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Procalcitonin and CD64,CD69 Flowcytometry for Early Diagnosis of Bacterial Neonatal Sepsis with Genotyping Frequency of CD14 Gene

A Thesis

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بسم الله الرحمن الرحيم

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صدق الله العظيم
من سورة طه
الآيات(25-28)

Dedication

To those who support me
All the time…..

My family……

orass

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Summary

Neonatal sepsis remains a global health problem owing to its significant contribution to morbidity and mortality. Its early diagnosis presents a clinical dilemma because of the variable and non-specific clinical presentation. This cross-section study was based on the analysis of 2 study groups of newly born infants who were admitted in Neonatal Intensive Care Unit (NICU) of Maternity and Children Teaching Hospital in AL-Diwaniya city. The cases group was 75 infants with a clinical diagnosis of neonatal sepsis. This group was further classified into 2 groups based on the results of blood culture. A positive bacterial culture was identified in 30 cases and these were labeled as "Proven sepsis" group. The culture negative cases were 45 cases in number and were thus labeled as "Probable sepsis" group. In addition, a random sample of 75 healthy control neonates was group matched to the cases group on gender, birth weight and gestational age in the period from 1st April to end December 2015.

The aim of our study were to evaluate the role of activated surface marker CD64 on neutrophils and CD69 on lymphocytes as a sensitive and specific indicator, to assess the role of procalcitonin (PCT) as a marker in the early diagnosis of neonatal sepsis and to analyze the genotypic and allelic frequency for *CD14* gene and the association of C/T at position – 159 in *CD14* gene and its susceptibility to sepsis in neonate.

There was a low culture positivity rate 30 (40%) among suspected sepsis neonates, the females less affected than males constituted around a third of both proven and probable sepsis study groups with ratio 3:2. No

obvious or statistically significant differences in gender composition were observed between the 3 study groups. In addition, no statistically significant differences were observed between the 3 study groups in proportion of low birth weight and preterm. The majority of sepsis cases had non-specific signs and symptoms such as hyperthermia which were the most frequently reported feature, with more than two thirds of the cases group showing this feature. Tachypnea, poor feeding and tachycardia ranked second (less than a half of cases showing these features).Then followed by vomitting and lethargy.

The gram-positive organisms accounted for the majority causative agent of neonatal sepsis cases (80%) while sepsis due to gram-negative organisms accounted for (20%).The most frequently bacteria was *Staphylococcus aureus* (50%) and *Staphylococcus epidermidis* (30%) for gram positive while *Klebsiella pnuemoniae* (13.3%) and *Escherichia coli* (6.7%) for gram negative.

The performance characteristics (validity) of a tests including sensitivity, specificity, positive predictive value and negative predictive value and receiver-operating characteristic (ROC) curves were used for the determination of thresholds for the proven sepsis(culture positive) group versus healthy neonate group and for the proven sepsis group versus probable sepsis(culture negative) group. Among selected hematological test, it was found that there was a significantly higher proportion of neonates with sepsis had leukocytosis (36.7% and 24.4% among proven and probable sepsis groups respectively) compared to healthy controls. In the same way, a very low blood neutrophil count was observed in a significantly higher percentage of neonates with sepsis (60% and 24.4% among proven and probable sepsis groups respectively) compared to healthy controls.

Performed Receiver-Operating Characteristic curve analysis to determine the diagnostic usefulness of Procalcitonin, CD64 neutrophil expression, CD69 lymphocyte expression, blood neutrophil count and Blood

WBC count for detecting neonatal sepsis. The validity of Procalcitonin was 100% for its sensitivity, specificity, positive predictive value, and its negative predictive value. The validity of CD64 neutrophil expression was its sensitivity 96.7%, its specificity 100%, its positive predictive value was 100% and its negative predictive value was 99.6 %. The validity of CD69 lymphocyte expression was its sensitivity 86.7%, its specificity 93.3%, its positive predictive value was 99.2 % and its negative predictive value was 98.4%.

Genotype analysis revealed 3 genotypes TT, CC, TC, when genotype distribution of the CD14(C-159T) polymorphism in septic neonate, TT genotype was the most frequent in both proven and probable sepsis groups (53.3% and 55.6%) respectively. On the other hand, CC genotype was the predominant in control group (56%). There was a statistical significant higher rate of TT and lower CC genotypes in proven septic neonates as compared to control group ($p<0.001$), OR = 13.14 and 95% CI (4.37 – 39.48). As there was a significant result with TT genotypes in relation to sepsis, it was verified that TT genotype represents a risk factor for sepsis. In conclusion, flowcytometric assessment of neutrophil CD64 can be considered a valuable marker for diagnosis of bacterial neonatal sepsis. PCT may be used not just as a marker of infection but more importantly as a marker of severity of infection.

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List of Abbreviations

Code	Meaning
ABEI	**Aminobutyl-N-ethylisoluminol**
ANC	**Absolute neutrophil count**
BSI	**Blood stream infection**
CD	**Cluster differentiation**
CDC	**Center of disease and control**
CLABSIs	**Central line–associated bloodstream infections**
CONS	**Coagulase negative staphylococci**
CBC	**Complete Blood Cell Count**
CI	**Confidence interval**
CRP	**C-reactive protein**
DNA	**Dioxy nucleic acid**
EOS	**Early onset sepsis**
ESR	**Erythrocytes sedimentation ratio**
E. coli	**Escherichia coli**
EF	**Etiological fraction**
ELBW	**Extremely low birth weight**
FITC	**Fluorescein isothiocyanate**

FCM	**Flowcytometry**
FRET	**Fluorescence resonance energy transfer**
GCSF	**Granulocyte colony-stimulating factor**
GFP	**Green fluorescent protein**
GBS	**Group B Streptococcus**
HAI	**Health-care associated infection**
HSV	**Herpes simplex virus**
IgA	**Immunoglobulin Alpha**
IgG	**Immunoglobulin Gamma**
IMCI	**Integrated Management of Childhood Illness**
IFN	**Interferon gamma**
IL	**Interleukin**
LOS	**Late-onset sepsis**
LPS	**Lipopolysaccharide**
MBL	**Mannose-binding lectin**
MRSA	**Methicillin resistance Staphylococcus aureus**
NICHD	**National Institute of Child Health and Human**
NPV	**Negative predictive value**
NICU	**Neonatal intensive care unit**
NRN	**Neonatal Research Network**
NS	**Neonatal sepsis**
NS	**Not significant**
No.	**Number**
O.D	**Optical density**
OR	**Odds ratio**
PAMPs	**Pathogen-associated molecular patterns**
PNR	**Patient–nurse ratio**
PCR	**Polymerase chain reaction**
PPV	**Positive predictive value**
PROM	**premature rupture of membranes**
P	**Probability value**
PCT	**Procalcitonin**
PE	**Phycoerythrin**
RLU	**Relative light unite**
ROC	**Receiver operating characteristic**

SIRS	**Systemic inflammatory response syndrome**
TLC	**Total leukocyte count**
TNF	**Tumor necrosis factor**
TNF-α	**Tumor necrosis factor α**
VLOS	**Very late-onset sepsis**
WBC	**white blood cell**
WCC	**White cell count**
WHO	**World Health Organization**

1.1 Introduction

Neonatal sepsis (NS) is a clinical syndrome of systemic illness accompanied by bacteremia occurring in the first month of life. Neonatal sepsis remains one of the main causes of mortality and morbidity despite the progress in hygiene, introduction of new and potent antimicrobial agents for treatment, and advanced measures for diagnosis **(Naher and Khamael, 2013).** So it is responsible for 30- 50% of the total neonatal deaths in developing countries. It is estimated that up to 20% of the neonates develop sepsis and approximately 1% die of sepsis related causes **(Gandhi *et al.*, 2013).**

World Health Organization (WHO) estimates that out of the four million neonatal deaths all over the world every year, over 35% are due to infection in the neonatal period **(Lawn *et al.*, 2006)**. The prognosis and outcome of neonatal sepsis depend on early diagnosis and efficient antibiotic therapy **(Stoll, 2007),** so early diagnosis and treatment of neonatal sepsis may help to decrease neonatal mortality. However early identification of neonatal sepsis is difficult because of the nonspecific or minimal clinical presentations. The clinical course of neonatal sepsis can be fulminant within hours of onset **(Ng and Lam, 2006)**, and infection in the newborn period is associated with 10% of neonatal death **(Stoll, 2007).** Thus, it is extremely important to make an early and accurate identification of neonatal sepsis for prompt antimicrobial therapy and better outcomes.

The gold standard for confirming diagnosis of neonatal sepsis is blood culture **(Weinberg *et al.*, 2006).** However, as pathogens in blood cultures are

only detected in approximately 25% of patients, the sensitivity of blood culture is suspected to be low **(Arnon and Litmanovitz, 2008)**. Also the blood culture results are not available for 48 hours for preliminary report to 7 days for final report after starting the culture, the possibility of sepsis in the presence of negative blood culture is noted in neonates who are exposed to antibiotics in utero **(Bhandari *et al.*, 2008),** and if blood cultures are drawn after administration of antibiotics, growth of microorganisms can be suppressed **(Icardi *et al.*, 2009).** The negative microbiological cultures do not always exclude the presence of bacterial sepsis **(Ng *et al.*, 2004).** So the other tests in diagnosis of neonatal sepsis are warranted. Hence, a reliable inflammatory marker or set of markers is required for prompt and accurate identification of neonatal sepsis, so that delayed or unnecessary treatment can be avoided **(Young *et al.*, 2012).**

The diagnosis of sepsis is difficult because of non-specificity of clinical signs and symptoms and overlapping of symptoms with other noninfectious causes of systemic inflammation **(Kaur *et al.*, 2013)**. Although neutrophil, total white blood cell (WBC), absolute neutrophil count (ANC), and platelet counts and blood culture are ordered to screen for suspected sepsis, these values are ineligible as infection markers due to insufficient sensitivity and specificity **(Khaleda *et al.*, 2010 ; Stoll, 2011)**.

Thus, most hospitals commonly use C-reactive protein (CRP) levels as markers. However, elevated CRP levels are also seen in several other clinical cases such as autoimmune disease, surgery, meconium aspiration and recent vaccination. Moreover their values do not rise significantly until almost 24-48 hr after the onset of infection **(Weinschenk *et al.*, 2000; Ng, 2004).**

Therefore Procalcitonin (PCT) has been candidate as anew marker in recent years for the diagnosis of systemic inflammation, infection, and sepsis; both in children and adults **(Manzano *et al.*, 2011; Kaur *et al.*, 2013)**. The effectiveness of PCT as an early diagnostic tool for neonatal sepsis has been

reported. Research studies reported that PCT is more effective than CRP at follow-up, as PCT levels rise earlier and return to normal levels more rapidly than CRP levels **(Naher *et al.*, 2011; Adib *et al.*, 2012; Sucilathangam *et al.*, 2012)**. Many studies also considered PCT to be a superior marker of neonatal sepsis compared to CRP with the added advantage of differentiating bacterial and viral infection, thus unnecessary use of antibiotics can be avoided **(Nnanna *et al.*, 2011; Sucilathangam *et al.*, 2012; Adib *et al.*, 2012).**

Since diagnosis of neonatal sepsis is one of the most difficult tasks in clinical practice, as the disease progress more rapidly than adult and the mortality rate is higher in neonates **(Zaki *et al.*, 2009)**, several different laboratory determinations are helpful in diagnosis of neonatal sepsis for instances; numerous cell surface antigens have been studied as potentially promising biomarkers of infection, including CD69 and CD64 **(Ng & Lam, 2006).** Flow cytometric analysis of cell surface antigens (CD11b, CD64, CD32 CD16, CD69, CD25 and CD45) has been performed to detect neonatal sepsis **(Hodge *et al.*, 2004)** .Other surface markers that have been investigated in different studies include CD69 on peripheral T and B lymphocytes may also have a role **(Lekkou *et al.*, 2004).** Furthermore, several studies have indicated that quantitation of the neutrophil CD64 is a worthwhile candidate for evaluation as a more sensitive and specific indicator of sepsis than the other available diagnostics tests **(Davis *et al.*, 2006),** that has high diagnostic specificity and sensitivity of neonatal sepsis **(Cardelli *et al.*, 2008, Bhandari *et al.*, 2008).**

On the other hand, variation in the ability to recognize pathogens may influence the risk of infection. One of the primary molecules that functions in recognition of pathogen is CD14 **(Underhill and Ozinsky, 2002)**. CD14 is a pattern recognition receptor that plays a central role in innate immunity

through recognition of bacterial lipoglycans, primarily lipopolysaccharide (LPS). CD14 has a unique ability to discriminate non-self lipoglycans of infectious pathogens first and for most LPS from non - infectious self **(Shimada *et al.*, 2000).** Since until now no single laboratory test has provided rapid and reliable identification of infected neonates. This inability has led to a search for new diagnostic technique**.**

Aim of the Study

The aim of the present study was designed to investigate the diagnostic value and determine whether any of serum procalcitonin level, neutrophils CD64 and lymphocytes CD69 markers as predictor for neonatal sepsis prior to the blood culture, as well as identify the genetic susceptibility of CD14/C-159T gene polymorphism to sepsis in neonates.

This aim will fulfill through the following objectives.

- Determination the Procalcitonin concentration in the serum of healthy subjects and those with neonatal sepsis in order to evaluate its benefit in early diagnosis of this disease.
- Identification the CD64 and CD69 markers on the surface of neutrophils and lymphocytes respectively by using flowcytometry assay to evaluate their role in the diagnosis of bacterial neonatal sepsis.
- Comparing both recent methods with conventional blood culture method.
- Identification the genotypic and allelic frequency of CD14/C-159T polymorphism and the association of its susceptibility to sepsis in neonate.

1.2 Literature Review

1.2.1. Neonatal Sepsis

Sepsis is defined as a systemic inflammatory response syndrome (SIRS) associated with infection on the basis of either microbiological cultures or strong clinical evidence of the presence of an infection. Severe sepsis is defined as sepsis plus evidence of organ dysfunction defined around pediatric parameters **(Wynn *et al.*, 2010).** However, neonatal sepsis is a clinical syndrome of bacteremia characterized by systemic signs and symptoms of infection in the first month of life. This syndrome encompasses systemic infections of the newborn including septicemia, meningitis, pneumonia, arthritis, osteomyelitis and urinary tract infection of the newborn **(Richard *et al.*, 2004).**

1.2.2 The Prevalence and Mortality Rate of Neonatal Sepsis

Neonatal sepsis is one of the most common causes of neonatal morbidity and mortality. It is estimated to cause 26% of all neonatal deaths worldwide **(Lawn *et al.*, 2005).** Its incidence is much higher in developing countries **(Kaistha *et al.*, 2009),** and responsible for about 30-50% of the total of neonatal death (**Bang *et al.*, 1999).** It is one of the most common reasons for admission to neonatal units in developing countries **(Shah *et al.*, 2012).** It refers to generalized bacterial infection documented by a positive blood culture in the first 28 days of life. The national neonatal-perinatal database reported that superficial infections like conjunctivitis and oral thrush are not usually included under neonatal sepsis **(Davies and Davies, 2010)**.

