukin 8 levels in Sudanese Neonatal sepsis patients

- Date: / /2020.

- ID number : ……

- Age :…… Day. Weight: ……/g

- Gender : Male ( ). Female : ( ).

- Causes of sepsis:

……
……
……
……
……
……
……
……
……
……
……

- Duration of Sepsis :

- Less than 24 hrs( ). - 24-72 hrs( ).

-More than 72 hrs ( ).

- Other disease :

……

……

……

Appendix (2)

IL-8 ELISA Kits

Appendix (3)

Human IL-8 ELISA MAX™ Deluxe Set

ertificate of Analysis

oduct Name: Human IL-8 ELISA MAX™ Deluxe Set

oduct Cat. No: 431504 (5 plates) / 431505 (10 plates) / 431506 (20 plates)

t No: B263299

piration Date: 31-MAY-2020

ontents Description	Quantity (5 plates)	Volume (per bottle)	Part No.	Lot No.
uman IL-8 ELISA MAX™ apture Antibody (200X)	1 vial	300 µL	78141	B261651
uman IL-8 ELISA MAX™ etection Antibody (200X)	1 vial	300 µL	78142	B261650
uman IL-8 Standard	2 vials	18 ng	78143	B261587
idin-HRP (1,000X)	1 vial	60 µL	79004	B261089
bstrate Solution C	1 bottle	30 mL	78105	B260164
ating Buffer A (5X)	1 bottle	30 mL	79008	B261830
atrix Diluent A (for serum d plasma samples)	1 bottle	10 mL	79056	B263279
say Diluent A (5X)	1 bottle	60 mL	78888	B261642
inc™ MaxiSorp™ ELISA ates, Uncoated	5 plates	-	423501	-

rage Conditions

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

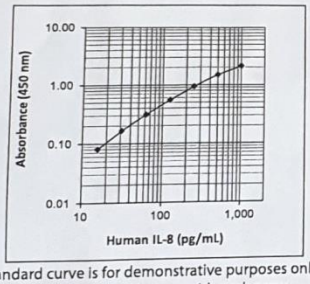
Opened or reconstituted components:

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.

e: 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 #: B263299



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

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

ture: (Quality Control) Date: 06/15/2018

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BioLegend®

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-8 Standard by adding 0.2 mL of 1X Assay Diluent A to make the 90 ng/mL standard stock solution. Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.

- **For measuring cell culture medium sample:**
  Prepare 1,000 µL of the top standard at 1,000 pg/mL by adding 11.1 µL of reconstituted standard stock solution to 988.9 µL 1X Assay Diluent A.
- **For measuring serum or plasma samples:**
  Prepare 1,000 µL of the top standard at 2,000 pg/mL by adding 22.2 µL of reconstituted standard stock solution to 977.8 µL 1X Assay Diluent A.

Perform six two-fold serial dilutions of above 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, cell culture supernatant samples should be diluted in 1X Assay Diluent A and serum or plasma samples should be diluted in Matrix Diluent A.

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 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.
2. 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.
3. Wash plate 4 times with Wash Buffer.
   For measuring cell culture medium sample:
   - Add 100 µL/well of standards and cell culture medium samples to the appropriate wells.

   For measuring serum or plasma samples:
   - Add 50 µL/well of Matrix Diluent A to the standard wells. Add 50 µL/well of 1X Assay Diluent A to the sample wells.
   - Add 50 uL/well of standards prepared above to the standard wells. Add 50 uL/well of serum or plasma samples to the sample wells.
4. Seal the plate and incubate at room temperature for 2 hours with shaking.
5. Wash plate 4 times with Wash Buffer, add 100 µL diluted Detection Antibody solution to each well, seal the plate, and incubate at room temperature for 1 hour with shaking.
6. Wash plate 4 times with Wash Buffer, add 100 µL diluted Avidin-HRP solution to each well, seal the plate, and incubate at room temperature for 30 minutes with shaking.
7. Wash plate 5 times with Wash Buffer, soaking for 30 seconds to 1 minute per wash. Add 100 µL of Substrate Solution C to each well and incubate in the dark for 15 minutes.
8. 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.

For more detailed set information, please refer to the online manual at:
www.biolegend.com/media_assets/pro_detail/datasheets/431504.pdf

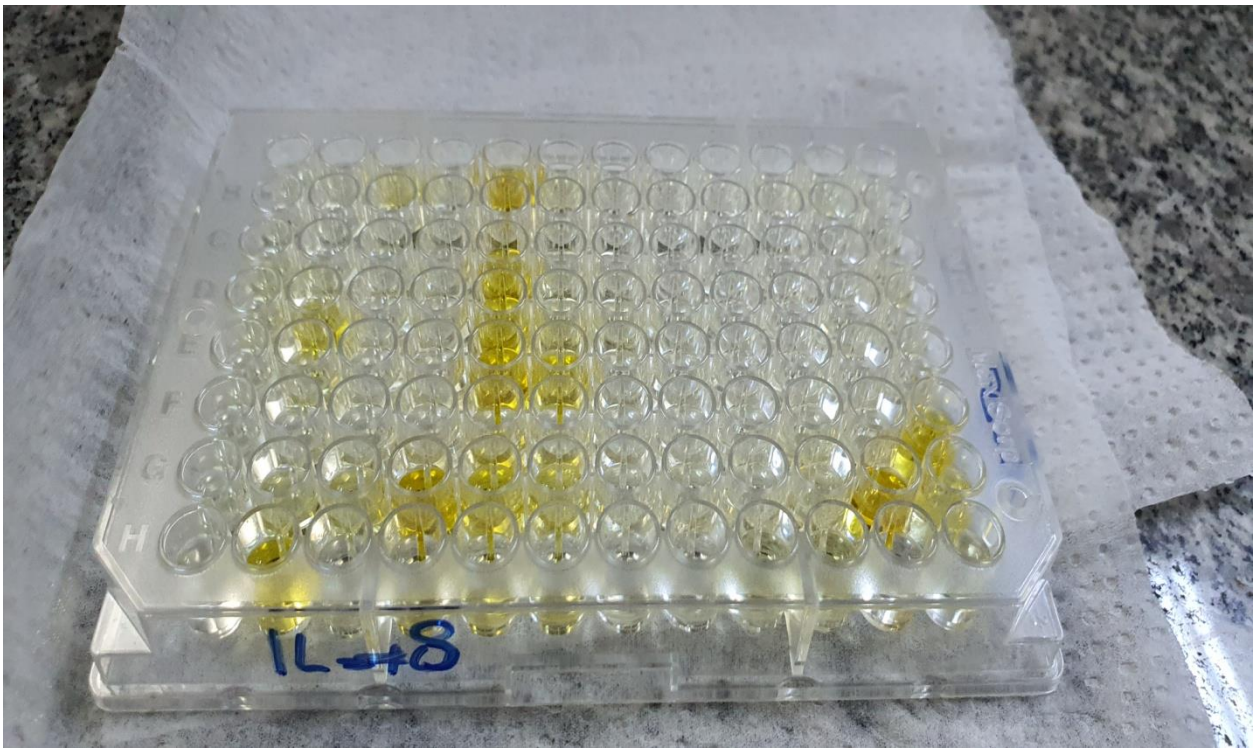
Part No. 78514_V03

Appendix (4)

a- ELISA Washer

b-ELISA reader

Appendix (5)



ELISA micro plate