The spectrum of organisms that causes neonatal sepsis varies over time and even within the same hospital which is due to the changing pattern of antibiotic use and changes in lifestyle **(Jyothi *et al.*, 2013).**

The number of neonatal patients at risk of acquiring nosocomial infections is increasing because of the improved survival of very low birth weight infants and their need for invasive monitoring and supportive care **(Couto *et al,* 2007).** The World Health organization (WHO) reported in 2005 that over 70% of death in children under age five occur within the first year of life and 40% occur within the first month **(WHO, 2005).** Neonatal infections currently cause 1.6 million deaths annually in developing countries. Sepsis and meningitis are responsible for most of these deaths**. (Vergnano *et al*., 2005)**.

According to WHO in 2010, 3.7 million newborns died before reaching 28 days of age in the United States, and 37% were due to infectious causes. However, neonatal mortality is about 34 per 1000 live births in Asia, 42 per 1000 live births in Africa and 17 per 1000 live births in Latin America **(Vergnano *et al.*, 2005)**, while relatively low rates being reported in the United States and Australasia which is about 6–9 per 1000 live birth and in Europe only 0.3-3 per 1000 live births **(Heath *et al.*, 2003).**

According to the geographic variations in Iraq the distribution of under-five death by age-groups was 55% of all neonatal death in age of 0-28 days of life, and neonatal sepsis is responsible for about 16% of the total of neonatal deaths under-five death per 1000 live births, as shown in **(Figure 1-1).** In AL- Qadisiya province was 44% in comparison with 29% ,35% and 37% in Wasit, Karbala and Al-Najaf respectively **(WHO, 2014)**.

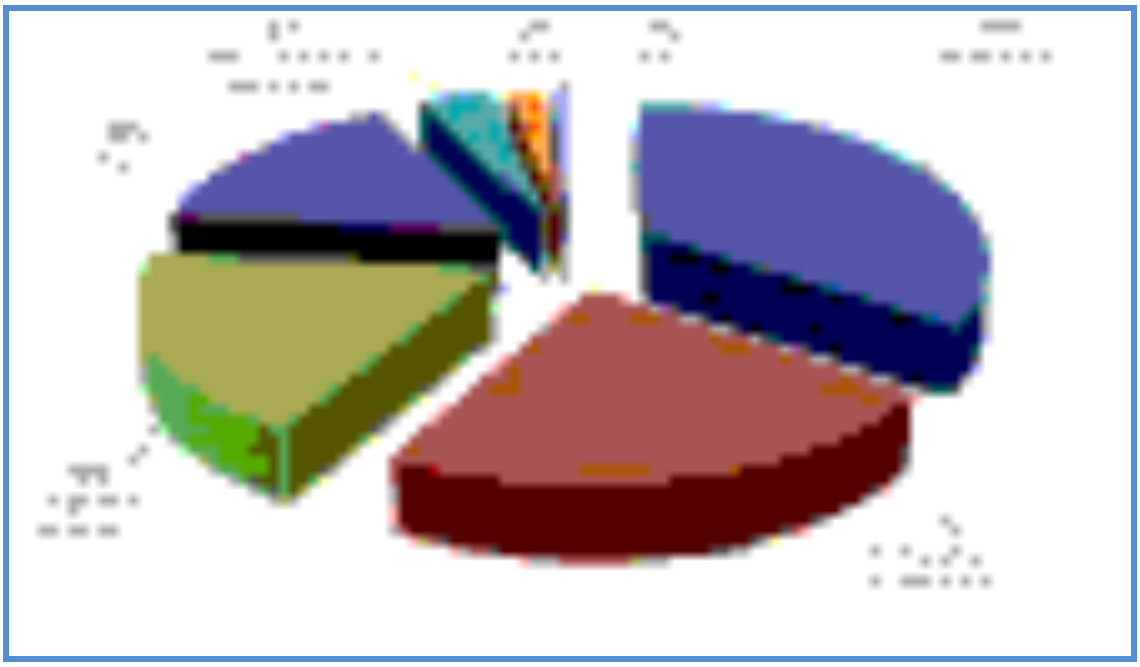

Figure (1-1): Distribution of under-five death of Neonates according to the causative agents (WHO, 2014).

1.2.3. Classification of neonatal sepsis

Neonatal sepsis classified with regard to its onset into three groups: Early onset sepsis (EOS), late-onset sepsis (LOS) and very late-onset sepsis (VLOS) **(Jaiswal *et al.*, 2011).**

1.2.3.1 Early onset sepsis:

Occurs within the first 7 days of life which is generally associated with the acquisition of microorganisms from the mother and usually presents with respiratory distress and pneumonia **(Stoll *et al.*, 2011)**. The source of the infection is commonly the maternal genital tract **(Kaufman and Fairchild, 2004)**.

The main risk factors for EOS include prematurity, low birth weight, febrile illness in the mother within 2 weeks of delivery, foul smelling and/or meconium stained liquor, premature rupture of membranes (PROM), prolonged labor, and perinatal asphyxia **(Stoll *et al.*, 2005; Stoll *et al.*, 2011)**.

1.2.3.2 Late-onset sepsis

Usually presents within 7-30days of birth and may either be caused by prenatally or postnatal-acquired organisms, while it usually occurs as a consequence of nosocomial transmission **(Benjamin and Stoll, 2006).** Organisms that have been implicated in LOS include coagulase negative staphylococci (CONS), *Staphylococcus aureus*, *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., *Enterobacter* spp., *Candida* spp., GBS, *Serratia* spp., *Acinetobacter* spp., and anaerobes **(Stoll *et al.*, 2002 a ; Stoll *et al.*, 2011**). The affected neonates usually present with septicemia and meningitis **(Sankar *et al.*, 2008)**. The main risk factors for LOS include prematurity and prolonged neonatal intensive care unit (NICU) stay. Other risk factors are central vascular access, invasive procedures, and the use of broad-spectrum antibiotics **(Stoll *et al.*, 2002 a ; Sankar *et al.*, 2008)**.

1.2.3.3 Very late-onset sepsis

This type of neonatal sepsis is usually diagnosed in extremely low birth weight (ELBW) infants who remain hospitalized for several weeks after birth. The major factors increasing the sepsis risk in these infants include the intravascular catheters required for their care, prolonged exposure to antimicrobial agents, and ongoing immature host defense mechanisms **(Edwards and Baker , 2004)**. The pattern of bacterial pathogens responsible from neonatal sepsis has changed with time and varies from place to place. There has also been a difference in the causative organisms of neonatal sepsis between the developed and developing countries **(Sivanandan *et al.*, 2011; Stoll *et al.*, 2011; Muller-Pebody *et al.*, 2011).**

The Percentages of approximate number of very low birth weight (VLBW) infants with septicemia which are illustrated in **(Figure 1-2)** indicated that EOS usually occurs via ascent of organisms from the birth canal to the amniotic fluid, with or without rupture of amniotic membranes, while LOS occurs with vertical and horizontal spread of organisms. In contrast the

vast majority of cases of sepsis in VLBW infants occur in the first 30 days of life, and VLBW infants requiring prolonged intensive care are at risk for VLOS beyond 2 months of age **(Kaufman and Fairchild, 2004).**

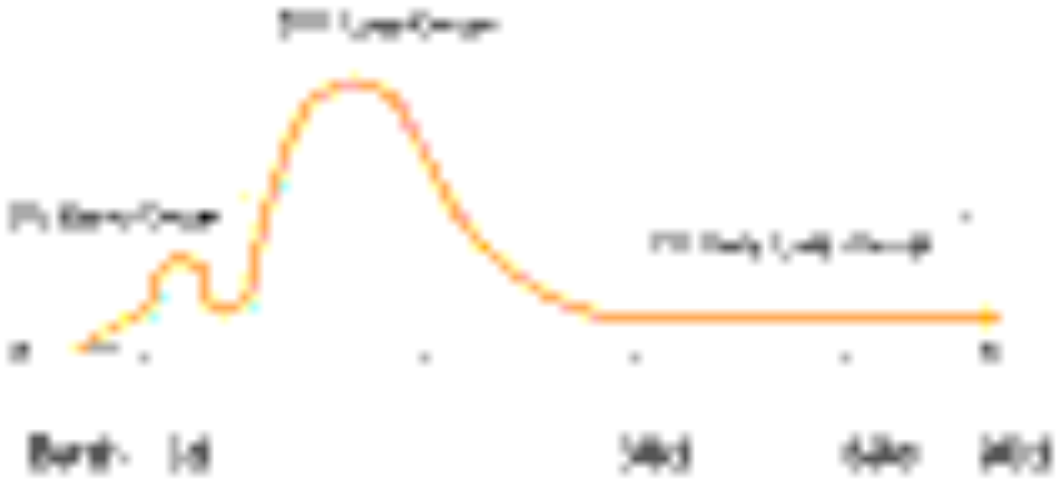

Figure (1-2): Timing of bacterial and fungal sepsis in VLBW infants (Kaufman and Fairchild, 2004).

1.2.4 Basic Physiology of Neonatal Infection

Throughout pregnancy and until the membranes rupture, the fetus is relatively protected from the microbial flora of the mother by the chorioamniotic membranes, the placenta, and poorly understood antibacterial factors in amniotic fluid **(Aletayeb *et al.*, 2011).** However, there are many ways that infectious agents can reach the fetus or newborn to cause infection. Procedures disturbing the integrity of the uterine contents, such as amniocentesis, cervical cerclage, transcervical chorionic villus sampling, or percutaneous blood sampling, can permit entry of skin or vaginal organisms, causing amnionitis and secondary fetal infection**.** Certain bacteria, particularly *Treponema pallidum* and *Listeria monocytogenes*, can reach the fetus through the maternal blood stream despite placental protective mechanisms, causing transplacental infection. This process is uncommon, but it leads to either congenital infection unlike infections caused by certain viruses or

Toxoplasma or to stillbirth resulting from overwhelming infection **(Schrag *et al.*, 2002).**

There are four separated mechanisms by which bacteria reach the fetus or newborn to cause infection:

1.2.4.1 Ascending Infection

Initial colonization of the neonate usually takes place after rupture of the maternal membranes. In most cases, the infant is colonized with the micro flora of the birth canal during delivery. However, particularly when the rupture of membranes lasts longer than 24h, vaginal bacteria may ascend and in some cases produce inflammation of the fetal membranes, umbilical cord, and placenta. Fetal infection can result from aspiration of infected amniotic fluid, leading to stillbirth, premature delivery, or neonatal sepsis. The organisms most commonly isolated from infected amniotic fluid are anaerobic bacteria, GBS**,** *E.coli*, and genital *Mycoplasmas* **(Wendel *et al.*, 2002)**.

1.2.4.2. Birth Canal

Infection of the mother at the time of birth, particularly genital infection, is the principal pathway of maternal transmission and can play an important role in the development of infection in the neonate, which is most likely caused by GBS which appear to be acquired from the vagina or cervix through ruptured membranes leading to amnionitis **(Stoll *et al.*, 2002 b).**

1.2.4.3. Transplacental (Hematogenous)

In Trans-placental hematogenous infection, spread may occur at different times during gestation or shortly before delivery (including the period of separation of the placenta) and it usually causes congenital infection. The main organisms which cause congenital infection include *Mycoplasma, Listeria monocytogenes, Chlamydia, and GBS* **(Kenneth *et al.*, 2007).**

1.2.4.4 Post-natal Infection

Finally, bacteria can be introduced after birth from the environment surrounding the baby, either in the nursery (nosocomial infection) or at home (community acquired). These two mechanisms are responsible for LOS, the most common organisms including; *Coagulase negative staphylococcus (CONS), Klebsiela pneumoniae , E.coli, Salmonella, Campylobacter, Enterobacter, Citrobacter, Pseudomonas aeruginosa, Serratia, Enterococci, Staphylococcus aureus, and Candida* **(Kenneth *et al.*, 2007).**

1.2.5 Neonate Host Defense

The development of the immune system entails a number of changes that occur during the first years of life. Neonates, especially preterm infants, are relatively immune- compromised because of immaturity of the immune system, as well as decreased placental passage of maternal antibodies. Several components of the neonatal immune system that are immature and contribute to increased susceptibility to serious bacterial, fungal, and viral infections **(Belderbos *et al.*, 2012),** that can be summarized as follows:

1.2.5.1 Innate Immune System

The innate immune system produces an immediate immunologic response and is capable of doing this without previous exposure to a specific pathogen. Recognition of pathogens occurs by identification of conserved biologic regions known as pathogen-associated molecular patterns (PAMPs). Recognition receptors, such as TOLL-like receptors, NOD-like receptors and RIG-like receptors, identify and respond to PAMPs with the production of cytokines and proinflammatory responses that activate the adaptive immune system **(Kumar *et al.*, 2012).** Studies comparing neonatal and adult innate immune functions show that neonatal cells have a decreased ability to produce inflammatory cytokines, especially tumor necrosis factor (TNF) and

interleukin (IL-6) **(Kollmann *et al.*, 2009).** In addition, they induce IL-10 production, which in itself is capable of inhibiting synthesis of proinflammatory cytokines **(Belderbos *et al.*, 2012).**

Neutrophil and dendritic cell functions are also reduced; neutrophils show a decreased expression of adhesion molecules, as well as a decreased response to chemotactic factors, and dendritic cells have a decreased capacity of producing IL-12 and gamma interferon (IFN-γ) **(Carr, 2000; Levy, 2007).** The overall reduction in cytokine production in neonates also results in decreased activation of natural killer cells **(Guilmot *et al.*, 2011).** Impairment of the innate immune system leads to an increased susceptibility to bacterial and viral infection in this population **(Guilmot *et al.*, 2011).**

1.2.5.2 Adaptive Immune System

The adaptive branch of the immune system is designed to eliminate specific pathogens. In newborns, the adaptive immune system slowly increases its function toward an adult like response, minimizing the otherwise overwhelming inflammatory response that would occur when infants transition from a sterile to a colonized environment **(Schelonka *et al.*, 2011).** Decreased cytotoxic function (strong T-helper 2 polarization with decreased IFN gamma production), lack of isotype switching, and overall immaturity and decreased memory (because of limited pathogen exposure at time of birth), reduce the neonate's ability to respond effectively to infections **(Tolar *et al.*, 2009; Takahashi *et al.*, 2009)**. For example, the reduction of cell-mediated immunity increases the risks of infections caused by intracellular pathogens, such as *Listeria*, *Salmonella,* herpes simplex virus (HSV), cytomegalovirus, and enteroviruses. Transplacental passage of maternal immunoglobulin G (IgG) is inversely related to gestational age and limits the functional ability of the neonate to respond to certain pathogens **(van den Berg *et al.*, 2011; Palmeira *et al.*, 2012).** Minimal IgG is transported to the fetus in the first trimester, whereas fetal IgG rises in the second trimester from

approximately 10% at 17 to 22 weeks gestation to 50% at 28 to 32 weeks gestation**.** Thus, preterm infants lack adequate humoral protection against a number of infant pathogens, whereas term infants will often be protected against most vaccine preventable neonatal infections through transplacental passage from the mother's serum **(Malek, 2003)**.

Furthermore, histological studies also demonstrated that the marginal zone of the spleen is not fully developed until 2 years of age, increasing the infant's susceptibility to encapsulated bacterial infections such as *Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitides* **(Zandvoort and Timens, 2002).** Finally, transfer of IgA, IgG, cytokines, and antibacterial peptides present in human milk may be compromised, especially in premature babies. The lack of secretory IgA decreases the ability of the neonate to respond to environmental pathogens **(Brandtzaeg, 2010).**

1.2.5.3 Complement

Complement levels increase with increasing gestational age, but are only about 50% of adult levels at term. Reduced complement levels are associated with deficient opsonization and impaired bacterial killing. Although both pathways seem to be capable of being activated, there may be variations in their activation level. In addition, profound C9 deficiency has been observed in neonates, reducing the ability to form bacteriolytic C5b-9 membrane attach complex (MAC), which will increase the risk of acquiring severe invasive bacterial infections **(Hogasen *et al.*, 2000).**

1.2.6 Risk Factors for Neonatal Sepsis

Health-care associated infection (HAI) rates on different neonatal units vary widely. There are several reasons for this variation, including differences in the way that sepsis is defined, differences in the types of babies treated and differences in standards of care. There is clear evidence that the contributors to HAI are indeed multifactorial. Clusters of infection with particular nosocomial pathogens inevitably occur on NICUs from time to time. An

understanding of risk factors for acquisition and/or infection with the outbreak strain can inform infection control measures to terminate the outbreak **(Gray, 2008).**

The determination of risk factors for all-cause sepsis is the most useful approach, where the aim of surveillance is to compare intra- or inter-unit infection rates, since the predominant microbial flora in units varies. The only exception to this might be CoNS, which account for the majority of blood culture isolates in NICUs. Infection rates with these bacteria might, therefore, represent a reasonable proxy for overall infection rates. However, such an approach might mask important differences in rates of infection with less common, but more virulent, pathogens **(Gray, 2007).** Risk factors for blood stream infection (BSI) fall into three main categories:

1.2.6.1 Intrinsic risk factors

The fetus and newborn face a complex set of immunological demands, including protection against infection, avoidance of harmful inflammatory immune responses that can lead to pre-term delivery, and balancing the transition from a sterile intra-uterine environment to a world that is rich in foreign antigens. These demands shape a distinct neonatal innate immune system that is biased against the production of pro-inflammatory cytokines. This bias renders newborns at risk of infection and impairs responses to many vaccines **(Levy, 2007).** Premature new born respond adequately to most protein antigens, but response to polysaccharide antigens is poor in the first 2 years of life. Opsonization activities of the alternate complement system and serum fibronectin levels are deficient in the term infant. The newborn has a high total T-lymphocyte count, but phenotypic surface markers differ from those in the older child. Cytotoxic T-cell activity is decreased as is T-cell helper function. T-cell dependent antigen specific response is delayed, and there is limited production of several cytokines. Natural killer cell activity

which is important in control of herpes group viral infections is also decreased **(Lewis and Wilson, 2001).**

It is universally agreed that the incidence of late onset sepsis is inversely proportional to birth weight and gestational age **(Gray, 2008).** The incidence of preterm births (<37 weeks gestation) is increasing in many countries around the world and has become a global health concern. More than 70% of preterm infants are born between 34 and 36 weeks gestation (late preterm). Most large series describing the epidemiology of neonatal sepsis are limited to infants with very low gestational age (<33 weeks) or VLBW (<1500 g birth weight) **(Cohen-Wolkowiez *et al.*, 2009).**

The maternal risk factors can be included in this category; maternal fever, pre-labor rupture of membranes of >18 hours, premature onset of labor, chorioamnionitis, urinary tract infections and group B streptococcus (GBS) colonization are the key maternal risk factors **(WHO, 2003).** Also the effect of gestational age on the risk of GBS disease for example could be explained by the amount of maternal IgG antibodies received by the infant, because susceptibility to invasive GBS disease has been correlated with deficiency in levels of maternal type-specific serum IgG antibodies **(Lin *et al.*, 2003).**

1.2.6.2.. Extrinsic risk factors (Invasive Procedures)

Any procedure that disrupts the normal barriers to infection is likely to present a higher risk of infection in the newborn than later in life. The normal newborn escapes most invasive procedures but may be subjected to scalp electrodes or percutaneous punctures for blood sampling. Scalp electrodes provide a portal of entry for maternal genital microorganisms. Infectious complications occur in less than 1% of infants and most are benign abscesses, but severe cellulitis, bacteremia, osteomyelitis, and disseminated HSV infection have been reported. Premature and ill newborns often require

feeding by nasogastric tubes, which provide a portal of entry and potentiate overgrowth of microbes in the upper gastrointestinal tract. Breast milk and formula feeds administered by continuous infusion remain at room temperature for several hours, allowing microbes to proliferate in the reservoir or tubing during infusion **(Mehall *et al.*, 2002).**

The most commonly reported causative pathogens of central line–associated bloodstream infections (CLABSIs) remain CoNS, *Staphylococcus aureus*, Enterococci, and *Candida* spp. Gram negative bacilli accounted for 19-21% of CLABSIs reported to center of disease and control (CDC) **(O'Grady *et al.*, 2002).**

1.2.6.3. Environment (infrastructure-related) risk factors

Some studies have examined the relationship between neonatal sepsis and organizational and structural factors on the NICU **(Gray, 2008)**. Infection rates in the NICU increase with overcrowding and understaffing **(Moore, 2004).** Increasing rates of endemic methicillin resistance *Staphylococcus aureus* (MRSA) was linked to overcrowding and understaffing , with eradication of MRSA when these conditions improved**.**

An outbreak of *Enterobacter cloacae* infection was associated with understaffing and overcrowding in another report; a decrease in percentage of HAIs from 5.8 to 1.8 was observed after moving to a new NICU with more nurses and space per infant, more accessible sinks, and improved ventilation**.** One of the most important predictors of patient well-being is the amount of direct nursing care patients receive per day, the measure of nursing provision used is the patient–nurse ratio (PNR); this is the average number of registered nurses (calculated over a 24 h period of shifts, including partial nurse shifts) and occupancy in terms of NICU census of midnight the previous day **(Profit *et al.*, 2010)**.

Evidence-based guidelines for healthcare workers' hand hygiene practices exist, but compliance with these is internationally low **(Creedon, 2005).** The hand-hygiene compliance observed among health care workers by many researchers doesn't exceed 50% **(Bischoff *et al.*, 2000).** Many factors have contributed to poor hand washing compliance among healthcare workers, including a lack of knowledge among personnel about the importance of hand hygiene in reducing the spread of infection and how hands become contaminated, lack of understanding of correct hand hygiene technique, understaffing and overcrowding, poor access to hand washing facilities, irritant contact dermatitis associated with frequent exposure to soap and water, and lack of institutional commitment to good hand hygiene **(Pittet and Boyce., 2001).** In order to be effective, efforts to improve compliance with hand washing guidelines must be multifaceted. Alcohol hand rubs (with emollients) need to be provided at each patient's bedside. Issues surrounding healthcare workers' skin irritation need to be addressed urgently **(Creedon, 2005).**

1.2.7 Microbiology of Neonatal Sepsis

The spectrum of organisms that cause neonatal sepsis changes over time and varies from region to region. In the past, Gram positive cocci, were the most common pathogens in the United States but this predominance shifted to Ggram negative enteric bacilli after antimicrobial agents were commonly used **(Stoll *et al.*, 2011)**. The microbial pathogens and risk factors associated with neonatal sepsis are shown in (Table 1-1).

Table (1-1): Microbial pathogens and risk factors associated with neonatal sepsis (Libster *et al.*, 2012).

Neonatal sepsis	Microbial pathogens	Risk factors
Early-onset	• Group B *streptococci* • *Escherichia coli* • *Streptococcus viridans* • *Enterococci* • *Staphylococcus aureus* • *Pseudomonas aeruginosa* • Other gram-negative bacilli	• Maternal Group B *streptococcal* colonization • Chorioamnionitis • Premature rupture of membranes • Prolonged rupture of membranes (> 18 h) • Preterm birth (< 37 weeks) • Multiple gestation
Late-onset	• Coagulase-negative *Staphylococci* • *Staphylococcus aureus* • *Candida albicans* • *Escherichia coli* • *Klebsiella pneumoniae* • *Enterococci* • *Pseudomonas aeruginosa* • *Group B streptococci*	• Prematurity • Low birth weight • Prolonged indwelling catheter use • Invasive procedures • Ventilator associated pneumonia • Prolonged antibiotics

1.2.8 Diagnostic tools used in neonatal sepsis

Neonatal sepsis is clinically diagnosed by a combination of clinical signs, nonspecific laboratory tests and microbiologically confirmed by blood culture which is the gold standard for diagnosis of septicemia **(Marchant *et al.*, 2013)**. Neonatal clinical sepsis syndrome identification is difficult as the clinical signs of neonatal septicaemia can be very similar to those of other life-threatening diseases such as necrotizing enterocolitis, hyaline membrane disease, and perinatal asphyxia **(English *et al.*, 2004).**

1.2.8.1 Clinical signs and symptoms

The early diagnosis of neonatal sepsis presents a clinical dilemma because of the variable and non-specific clinical presentation of this condition **(Ng *et al.*, 2002; Bhandari *et al.*, 2008).** However, studies in middle- and low-income countries have provided seven danger signs which can be used to identify infants with very severe disease including neonatal sepsis as shown in Table 1-2 **(Stoll and Shane, 2016)**.

Table (1-2): Initial Signs and Symptoms of Infection in Newborn Infants (Stoll and Shane, 2016).

GENERAL Fever, temperature instability "Not doing well" Poor feeding Edema	**RENAL SYSTEM** Oliguria
	CARDIOVASCULAR SYSTEM Pallor; mottling; cold, clammy skin Tachycardia Hypotension Bradycardia
GASTROINTESTINAL SYSTEM Abdominal distention Vomiting Diarrhea Hepatomegaly	**CENTRAL NERVOUS SYSTEM** Irritability, lethargy Tremors, seizures Hyporeflexia, hypotonia Abnormal Moro reflex Irregular respirations Full fontanel High-pitched cry

RESPIRATORY SYSTEM	HEMATOLOGIC SYSTEM
Apnea, dyspnea Tachypnea, retractions Flaring, grunting, Cyanosis	Jaundice Splenomegaly Pallor Petechiae, purpura,Bleeding

These signs provide high sensitivity and moderate specificity for detecting serious illness in newborns in low-resource settings and have now been incorporated into the new neonatal WHO Integrated Management of Childhood Illness (n-IMCI) guidelines, **Table 1-3 (Stoll and Shane, 2016)**.

Table (1-3): Clinical Criteria for the Diagnosis of Sepsis in the International Setting (Stoll and Shane, 2016).

NEUROLOGIC: convulsions, drowsy or unconscious, decreased activity, bulging fontanel
RESPIRATORY: respiratory rate >60 breaths/min, grunting, severe chest indrawing, central cyanosis
CARDIAC: poor perfusion, rapid and weak pulse
GASTROINTESTINAL: jaundice, poor feeding, abdominal distention
DERMATOLOGIC: skin pustules, periumbilical erythema or purulence
MUSCULOSKELETAL: edema or erythema overlying bones or joints
OTHER: Temperature >37.7°C (99.9°F; or feels hot) or <35.5°C (95.9°F; or feels cold)

1.2.8.2 Laboratory tests

1.2.8.2.1 White cell count (WBC), platelet count and differential count

Despite being widely acknowledged to lack specificity and sensitivity of the white cell count, platelet count and white differential count, but they are still used for the screening of neonates with sepsis **(Ng and Lam ,2006; Hoffmann, 2009)**. Limitations of the WBC include the following **(Hoffmann, 2009).**

- May be normal, high or low in infection

- May be high due to many conditions, such as stress of delivery, following surgery or trauma.
- There may be differences between arterial or venous sample values
- The differential count and assessment of the immature neutrophil cell count is operator dependent (subjective) and therefore not always reliable **(Ng and Lam , 2006).**

Thrombocytopenia (low platelet count) with counts of <100 $x10^9$/l (normal neonatal reference range for platelets is 150-450 $x10^9$/l, as obtained from Haematopathology Laboratory from Children Hospital, British Columbia, January 1990) may occur in neonatal sepsis but this is not specific to sepsis **(Murphy and Weiner, 2012).** Increased mean platelet volume and platelet distribution width has been noted in neonates with sepsis within 2-3 days of life **(Murphy and Weiner, 2012).**

Complete blood cell count, contrary to older children and adults, the white blood cell (WBC) count does not accurately predict infection in neonates. A recent multicenter review of CBCs and blood cultures in neonates admitted to 293 neonatal intensive care units (NICUs) in the United States, showed that low WBC and absolute neutrophil counts, as well as high immature-to-total neutrophil ratio (I : T ratio) were associated with increasing odds of infection (odds ratios 5.38, 6.84, and 7.90, respectively); however, the test sensitivities for detection of sepsis were low **(Hornik *et al.*, 2012).**

1.2.8.2.2 Microbiological cultures

Blood cultures are currently used as the gold standard for diagnosis of infection/sepsis and help in therapeutic decision-making, especially in choosing the appropriate antibiotics **(Icardi *et al.*, 2009; Jia *et al.*, 2013).** Microbiological cultures are fraught with difficulties, which include the following:

- There may be delay in final culture results for 48-72 hours after collection. This results in unnecessary exposure to antibiotics in neonates with clinical suspicion of sepsis and creates an environment for emergence of bacterial resistance **(Bhandari *et al.*, 2008**).
- Genuine bacteremia may remain undetected in a significant proportion of infected cases because of the small volume of blood taken from preterm infants (**Ng and Lam, 2006**).
- In neonatal sepsis blood cultures are often negative in some cases of pneumonia and meningitis **(Layseca *et al.*, 2002).** The negative **blood cultures** may even occur in fatal generalized bacterial infection **(Layseca *et al.*, 2002).**
- The possibility of sepsis in the presence of negative **blood culture** has been noted in neonates who had been exposed to antibiotics in utero, presumably due to antibiotic interference with growth of the organism in vitro **(Bhandari *et al.*, 2008**).
- Bacteremia may often be transient or intermittent, especially during the early stages of infection (**Ng and Lam, 2006**).

1.2.8.2.3 Sepsis screen

All neonates, suspected to have sepsis, should have a septic screen to corroborate the diagnosis. However, the decision to start antibiotics need not be conditional to the sepsis screen result if there is a strong clinical suspicion of sepsis **(Murphy and Weiner, 2012)**.

1.2.8.2.4 Acute phase reactants

Acute phase reactants are also frequently used in predicting neonatal sepsis. The most extensively used acute phase reactant is C-reactive protein (CRP). In a meta-analysis the sensitivity of this test was estimated to be 80%, but specificity was only 60% - 80% **(Hoffmann, 2009).** Several studies agree that serial measurements of CRP guide the duration of 5 antibiotic treatment

in neonates managed for suspected sepsis before the final blood culture results are available **(Chiesa *et al.*, 2004; Hofer *et al.*, 2012).**

The CRP was first described in the 1930s and since then multiple studies have shown elevation of the CRP in several infectious and noninfectious etiologies that share a common background of inflammation or tissue injury. In neonates, serial measurements of the CRP in the first 24 to 48 hours of symptoms increases the sensitivity of the test, with suggestion that normal CRP values during this period have a 99% negative predicted value for determination of infection **(Philip and Mills,2000; Hengst, 2003).**

In contrast, elevated levels of CRP may be more difficult to interpret, especially for diagnosis of EOS, because factors such as premature rupture of membranes (PROM), maternal fever, pregnancy-induced hypertension, prenatal steroid use, and fetal distress may also cause elevation of the CRP **(Chiesa *et al.*, 2001).** Additionally, studies have suggested a physiologic variation of the CRP during the first few days of life limiting the use of single values. Gestational age influences CRP kinetics, with preterm infants having a lower and shorter CRP response compared with healthy term infants **(Chiesa *et al.*, 2011; Hofer *et al.*, 2011)**. Studies suggest that CRP is best used as part of a group of ancillary diagnostic tests to help determine if an infant has infection, rather than as a single test. Furthermore, elevated CRP levels are seen in infection, in autoimmune disease, in surgery, meconium aspiration and recent vaccination. Also, the CRP values do not rise significantly until almost 14-48 hr after the onset of infection **(Koksal *et al.*, 2007; *Naher et al.*, 2011).**

One of the newer acute phase markers of infection is **Procalcitonin** (**PCT**), the prohormone of calcitonin, which occurs in very low concentrations in the serum of healthy people **(Ballot *et al.*, 2004).** PCT is claimed to be more specific for bacterial infections than viral infections, but it is not universally accepted as an improved diagnostic assay of infection

(Davis *et al.*, 2006). In the literature diagnostic accuracy of PCT appears to be superior to that of CRP **(Hoffmann, 2009)**.

Although PCT is reasonably predictive of neonatal sepsis, it is not sufficiently reliable to be used as a sole marker in evaluation of neonatal sepsis **(Ballot *et al.*, 2004; Koksal *et al.*, 2007).** Tissue release of PCT increases with infection, making it a potential marker for early detection of sepsis. PCT differs from CRP, in that PCT levels increase more rapidly and may be more useful for detection of EOS **(Auriti *et al.*, 2012)** ,in a multicenter, prospective observational study of 762 neonates, showed a significant increase in the median value of PCT level in neonates with sepsis compared with those without sepsis (3.58 vs 0.49 ng/mL; P<.001). In addition, a cutoff value of 2.4 ng/mL was suggested as the most accurate level for differentiation of sepsis in neonates regardless of gestational age, with a sensitivity of 62% and a specificity of 84%. A meta-analysis of 16 studies (1959 neonates), showed that PCT had a pooled sensitivity and specificity of 81% and 79% respectively **(Vouloumanou *et al.*, 2011).**

Indeed, bacteriologic results need time and may be negative in newborns. Besides, it is impractical to obtain blood sample for serial blood culture from infants (**Naher *et al.*, 2011).** Therefore, new laboratory methods for early diagnosis of the diseases, evaluation of prognosis and treatment efficiency are needed.

Moreover, PCT has been proposed as a marker of bacterial sepsis in critically ill patients. PCT is a precursor of calcitonin and a 116 amino acids protein. In contrast to calcitonin that has a short half-life of 10 min; PCT has a much longer half-life as 25-30 hr **(Sucilathangam *et al.*, 2012).** In healthy persons, PCT levels are barely detectable **(Altunhan *et al.*, 2011).** Although, the exact sites of production of PCT in sepsis have not been identified, monocytes and hepatic cells are believed to be potential sources **(Stocker *et al.*, 2010),** and bacterial lipopolysaccharide (LPS) has been shown to be a

potent inducer of PCT release into the systemic circulation **(Vouloumanou *et al.*, 2011).** PCT level increases more rapidly in neonatal infection than acute phase C-reactive protein (CRP) and its level also decreases more rapidly after successful treatment, PCT level increases 3-4 hours after exposure to bacterial agents and within 6 hours, it reaches to maximum level, while CRP increase requires 12-18 hours **(Adib *et al.*, 2012).**

Mannose-binding lectin (MBL) is a plasma protein, primarily produced by the liver, with an important role in the innate immune defense. MBL activates the lectin pathway of the complement system, increasing opsonization and enhancing phagocytosis **(Neth *et al.*, 2000).** Genetic polymorphisms in the MBL gene have been associated with an increased risk of sepsis. In a recent study in which MBL levels were measured in 93 neonates, development of sepsis was associated with lower levels of MBL and with the presence of BB genotype in exon 1 of the MBL gene. MBL remains a research tool with further studies needed to confirm diagnostic utility **(Ozkan *et al.*, 2012).**

1.2.8.2.5 Cytokine Profile

Interleukin-6 (IL-6), a pro-inflammatory cytokine, has been shown to be useful in the early diagnosis of neonatal infection **(Benitz, 2010).** The latter study also suggested that the best prediction of neonatal sepsis was obtained from the combined use of IL-6 and CRP, that is IL-6 initially and CRP at 24hours. However, IL-6 is the main stimulus involved in the induction of the acute phase reaction and enhancement of CRP synthesis. Hence, serial measurement of CRP , which is much simpler to do and more cost effective, is probably of more value than expensive and time consuming determination of IL-6 plasma concentration in the evaluation of neonatal sepsis **(Cernada *et al.*, 2012) .** Multiple cytokines have been studied for diagnosis of neonatal sepsis including IL-6, IL-8, IL-10, and TNF-alpha. IL-6 and IL-8 increase very rapidly with bacterial invasion, but they promptly normalize in serum

levels (within the first 24 hours), limiting their ability to be used as clinical markers. TNF-alpha has not shown to have high sensitivity, but the ratio of IL-10 and TNF-alpha has been used for diagnosis of LOS in VLBW neonates with some success **(Ng *et al.*, 2003; Resch *et al.*, 2003).**

Furthermore, granulocyte colony-stimulating factor (GCSF), a mediator produced by bone marrow, facilitates proliferation and differentiation of neutrophils, and has been proposed to be a reliable infection marker for early diagnosis of neonatal sepsis. A concentration ≥ 200 pg/ml has a high sensitivity (95%), and negative predictive value (99%) for predicting early onset neonatal bacterial and fungal infections **(Carr *et al.*, 2009).** Also tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that stimulates IL6 production and has a broad spectrum of biological actions on several types of target cells, both immune and non-immune. Newborns developing early-onset infections are born with higher TNF-α concentrations than non-infected infants **(Carr *et al.*, 2009).**

Other markers studied over the last few years include adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1, Eselectin, L-selectin), complement activation products (C3a-desArg, C3bBbP, SC5b-9), and IL-1alpha, IL-1beta, and IL-receptor antagonist (IL1RA), which have been found to significantly increase during sepsis, though these findings require further evaluation for clinical application in the diagnosis of newborns' infections **(Cernada *et al.*, 2012).** It has been demonstrated that median IL-6 and TNF-α levels were significantly higher in groups of patients with a diagnosis of clinical sepsis than in the controls. The optimal cutoff point was 32 pg /ml for IL-6 and 12 pg /ml for TNF-α. The combination of TNF-α and IL-6 provided a sensitivity of 98.5%, and it is a highly sensitive marker of sepsis in the immediate postnatal period **(Cernada *et al.*, 2012).** Therefore, **e**valuating a combination of cytokine profiles may

increase the likelihood of identifying infection more than single measurements.

1.2.8.2.6 Cell surface markers

Numerous cell surface antigens have been studied as potentially promising biomarkers of infection, including CD11b, CD69 and CD64 **(Ng and Lam, 2006).** Neutrophil CD64 and Neutrophil/Monocyte CD11B the specific markers, neutrophil CD64 and neutrophil/monocyte CD11B, are cell surface antigens whose production increases after activation of leukocytes by bacteria and therefore can potentially be used for diagnosis of neonatal sepsis. Their upregulation precedes that of CRP, suggesting potential use in EOS. A study conducted by **Genel *et al.*, 2012** showed that CD64 had a sensitivity and specificity to accurately identify neonatal sepsis of 81% and 77% respectively, with an NPV of 75%. Similarly, CD11b had a sensitivity and specificity of 66% and 71%. Cost and processing time may be barriers to use of these markers in clinical practice. Neutrophil CD11b and CD64 appear to be promising markers for the diagnosis of early- and late-onset infections. Other surface markers that have been investigated in different studies include CD69 on peripheral T and B lymphocytes may also have a role **(Lekkou *et al.*, 2004).** CD69, a protein expressed early on the surface of stimulated T cells, is used as a marker of activation and correlates with antigen specific proliferative response of lymphocytes **(Marzio *et al.*, 1999).** CD69 is an activation marker upregulated upon stimulation of NK cells. We recently observed that the expression of TLR2, TLR4, and the early activation marker CD69 was upregulated in NK cells of septic patients compared to those of healthy volunteers, suggesting blood NK cells are activated during the early stages of sepsis. Interestingly, the expression of CD69 was even higher for SIRS patients, who have sterile infl ammation, suggesting that CD69 might be a marker of acute inflammation rather than infection **(Guimaraes *et al.*, 2012).**

Advantages of using CD64 and CD69 as a diagnostic markers analyzed by flowcytometry which represented in to:-

- The flow cytometric analysis can be performed with minimal blood volume (50 µL of whole blood).
- The result is available within 4 hours after the specimen reaches the laboratory.
- The measurement can be quantitative and thus enables comparison of results among different centers.
- Unlike cytokines, which are usually assayed in patches, the measurement of cell surface antigens is performed on an ad hoc basis.
- The persistent expression of CD 64 for at least 24 hours gives the marker a wide diagnostic window; and
- The very favorable diagnostic utilities in different studies render CD64 one of the best infection markers for the identification of early- and late-onset sepsis **(Ng *et al*.; 2004).**

1.2.8.2.7 Flowcytometry

Flowcytometry is a technique of measuring physical and chemical properties of live cells or other biological particles as they pass in a fluid, single-cell stream through a measuring apparatus. In the most common scenario, one or more lasers interrogate each particle and, at a minimum, the system measures the degree and direction of scattered light - indicators of the particle's size, shape and structure. If particles have been stained with one or more fluorescent dyes - known as fluorochromes, the light source excites these dyes to provide additional biological information about each particle, such as metabolic activity, DNA content and the presence of specific surface and intracellular markers. Precise optical and electronic elements collect the fluorescent pulses and scattered light, convert them into digital values and

send them to a computer for analysis. Some flow cytometers are also equipped to identify and sort user-specified particles into collection vessels. High-performance cell sorters can routinely reach rates of 70,000 cells per second **(Shapiro, 2003).**

The unique power of flow cytometers is that they can rapidly and quantitatively measure multiple simultaneous parameters on individual live cells and then isolate cells of interest. Additionally, the sensitivity and throughput rates achievable by high-performance commercial instruments enable detection of extremely rare populations and events (frequencies below 10-6), such as stem cells, dendritic cells, antigen specific T cells and genetic transfectants **(Ashcroft and Lopez, 2000).**

As a result, applications for flow cytometers continue to grow. In addition to traditional immunology and pathology applications involving particles such as lymphocytes, macrophages, monocytes and tumor cells, flow cytometers are widely used in conjunction with fluorescence-based protein reporters, such as green fluorescent protein (GFP). In this arena, flow cytometers can monitor both transfection efficiency and protein expression levels **(Mao *et al.*, 2001; Rosen *et al.*, 2002).** They also can detect fluorescence resonance energy transfer (FRET), which provides information about molecular interactions, protein structure and DNA sequence **(Roncarati *et al.*, 2002; Scheinfeld *et al.*, 2002).** Interest is growing in the use of flow cytometers to screen cell- or bead-based combinatorial libraries **(Daugherty *et al.*, 2000).**

Increasingly, flowcytometric assays are used to detect molecules that bind to a target protein *in vitro* or exhibit a particular activity in a cell- based assay. Flow cytometry also enables screening of protein libraries expressed in cells or displayed on the surface of bacteria or beads. A flow cytometer, for instance, can detect modulation of a signal transduction pathway by a

particular small molecule and identify proteins with a particular binding specificity, enzymatic activity, expression level and stability. With any application, cells exhibiting desired activity profiles can be sorted into test tubes, multiwell plates or microscope slides **(Lorens *et al.*, 2000; Wentzel *et al.*, 2001).**

At purity rates greater than 99%, these sorted particles are then available for further studies such as PCR analysis or *in situ* hybridization, and the cells are fully functional for long-term *ex vivo* culture, expansion, transplantation or other subsequent applications **(Battye *et al.*, 2000).** Ongoing development efforts in the flow cytometry industry are aimed at automation and laboratory integration. Input/output robotics, pushbutton operation and automated sample preparation will increase throughput rates and make the technology more accessible to a wider user base, as new fluorescent dyes and creative screening approaches expand applications into the proteomic arena. Eventually, software advances will seamlessly network instruments into comprehensive analytical and diagnostic systems, and the industry may marry its technology with imaging and microfluidics **(Shapiro, 2003).**

A suspension of stained cells is presented in single file to an intense light source for measurement of inherent cellular features. On instruments with sort capability, as each cell is characterized, it may be separated from the main population by electrostatic droplet deflection **(Figure 1-3).**

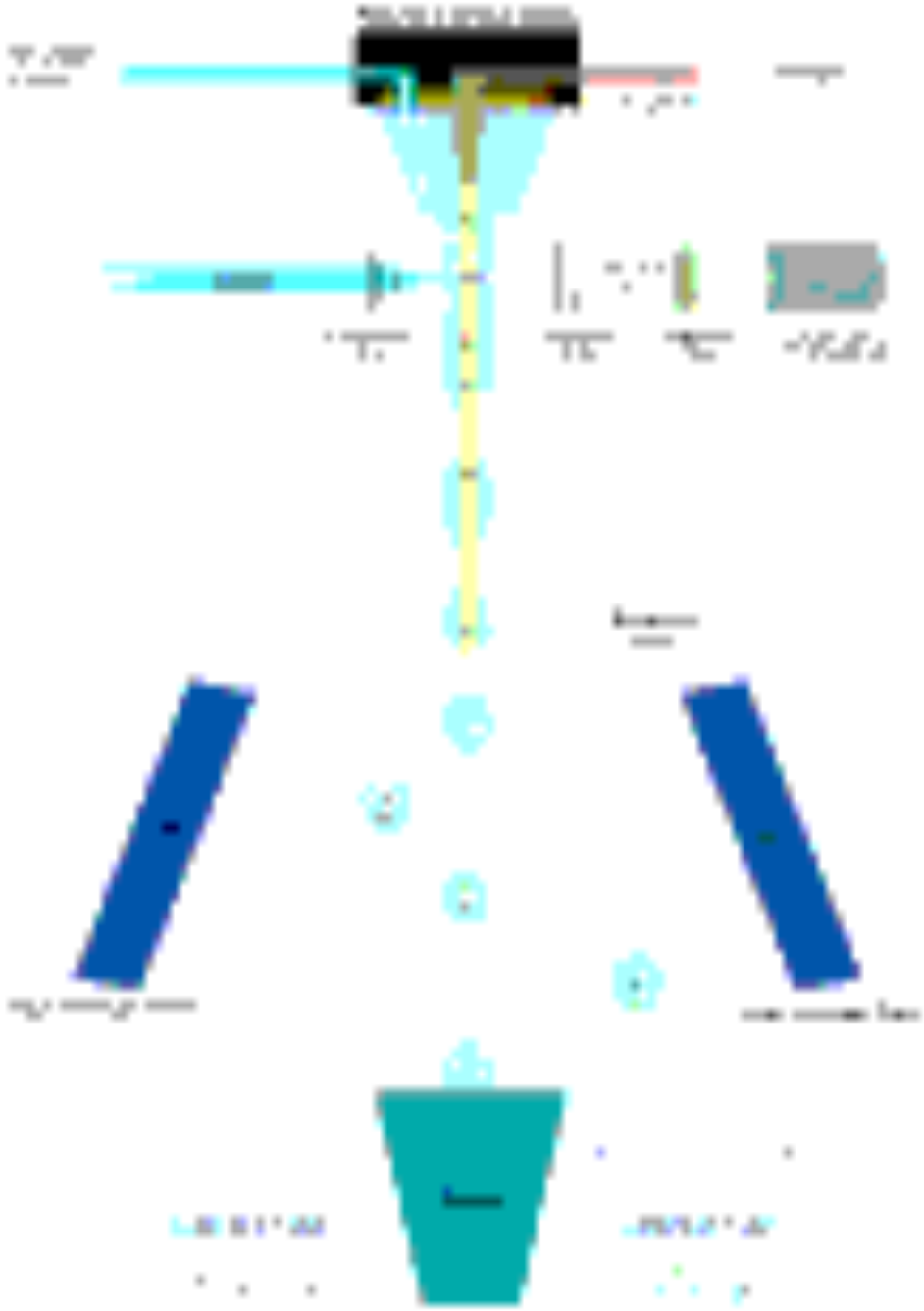

Figure (1-3): Fluorescence Activated Sorting Flow Cytometer Operation. (Shapiro, 2003).

1.2.8.2.8 Molecular Detection of Neonatal Sepsis Pathogens

Polymerase chain reaction (PCR) analysis relies on the fact that the bacteria specific 16S rRNA gene is highly conserved in all bacterial genomes,

and so it can be useful for identification of bacteria in clinical samples. Amplification targeting of this 16S rRNA gene is a potentially valuable clinical tool in samples with low copy numbers of bacterial DNA, as this gene is present in 1 to more than 10 copies in all bacterial genomes. The gene also has a number of divergent regions nested within it, so PCR can be targeted for species-specific detection of bacteria in clinical samples. This technology has also been reported to be a very sensitive (100%), and rapid method for detecting potential pathogens in amniotic fluid, commonly involved in the pathogenesis of pre-term labor and adverse neonatal outcomes **(Maiwald, 2004; Esparcia *et al.*, 2011).**

However, the performance of broad-range PCR analysis at a level of high analytical sensitivity is complex, and remains one of the most challenging PCR applications in the diagnostic laboratories. For instance, as 16S rRNA gene amplification targets all bacterial species, small amounts of inherent residual DNA present in the reagents may be co-amplified, resulting in false positivity. Methods for the removal of potential background contaminations include long wave UV light gamma irradiation DNase, restriction endonuclease digestion, ultra filtration, and low DNA polymerases.

However, many of these methods result in a reduced sensitivity in detecting target DNA, with a detection limit range of 10^3–10^4 copies/ml, which is not ideal for diagnosing sepsis in clinical settings. It was found that a combination of pre-PCR culture with the use of AmpliTaq Low DNA achieves an acceptable level of sensitivity (5–50 copies/ml in a turnaround time of eight hours) for the real time amplification of bacteria in blood samples, without the need to remove any inherent DNA contamination. Detection by PCR does not yield the antimicrobial sensitivity pattern of the pathogen. Early exclusion of bacterial infection could help to reduce overuse of antibiotics. It is predicted that eventually real time PCR combined with

DNA Micro Array technology will allow not only identification of the organism but also the antimicrobial sensitivity pattern, which is so critical to clinical care. It has been revealed from an Indian study that PCR is useful, and superior to blood culture for early diagnosis of sepsis in neonates with 100% sensitivity and 100% specificity. Once available in most tertiary centers, PCR can help in early and accurate diagnosis **(Straka *et al.*, 2004; Kasper *et al.*, 2013).**

1.2.8.2.9 Association Between CD14/C-159T Gene Polymorphism and Risk of Neonatal Sesis

The CD14 pattern recognition receptor has a unique ability to discriminate nonself lipoglycans of infectious pathogens— first and foremost, LPS—from noninfectious self. At the molecular level, CD14 acts by transferring LPS and other bacterial ligands from circulating LPS-binding protein to the Toll-like receptor 4/MD-2 signaling complex (**Leung *et al.*, 2005; Klein *et al.*, 2003**).

Engagement of this complex results in the activation of innate host defense mechanisms such as release of inflammatory cytokines, and in up-regulation of costimulatory molecules, thus providing cues that are essential to directing adaptive immune responses (**Jacque *et al.*, 2006; Zhao and Bracken, 2011).**

Although *CD14* exists as a single-copy gene, CD14 protein is found in two distinct forms: a 50- to 55-kDa glycosylphosphatidylinositol- anchored membrane molecule, membrane CD14 (mCD14) expressed primarily on the surface of monocyte/macrophages and neutrophils and a soluble form lacking the glycosylphosphatidylinositol anchor. Soluble CD14 (sCD14) appears to derive from monocytes, as well as the liver. And is found in normal serum at microgram concentrations. Both mCD14 and sCD14 are critical for LPS-dependent signal transduction. LPS-responsive cells that lack mCD14, such as

endothelial cells, epithelial cells, and astrocytes, become sensitive to low concentrations of LPS in the presence of sCD14 **(Zhao and Bracken, 2011).**

Regulation of *CD14* gene expression appears to be important in several disease states. Increased serum levels of sCD14 are associated with high mortality in Gram-negative septic shock. The human CD14 gene lies on the long arm of chromosome 5 (5q31.1). Antigenic molecules on the surface of both Gram-positive (peptidoglycans and lipoteichoic acid) and Gram-negative (lipopolysaccharide, LPS) bacteria can be detected by many pattern recognition receptors that include Toll-like and CD14 receptors. and CD14 binds LPS-binding protein and apoptotic cells **(Zhao and Bracken, 2011).**

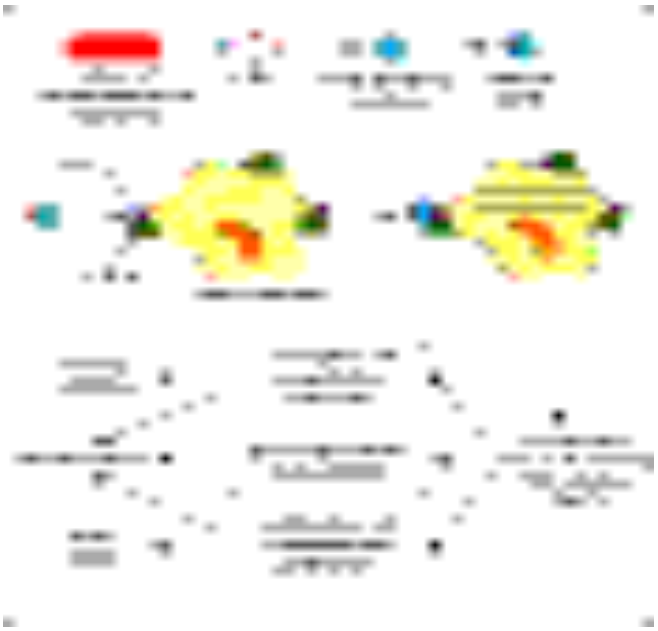

Figure (1-4): Physiologic Action of Lipopolysaccharide (LPS) from the Gram-Negative Cell Wall (Jeffrey and Henri, 2006).

1.2.9 Advances in Prevention

1.2.9.1 Before Delivery

Maternal immunization is an important method of providing neonates with appropriate antibodies, as soon as they are born .This approach, in comparison with other approaches, is less sensitive to obstacles in accessing

the health care system. Examples of successful interventions include maternal tetanus toxoid, and influenza immunizations. Studies of maternal immunization with *S. agalactiae* type III conjugate vaccine have demonstrated excellent placental transfer and persistence of protective levels in 2-month-old infants **(Healy and Baker, 2007).** Encouraging results are also emerging from studies of maternal immunization with pneumococcal polysaccharide and conjugate vaccines **(Vergnano *et al.*, 2005).** The vaccines all have excellent safety profiles. However, barriers to maternal immunization include: liability issues for vaccine manufacturers in developed countries; education of the public and health care providers regarding the benefits of maternal immunization; and poor ascertainment of data from low-income countries **(Healy and Baker, 2007).** The development of group B streptococcal (GBS) vaccines has been promising. The vaccines target conserved surface antigenic proteins, such as the Sip protein located on the cell surface, or immunogenic proteins from GBS pili. If a protective immune response is achieved, it would inhibit GBS adhesion to host tissue and prevent trans-epithelial migration. Although they are not yet commercially available, several vaccines are close to being released and will hopefully prove to be efficacious in decreasing the rates of EOS and LOS, caused by GBS **(Melin, 2011).**

1.2.9.2 During Labor and Delivery

There is strong evidence that clean delivery practices and hand washing during delivery reduces rates of neonatal sepsis both at home and in health facility settings **(Bhutta *et al.*, 2005).** Interventions to improve hand washing rates have been remarkably successful in research settings **(Bhutta *et al.*, 2005).** New studies from Malawi and Nepal indicate that maternal antisepsis interventions such as vaginal chlorhexidine during labor may have a significant impact on rates of neonatal mortality and sepsis in developing countries **(McClure *et al.*, 2007).** Intrapartum antibiotic prophylaxis has been

highly effective in reducing both early-onset neonatal bacterial and maternal sepsis in developed countries**.** Risk factors for early-onset neonatal bacterial sepsis in low-income settings are probably similar to resource-rich settings, but have not been evaluated in the context of high rates of maternal under nutrition, anemia, HIV, and malaria **(Ohlsson and Shah, 2009)**.

1.2.9.3. After Delivery

There is also strong evidence that hand washing by health care providers after delivery can reduce neonatal sepsis and infection rates, especially in hospitals **(Bhutta *et al.*, 2005).** Umbilical stump chlorhexidine cleansing has recently been shown to substantially reduce neonatal deaths in Nepal **(Mullany *et al.*, 2006).** There is emerging evidence that neonatal skin antisepsis preparations, such as sunflower seed oil provides cheap, safe, and effective protection against nosocomial infections in hospitalized pre-term neonates, and infants in studies in South Asia. **(Mullany *et al.*, 2006).**

Neonatal immunization has long been considered an important method of reducing neonatal infections. However, the response varies according to the antigen **(Levy, 2007).** BCG, polio, and hepatitis B vaccines are highly immunogenic when given at birth **(Siegrist, 2003).** However, maternal antibodies interfere with a neonate's response to measles vaccine when administered less than six months of age. Protein antigen vaccines (e.g. pertussis and tetanus toxoid) given at birth, have been shown to produce poor responses compared to the same antigen given at two months of age, and are associated with later tolerance **(Siegrist, 2003).**

Studies also indicate that *S. agalactiae* and *Streptococcus pneumoniae* vaccines are both likely to be ineffective when given in the neonatal period **(Levy, 2007).** Breast milk contains secretory IgA, lysozymes, white blood cells, and lactoferrin, and has been shown to encourage the growth of healthy

lactobacilli and reduce the growth of *E. coli* and other Gram-negative pathogenic bacteria **(Levy, 2007).**

Neonatal micronutrient supplementation trials have focused on vitamin A supplementation. Older studies have shown significant reductions in respiratory disease in low-birth-weight infants after the administration of parenteral vitamin-A **(Darlow and Graham, 2007).** More recently, trials of vitamin A supplementation in newborns have shown encouraging reductions in neonatal mortality, and more trials are underway **(Gogia and Sachdev, 2009).**

2. Materials and Methods

2.1. Patients

2.1.1. Study Design

This prospective study was conducted on 75 neonates who were admitted to Maternity and Childhood Teaching Hospital at Neonatal Intensive Care Unit (NICU) at AL-Diwaniya city, and 75 healthy neonates as a (control group) in the period from 1st April to end December 2015. They were evaluated for neonatal sepsis with sepsis screen tests, Blood culture, flowcytometry analysis, procalcitonin test and subjected to PCR based technique for CD14 polymorphism gene. Members of all groups were informed and instructed about the aims of the study and their verbal acceptance was obtained before taking samples.

2.1.2. Selection Criteria for Subjects

2.1 2.1. Inclusion Criteria:

1. Neonates of both sexes were included in this study.

2. Any suspected case of neonatal sepsis with maternal risk factors for sepsis e.g. prolonged labor, premature rupture of membrane (PROM) or prolonged PROM >18 hours, maternal intrapartum fever, urinary tract infection, chorioamnionitis and sepsis related clinical signs: (temperature instability, apnea, need for supplemental oxygen, bradycardia, tachycardia, hypotension /hypoperfusion, feeding intolerance, abdominal distension). Initially, Apgar score was used to indicate the infant condition in the first and fifth minutes after birth that includes: (appearance, heart rate, muscle tone, respiratory effort).

2.1.2.2. Exclusion Criteria:

There were administration of antibiotic therapy prior to admission, birth asphyxia, hyaline membrane disease, documented necrotizing enterocolitis (NEC), aspiration syndromes, laboratory finding suggestive of inborn error of metabolism, infant of diabetic mother, and congenital anomalies including congenital heart disease.

2.1.3. Clinical Assessment of Patients

The clinical criteria taken as indicative of sepsis were:

I. Maternal risk factor such as fever, prolonged rupture of amniotic membrane >24 hr.

II. Neonatal history: low birth weight (<2500 grams), premature birth (<37 weeks) and full term neonates.

III. Signs and symptoms of sepsis: feeding intolerance, lethargy, temperature instability, apnea, respiratory distress, poor perfusion, seizures, tachypnea, bradycardia, abdominal distension or vomits.

Neonates who had any features from **I and II** associated with two or more clinical symptoms of sepsis would warrant a septic screen ***(Lachowska & Gajewska, 2004).***

2.1.4. Collection of Samples:

Blood samples were collected from neonates included in present study by venipuncture. A total of 5 milliliters of blood were drawn aseptically using the following procedure: Gloves were worn and extraction sites scrubbed in an expanding circular motion first with 70% isopropyl alcohol which was allowed to dry, then with iodine tincture (iodine in alcohol), allowed to dry before needle insertion ***(Barenfanger, 2004).***

Before initiation of antibiotic therapy in infants suspected of sepsis, blood samples for blood culture (1 ml), flowcytometry analysis (1 ml), PCT

measurements (2 ml) and genetic polymorphism (1 ml) were obtained by peripheral venous puncture.

The study design is illustrated in the flow chart below, **figure (2-1).**

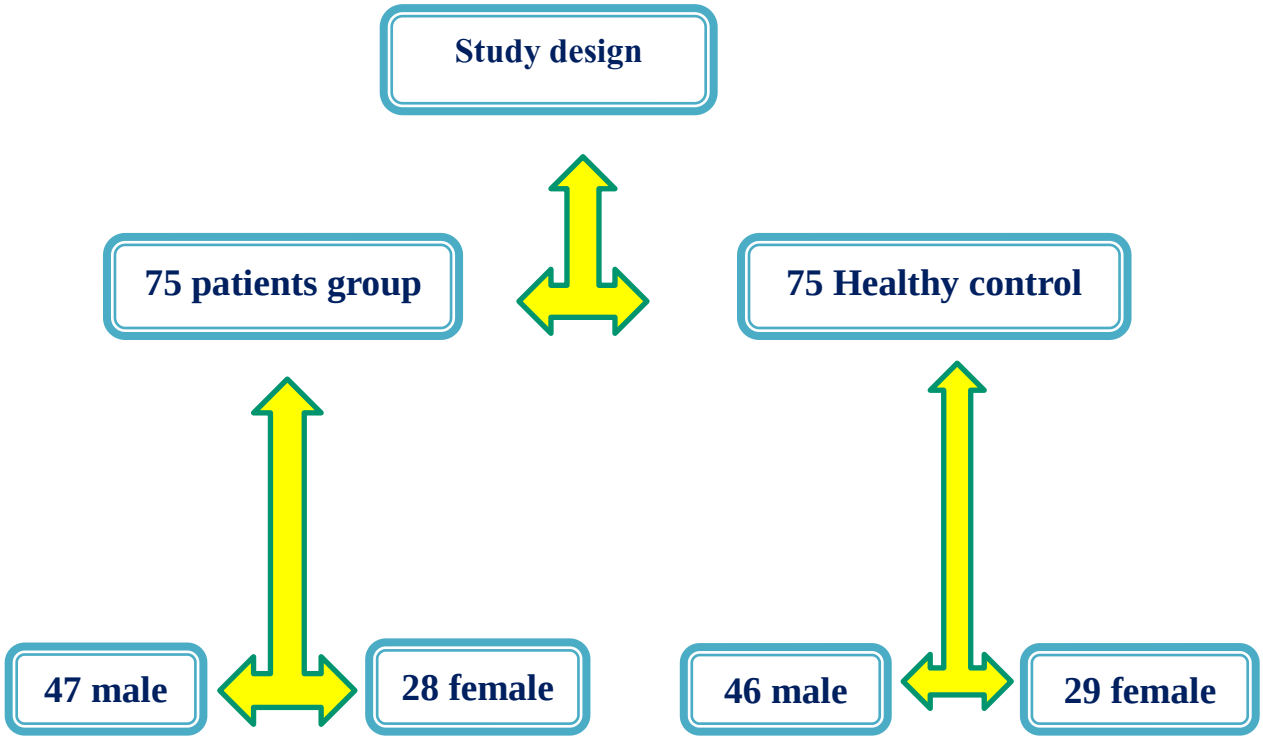

Figure (2-1): A Flow Chart Illustrating the study design.

All patients' candidates were assessed and diagnosed by consultant pediatricians and a diagnosis of neonatal sepsis was established depending according to screening criteria for sepsis

The sample collection is illustrated in the flow chart below, **figure (2-2).**

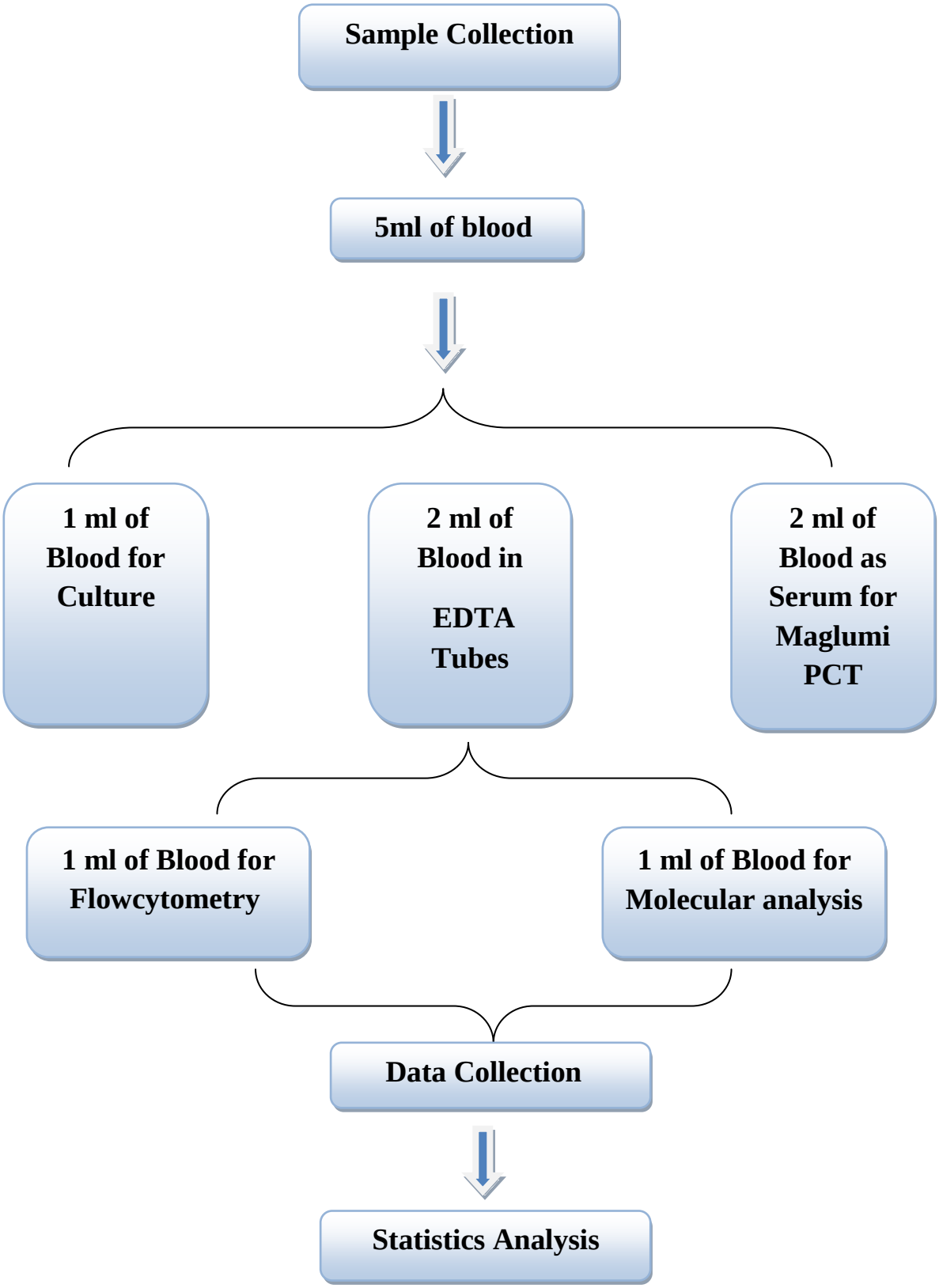
Sample Collection
5ml of blood
1 ml of Blood for Culture
2 ml of Blood in EDTA Tubes
2 ml of Blood as Serum for Maglumi PCT
1 ml of Blood for Flowcytometry
1 ml of Blood for Molecular analysis
Data Collection
Statistics Analysis

Figure (2-2): A Flow Chart Illustrating the sample collection.

Blood samples were immediately transported to the laboratory, and they were processed upon arrival; blood samples inoculated immediately and aseptically into blood culture bottle, serum was obtained from blood by clotting then centrifugation and stored at -20°C for (PCT) estimation, the rest of blood sample was taken in 2 tubes containing EDTA one to detect the expression of CD64 on neutrophils and CD69 on lymphocytes by flowcytometry and the other one for PCR techniques to analysis of *SNP* of *CD14 / C-159T* gene, Finally, according to clinical symptoms of sepsis, microbiologic and laboratory results, neonates classified in to three groups:

Group I: Proven Sepsis

Consisted of 30 newborn infants (8 fullterm and 22 preterm) with obvious clinical signs of infection and positive bacterial blood culture. Their gestational age ranged from (36- 39 wks.), their age ranged from (1-28 days) ,their weight ranged between (1-3.5 kg), their sex are 19 males, 11 females. 12 of them were born by caesarian section, 18 were born by normal vaginal delivery.

Group II: Probable Sepsis

Included 45 neonates (14 fullterm and 31 preterm) with negative bacterial culture but have three or more clinical signs of infection such as fever, temperature instability, tachycardia, tachypnea, abdominal distention, respiratory distress, seizures, apnea, cyanosis, oliguria, gastrointestinal bleeding or petechiae. Their gestational age ranged from (36-39 wks), their age ranged from (1-28 days), their weight ranged from (1– 3.5 kg) 28 was males and 17 females, 25 of them were born by normal vaginal delivery and 20 by caesarian section.

Group III: Control Group

Included 75 neonates were enrolled in this study as a control group during their attendance for usual routine assessment, all considered as healthy neonates with matched gestational age, age and weight as control group (29 female and 46 male). They were under investigation because of a suspicion of different diseases, but all had normal results and no illness was subsequently.

2.2. Materials

2.2.1. Equipments and Instruments

Table (2-1): Instrument & Equipment's with their Remarks.

Equipment & Instrument	Company
Autoclave	Tuttnauer (USA)
Digital camera	Samsung/ china
Disposable syringe 5 ml	Sterile EO. / China
Eppendorf tubes	Biobasic/ Canada
Exispin centrifuge	Bioneer/ Korea
Flowcytometry	Partec /Germany
Gel electrophoresis	Bioneer/ Korea
High Speed Cold centrifuge	Eppendorf /Germany
Incubator	Mammert/Germany
Maglumi 800	Snibe/UK
Micropipettes 5-50, 0.5-10, 100-1000µl	CYAN/ Belgium
Oven	Mammert/Germany
Refrigerator	Concord /Lebanon
Sensitive Balance	Sartorius/Germany
Sterile test tube	Superestar/ India
Thermocycler PCR	Mygene /Bioneer
UV Transilluminator	ATTA/ Korea

Vortex	CYAN/ Belgium
Water Bath	Mammert/Germany

2.2.2. Reagents and Stains

Table (2-2): The Reagents and Stains Used in this Work with Manufacturer Company and Country

Reagents	**Company**
API- 20E	(BioMérieux- France)
API- staph	(BioMérieux- France)
Gram stain kit	(HiMedia- India)
Hydrogen peroxide (3% H2O2)	(HiMedia- India)
Oxidase discs	(HiMedia- India)

2.2.3. Culture Media

Table (2-3): The Culture Media Used in this Work with Manufacturer Company and Country

Culture Media	**Company**
Blood agar	(HiMedia- India)
Blood culture bottles(BHI)	(HiMedia- India)
Chocolate agar	(HiMedia- India)
MacConkey agar	(HiMedia- India)

Manitol Salt agar	(HiMedia- India)

2.3. Methods

2.3.1. Hematological Tests

Total leukocyte count and absolute neutrophil count were done for each neonates .Abnormal values of these tests (white blood cell count < 4000 or > 15000 mm^3, neutrophil < 1000mm^3) were considered as supportive for diagnosis of sepsis ***(Philip and Hewitt, 2000).***

2.3.2. Blood Culture

A total of 1 ml. of blood was obtained from two different sit of selected neonates with suspected septicemia by venipuncture under sterile condition, each 0.5ml. Of blood inoculated immediately into 60 ml. of brain –heart infusion (BHI) broth contain 0.05% sodium polyanethol sulphonate. The rubber diaphragm of the culture bottle was likewise scrubbed with povidone-iodine solution prior to needle insertion. The cultures for aerobic bacteria were incubated at 37^{o}C for 7 days under aerobic conditions, and inspected visually every morning. Subcultures were performed 24 hours after inoculation, the second on third day and a final on the seventh day. Samples were removed from each culture bottle aseptically with a sterile 1 ml syringe, and one drop was subcultured onto 5% blood agar, Chocolate agar and MacConkey agar plates, which were then incubated at 37^{o}C for 24 hours. One blood agar plate, along with the Chocolate agar, was incubated in 5%CO **2** ***(Vandepitte, et.al. 2003).***

After 18 hours plates were observed for growth and colonies were processed according to standard Microbiological procedures. ***(Bailey & Scott's, 2007; Mackie & McCartney, 2007).***

The growth was identified by colony characteristics, gram stain and standard biochemical tests in Bailey and scott's Diagnostic Microbiology. Depending on microscopic examination, organisms were divided into gram positive and gram negative organisms are further investigated by the Api staph kit or Api 20 kit, and biochemical tests accordingly. Cultures which did not yield any growth following three subculture were reported negative at the end of 7 days.

2.3.3. Serological Test

- ***Maglumi PCT (CLIA) Kit***

The kit has been designed for the quantitative determination of procalcitonin (PCT) in human serum, plasma and whole blood. The method can be used for samples over the range of 0.13-100.0ng/ml. The test has to be performed on the Fully-auto chemiluminescence immunoassay (CLIA) analyzer Maglumi (Including Maglumi 600,Maglumi 1000,Maglumi 1000 Plus, Maglumi 2000,Maglumi 2000 Plus,Maglumi 3000 and Maglumi 4000).

- ***Principle of the Test***

Sandwich immunoluminometric assay:

Use an anti-PCT monoclonal antibody to label N-4-aminobutl-N-ethylisoluminol (ABEI), and use another monoclonal antibody to label magnetic microbeads. Sample, Calibrator or Control with ABEI Label, and magnetic microbeads coated with monoclonal antibody are mixed thoroughly and incubated at 37℃ to form a sandwich then cycle washing for 1 time. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated. The light signal is measured by a

photomultiplier as Relative light unite (RLU) within 3 seconds and is proportional to the concentration of PCT present in samples.

- ***Kit Components (Material Supplies)***

Table (2-4): Maglumi PCT (CLIA) kit Used in this Study:

Reagent Integral for 100 Determinations	
10.5ml	**ABEI Label:** anti-PCT monoclonal antibody labeled ABEI, contains BSA, 0.2%NaN3
2.5ml	**Calibrator High:** bovine serum, 0.2%NaN3
2.5ml	**Calibrator Low:** bovine serum, 0.2%NaN3
25 ml	**Diluent:** buffer, contains BSA, 0.2%NaN3
2.5ml	**Nano magnetic microbeads:** TRIS buffer, 1.2% (W/V), 0.2%NaN3, coated with anti-PCT monoclonal antibody.
All reagents are provided ready-to-use.	
Reagent Vials in kit box : Internal Quality Control	
2.0ml	**Level 1:** containing BSA, 0.2%NaN3
2.0ml	**Level 2:** containing BSA, 0.2%NaN3
Target value refer to Quality Control Information date sheet	

Internal quality control is only applicable with Maglumi system. Instructions for use and target value refer to Quality Control Information

date sheet. User needs to judge results with their own standards and knowledge.

- ***Test Procedure***

To ensure proper test performance, strictly adhere to the operating instructions of the Fully-auto chemiluminescence immunoassay (CLIA) analyzer Maglumi. Each test parameter is identified via a RFID tag on the Reagent Integral. For further information please refer to the Fully-auto chemiluminescence immunoassay (CLIA) analyzer Maglumi Operating Instructions.

40µl	Sample, calibrator
+80µl	ABEI Label
+20µl	Nano magnetic microbeads
15 min	Incubation
400µl	Cycle washing
3s	Measurement

- ***Calculation of Results***

The analyzer automatically calculates the PCT concentration in each sample by means of a calibration curve which is generated by a 2-point calibration master curve procedure. The results are expressed in ng/ml. For further information please refer to the Fully-auto chemiluminescence immunoassay (CLIA) analyzer Maglumi Operating Instructions.

Reference values:

Serum and plasma: <0.5ng/ml

Whole blood: <1.0ng/ml

Results may differ between laboratories due to variations in population and test method. If necessary, each laboratory should establish its own reference range.

2.3.4. Flow Cytometry

Flowcytometric analysis was done for detection of neutrophils CD64 and lymphocytes CD69 according to the **(Bain, 2010).** One ml of EDTA treated blood was freshly processed and analyzed by flowcytometry within 24 hours according to the kit manual.

2.3.4.1.Reagents of CD64 and CD69 Kits (Becton-Dickinson- USA):

200 tests consisting of:

- Reagent A(leukocyte fixation) *25 ml*
- Reagent B(erythrocyte lysing) *500ml*
- PE Mouse Anti –Human CD69 100 tests /20 ul
- FITC Mouse Anti- Human CD64 100 tests /20 ul

2.3.4.2.Procedure of Flowcytometry:

- **Antibody labeling** : Blood samples were then liquated into required tubes (code no. 04-2000) of 100 ul each and stained with 20 ul of appropriate combination of monoclonal antibodies to membrane markers (**FITC** Mouse anti-human CD64 and **PE** Mouse anti-human CD69) (Becton Dickinson Pharmingen, USA).
- Mix thoroughly. Incubate for 15 minutes in the dark at room temperature.
- **Leukocyte Fixation:** A100 ul of **reagent A (leukocyte fixation)** was add and mixed thoroughly and incubated for 10 minutes in the dark.
- **Eeythrocyte lysis:** A 2.5 ml of **reagent B (erythrocyte lysing)** was add and shake gentle and incubated for 20 minutes in the dark.
- **Analysis:** The sample was analysis on the flow cytometer (Partec ,Cylyse,Germany).

- Neutrophils and lymphocytes phenotyping was done by gating according to forward scatter FSC (size) and side scatter SSC (granularity) histogram.
- Results were expressed as percentages of cells positive for CD64 and CD69.
- Neutrophils were electronically selected on the basis of their side – and forward –scatter characteristics and 10.000 cells were analyzed in each sample. Results were expressed as a percentage of positive cells. Considering the percentage of ≥ 20% as positive result while the percentage of < 20% as negative result according to **(Bain, 2010).**

2.3.5. Molecular Analysis of CD14/C-159T Gene Polymorphism

2.3.5.1 kits

Table (2-5): The kits Used in this Study with their Companies and Countries of Origin, Reagent Integral for 192 Reations.

Kit		Company	Country
AccuPrep®Genomic DNA extraction kit		**Bioneer**	**Korea**
Binding buffer (GC)	25ml x 2		
Binding column	100 x2 pack		
Collection 2ml tube	100 x2 pack		
Elution buffer(EL)	30 ml x2		
Proteinase K 25 mg	25 ml x2		
Wash buffer 1(W1)	40 ml x2		
Wash buffer 2(W2)	20 ml x2		

AccuPower™ PCR PreMix /	20 µl reaction	Bioneer	Korea
dNTPs (dATP, dCTP, dGTP, dTTP)	250 µM		
KCl	30 mM		
$MgCl_2$	1.5 mM		
Tris-HCl pH 9.0	10 mM		
Taq DNA polymerase	1 U		
Stabilizer and Tracking dye			

2.3.5.2 Primers

The CD14/ C-159T Polymorphism that used in this study were according to (Baladini *et* al., 1999) and these primers were provided from (Bioneer company, Korea) as following tables:

Table (2-6): The PCR Primers with their Sequence and Amplicon Size

Primer	Sequence		Amplicon
P1 primer	**F**	5' GTGCCAACAGATGAGGTTCAC 3'	497bp
	R	5' CCTCTGTGAACCCTGATCAC 3'	
P2 primer	**F**	5' CCTGAAACATCCTTCATTGC 3'	442bp
	R	5' CGCAGCGGAAATCTTCATC 3'	

2.3.5.3 Restriction Enzyme

Table (2-7): The Restriction Enzymes were Used in RFLP-PCR Assay with their Company and Country of Origin

Restriction enzymes	Target gene	Company/Country

AVAII	**CD14**	**Biolabs/ U.K**

2.3.5.4 Molecular Weight Markers

The molecular weight marker used in this work, its description and source are depicted in table (2-8) bellow**:**

Table (2-8): Molecular Weight Marker with their Remarks

DNA Ladder	Description	Source
100 bp	100-2000 base pairs. The ladder consists of 13 double strand DNA fragment with size of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000,1200, 1600,2000. The 500,1000,1200 bp present at triple the intensity of other fragments and serve as a reference. All other fragments appear with equal intensity on gel.	Bioneer (Korea)

2.3.5.5 Chemicals

Table (2-9): All the Chemicals Materials that Used in this with their Company and Country of Origin

Chemical	Company and Origin
Absolute Ethanol	Scharlau (Spain)
Agarose	BioBasic (Canada)
Ehidium Bromide	BioBasic (Canada)
Free nuclease water	Bioneer (Korea)
Isopropanol	Lobachemie/ India
TBE buffer	BioBasic (Canada)

2.3.5.6. Solution Preparation

All solutions and buffers were prepared according to **Su *et al*., (2008).**

A. TBE (1X) Buffer:

This solution was prepared by mixing 10 ml of stock TBE-10X buffer with 90 ml of distilled water, and then stored at 4^0C until used in electrophoresis.

B. DNA Loading Dye:

This buffer was prepared by dissolving and mixing 40g of sucrose and 0.25g of bromophenol blue in 100ml of sterilized distilled water then stored in sterilized flask at room temperature until used in electro-phoresis.

C. Ethidium Bromide Solution (0.5 %):

This solution was prepared by dissolving 0.25g of ethidium bromide stain in 50 ml sterilized distilled water, stored in sterilized flask, final concentration 0.5 mg / ml. It was used in electrophoresis as specific DNA stain.

2.3.5.7. Genomic DNA Extraction

Genomic DNA was extracted from frozen blood of 75 neonatal sepsis patients and 75 healthy control samples by using AccuPrep®Genomic DNA extraction kit (Bioneer. Korea), and done according to company instructions as following steps:

1. A 200µl frozen blood sample was transferred to sterile 1.5ml microcentrifuge tube, and then we added 20µl of proteinase K and mixed by vortex and incubated at 60℃ for 10 minutes.

2. After that, 200µl of Binding buffer (GC buffer) was added to all tubes samples and mixed well by vortex to achieve maximum lysis efficiency, and then all tubes were incubated at 60℃ for 10 minutes.

3. A 100µl of isopropanol was added to mixture and mixed well by pipetting, and then the lysate was carefully transferred into (GD) Binding filter column that fitted in a 2 ml collection tube, and then closed the tubes and centrifuged at 8000 rpm for 1 minute.

4. The throughout samples lysates were discarded in disposal bottle, and then 500µl Washing buffer 1 (W1) was added to each Binding filter column, and centrifuged at 8000 rpm for 1 minute.

5. The throughout washing buffer 1 was discarded in disposal bottle, and then 500µl Washing buffer 2 (W2) was added to each Binding filter column, and centrifuged at 8000 rpm for 1 minute.

6. The throughout washing buffer 2 was discarded in disposal bottle, and then the tubes were centrifuged once more at 12000 rpm for 1 minute to remove ethanol completely.

7. After that, GD Binding filter column that containing genomic DNA was transferred to sterile 1.5ml microcentrifuge tube and then 50µl of Elution buffer was added and left stand the tubes for 3 minutes at room temperature until the buffer is completely absorbed into the binding filter for elution the extracted DNA.

8. Finally, all tubes were centrifuged at 8000 rpm for 1 minute to elute DNA, and store at -20℃freezer until used.

2.3.5.8. Determination of DNA Concentration and Purity

The extracted blood genomic DNA was checked by using Nanodrop spectrophotometer (THERMO. USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system.
3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of blood genomic DNA was added to measurement.

2.3.5.9. Polymerase Chain Reaction–Restriction Fragment Length Polymorphism

Technique of PCR-RFLP was performed for detection genotype in CD14*/ C-159T* Polymorphism neonatal sepsis patient's samples as well as in healthy blood samples as control groups. Methods were carried out according to method described by **(Sharaf *et al.*, 2010)** respectively as following steps:

2.3.5.9.1. Polymerase Chain Reaction Amplification

Conventional PCR approach was done to amplify the target DNA. PCR master mix was prepared for detection of *CD14* gene by using (AccuPower PCR PreMix Kit, Bioneer / Korea) using two overlapping primers P1 and P2 region primers and this master mix done according to company instructions as following, Table(2-10).

Table (2-10): Component of PCR Master Mix Used in a Study.

PCR Master mix	Volume
DNA template	5µl
P1 or P2 Forward primer (10pmol)	1µl
P1 or P2 Reveres primer (10pmol)	1µl
PCR water	13µl
Total volume	20µl

After that, these PCR master mix component that mentioned above placed in standard AccuPower PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (Mygene. Korea).

2.3.5.9.2.Polymerase Chain Reaction–Restriction Fragment Length Polymorphism Thermocycler Program

Thermal cycling program of PCR were done for each gene independent using Thermocycler PCR Mygene /Korea, as following, Tables (2-11).

Table (2-11): Polymerase Chain Reaction Thermal Cycling Program Used in a Study

PCR step	Temp.	Time	repeat
Initial denaturation	96°C	3min.	1
Denaturation	96°C	40 sec.	38cycle

Annealing	56°C-P1 58°C-P2	40 sec.	
Extension	72°C	50 sec.	
Final extension	72°C	10 min	1
Hold	4°C	Forever	-

2.3.5.9.3. Polymerase Chain Reaction Product Analysis

The PCR products were analyzed by agarose gel electrophoresis following steps:

1- Agarose gel of 1% was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 3µL of ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well after mixing with bromophenol blue in the ratio of 3:1 and 5ul of (100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.

5- Products of PCR were visualized by using UV Transilluminator.

2.3.5.9.4. Polymerase Chain Reaction–Restriction Fragment Length Master Mix Preparation

Master mix of PCR-REFLP was prepared for CD14 gene by using AVAII restriction enzyme in restriction site (G↓G (A, T) CC) and this master mix done independent according to company instructions as following, table (2-12).

Table (2-12): Polymerase Chain Reaction–Restriction Fragment Length Master Mix Used in a Study

REFLP-PCR Master mix	Volume
AVAII (10 unit)	1 µl
Free nuclease water	7 µl
PCR product	10µl
Restriction enzyme buffer 10X	2 µl
Total volume	20 µl

After that, this master mix placed in Exispin vortex centrifuge at 3000rpm for 2 minutes, then transferred into incubation at 37°C for 1houre. After that, the AVAII restriction enzyme inactivated at 80°C for 20 min. Then PCR-RFLP product was analysis by 2% agarose gel electrophoresis methods that mention in PCR product analysis. The result of AVAII restriction enzyme could be cut into 353 and 144bp as homozygote (TT), and into 497bp, 353 and 144bp as heterozygous (CT), and 497bp still undigested as homozygote (CC).

2.4. Statistical Analysis

Data were translated into a computerized database structure. The database was examined for errors using range and logical data cleaning methods, and inconsistencies were remedied. An expert statistical advice was sought for. Statistical analyses were done using IBMSPSS version 23 computer software (Statistical Package for Social Sciences) in association with Microsoft Excel 2016.

Compliance of quantitative random variables with Gaussian curve (normal distribution) was analyzed using the Kolmogorov-Smirnov test. All the outcome (response) variables were non-normally distributed variables. Such variables can be described by median and interquartile range. The difference in median between 2 groups was assessed by non-parametric test (Mann-Whitney), while between 3 groups Kruskal-Wallis test was used. The statistical significance, direction and strength of linear correlation between 2 quantitative variables, one of which being non-normally distributed was measured by Spearman's rank linear correlation coefficient **(Sorlie, 1995)**

An estimate was considered statistically significant if its P value was less than an α level of significance of 0.05. The statistical significance of the measured OR is assessed by a special χ^2 (Chi-square) formula **(Weng *et al.*, 2006).**

In signal detection theory, a receiver operating characteristic (ROC), or simply ROC curve, is a graphical plot which illustrates the performance of a binary classifier system as its discrimination threshold is varied. It is created by plotting the fraction of true positives out of the positives (TPR = true positive rate) vs. the fraction of false positives out of the negatives (FPR = false positive rate), at various threshold settings. TPR is also known as sensitivity (also called recall in some fields), and FPR is one minus the specificity or true negative rate **(Weng *et al.*, 2006).**

3.Results

3.1. Demographic characteristic of the selected groups included in study

The results presented in this chapter were based on the analysis of 2 study groups of newly born infants. The cases group were 75 infants with a clinical diagnosis of neonatal sepsis. This group was further classified into 2 groups based on the results of blood culture. A positive bacterial culture was identified in 30 cases and these were labeled as “Proven sepsis” group. The culture negative cases were 45 in number and were thus labeled as “Probable sepsis” group. In addition, a random sample of 75 healthy control neonates was group matched to the cases group on gender, birth weight and gestational age. As shown in **Table (3-1),** females constituted around a third of all the 3 study groups. No obvious or statistically significant differences in gender composition was observed between the 3 study groups. In addition, no statistically significant differences were observed between the 3 study groups in proportion of low birth weight and preterm.

As shown in **Table (3-2),** there were no important or statistically significant differences in median birth weight, gestational age and age of neonate between the 3 study groups.

Table (3-1): Description of the 3 Study Groups by Gender, Birth Weight and Gestational Age.

	Study group						
	Proven sepsis (Culture positive)		**Probable sepsis (culture negative)**		**Healthy controls**		
	No	**%**	**No**	**%**	**No**	**%**	**P**
Gender							0.98[NS]
Male	19	63.3	28	62.2	46	61.3	
Female	11	36.7	17	37.8	29	38.7	
Total	30	100.0	45	100.0	75	100.0	
Birth weight categories (gm)							0.26[NS]
Normal birth weight (=>2500 gm)	7	23.3	8	17.8	25	33.3	
LBW (< 2499 gm)	11	36.7	23	51.1	31	41.3	
Extremely LBW (<1000gm)	12	40.0	14	31.1	19	25.3	
Total	30	100.0	45	100.0	75	100.0	
Gestational age categories (weeks)							0.72[NS]
At term (39-40)	8	26.7	14	31.1	26	34.7	
Preterm (Gestational age <38 weeks)	22	73.3	31	68.9	49	65.3	
Total	30	100.0	45	100.0	75	100.0	

* LBW=Low Birth Wight, NS= Non Significant, P= P-Value, No=Number

Table (3-2): The Difference between the Study Groups in Median of Selected Outcome Measurements.

	Study group			
	Proven sepsis (Culture positive)	**Probable sepsis (culture negative)**	**Healthy controls**	**P**
Birth weight (gm)				0.12[NS]
Range	(900 to 3000)	(900 to 4000)	(900 to 3600)	
Median	1500	1600	2000	
Inter-quartile range	(1100)	(1000)	(1550)	
No	30	45	75	
Mean Rank	65	70.9	82.5	
Gestational age (weeks)				0.82[NS]
Range	(29 to 40)	(28 to 40)	(28 to 40)	
Median	32	32	32	
Inter-quartile range	(7)	(7)	(7)	
No	30	45	75	
Mean Rank	75.2	72.4	77.5	
Age (days)				0.82[NS]
Range	(1 to 20)	(1 to 26)	(1 to 26)	
Median	4	6	6	
Inter-quartile range	(9)	(8)	(8)	
N	30	45	75	
Mean Rank	71.4	77.7	75.8	

*NS= Non Significant, P= P-Value, No=Number.

3.2 Distribution of Proven Sepsis Neonates According to the Onset of Disease

Maximum culture positive cases were seen in neonates more than three days old (LOS) as compared to neonate aged less than three days (EOS) in the present study, **Table (3-3).**

Table (3-3): Distribution of Proven Sepsis Neonates According to the Onset of Disease.

Onset of Sepsis	NO.	%
Early onset Sepsis	7	27%
Late onset Sepsis	23	73%
Total	30	100%

3.3 Blood Culture Isolates among Proven Sepsis Neonates Types

In this study, the total number of isolates was 30 (7 from EOS and 23 from LOS). In EOS, the isolated organism was Klebsiella (4/7), E. coli (2/7) and S.aureus (1/7). In LOS, the prevalent isolates were S.aureus (14/23), while S.epidermidis isolates were (9/23), **Table (3-4).**

Table (3−4): Isolated Organisms among Cases of EOS and LOS.

Type of Organism	Type of Neonatal Sepsis					
	EOS (n=7)		LOS (n=23)		Total (n=30)	
	No	%	No	%	No	%
S.aureus	1	14.3	14	60.9	15	50
S.epidermidis	0	0	9	39.1	9	30
E. coli	2	28.6	0	0	2	6.7
K.pnuemoniae	4	57.1	0	0	4	13.3

* EOS= Early Onset Sepsis, LOS=Late Onset Sepsis, No= Number

3.4. Positivity Rate of Common Signs and Symptoms between Proven and Probable Sepsis Neonates.

The most frequent clinical presentation and maternal risk factor of neonates with proven sepsis and Probable sepsis groups were fever , tachypnea , poor feeding , and tachycardia ,while the less frequent clinical presentation were vomiting, lethargy and PROM , **Figure(3-1).**

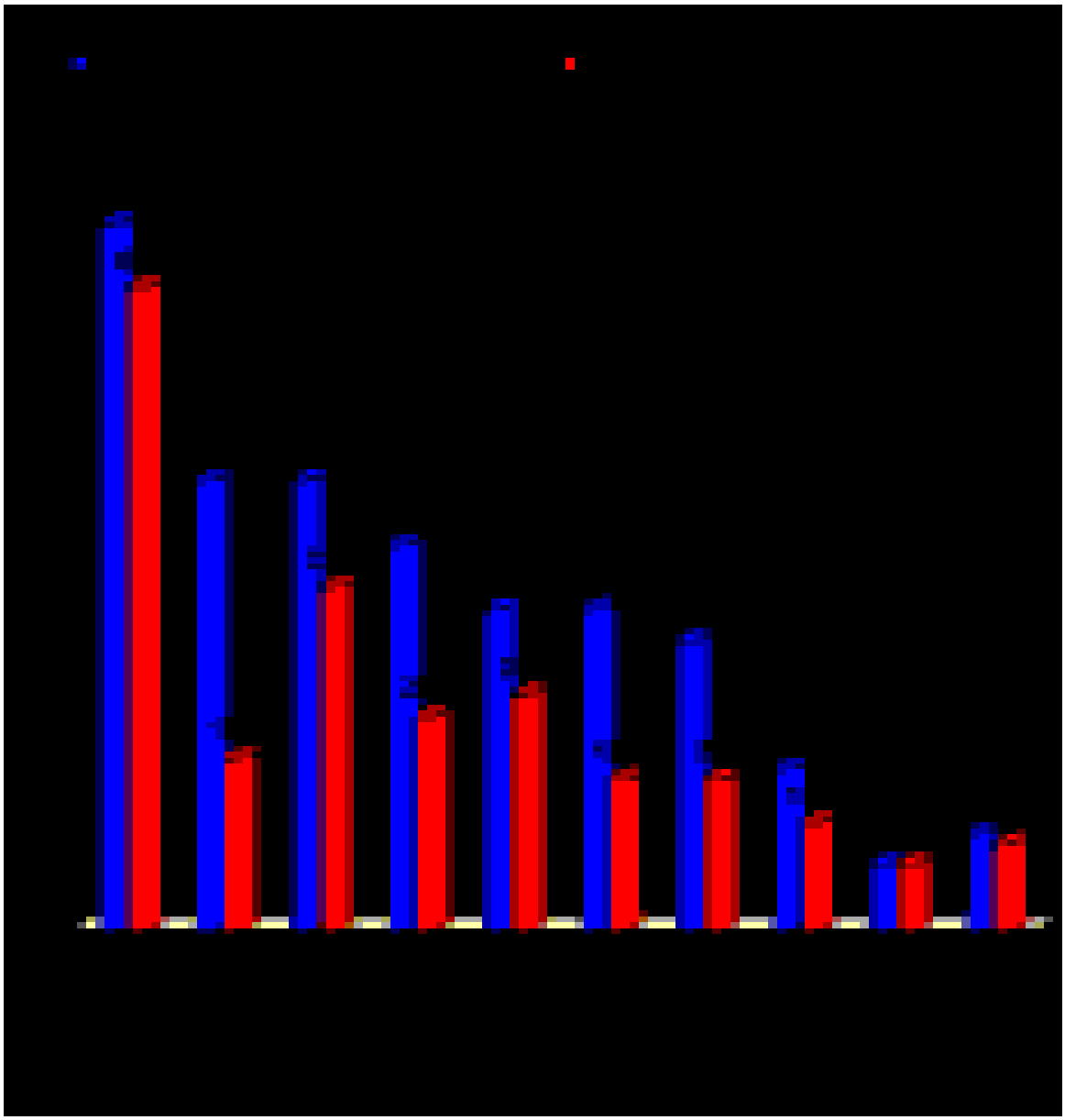

Figure (3-1): Bar Chart Showing the Relative Frequency of Selected Clinical Features and Maternal Risk Factor among Cases with Neonatal Sepsis.

3.5 Effect of Sepsis on Selected Measurements

As shown in **Table (3-5),** a significantly higher proportion of neonates with sepsis had leukocytosis (36.7% and 24.4% among proven and probable

sepsis groups respectively) compared to none of healthy controls. In the same way, a very low blood neutrophil count was observed in a significantly higher percentage of neonates with sepsis (60% and 24.4% among proven and probable sepsis groups respectively) compared to none of healthy controls.

Table (3-5): The Difference between the 3 Study Groups in White Blood Cells and Neutrophil Count.

	Study Group						
	Proven sepsis (Culture positive) (n=30)		Probable sepsis (culture negative) (n=45)		Healthy controls (n=75)		
	No	%	No	%	No	%	P
Leukocytosis (>15000/m^3 WBC count)	11	36.6	11	24.4	0	0.0	<0.001
Very Low Neutrophil count (<1000/m3)	18	60.0	11	24.4	0	0.0	<0.001

* WBC= White Blood Cells, P= P-Value, No=Number

The median WBC count was significantly different between study groups. It was significantly higher (8500 x10^6/mm^3) among probable sepsis group compared to healthy controls (6800 x10^6/mm^3). The median was also obviously higher among proven sepsis group (7750 x10^6/mm^3) among probable sepsis group compared to healthy controls, but the difference failed to reach the level of statistical significance (possibly because of small sample size). The two cases groups (proven and probable) showed no obvious differences in median blood WBC count, **Table(3-6).**

The median neutrophil count was significantly different between study groups. It was significantly lower (810 $x10^6/mm^3$) among proven sepsis group compared to healthy controls (1250 $x10^6/mm^3$). It was also obviously lower than that of probable sepsis group, but the difference failed to reach the level of statistical significance. In addition, the median count for probable sepsis group was not obviously different (1200 $x10^6/mm^3$) from that of healthy controls. The two cases groups (proven and probable) showed no obvious differences in median blood WBC count, **Table(3-6).**

Table (3-6): The Difference between the Study Groups in Median of White Blood Cells and Neutrophil Count.

	Study Group			
	Proven sepsis (Culture positive)	**Probable sepsis (culture negative)**	**Healthy controls**	**P**
Blood WBC count				0.016
Range	(2000 to 30000)	(2000 to 32000)	(5000 to 9000)	
Median	7750	8500	6800	
Inter-quartile range	(17000)	(6500)	(1600)	
No	30	45	75	
Mean Rank	78.7	89.1	66	
P (Mann-Whitney) for difference in median between				
Proven sepsis (Culture positive) X Probable sepsis (culture negative) =0.67[NS]				
Proven sepsis (Culture positive) X Healthy controls =0.33[NS]				
Probable sepsis (culture negative) X Healthy controls =0.002				
Blood neutrophil count				0.015
Range	(200 to 13000)	(200 to 23000)	(1000 to 1500)	
Median	810	1200	1250	
Inter-quartile range	(1050)	(500)	(400)	
No	30	45	75	
Mean Rank	55.8	76.5	82.8	
P (Mann-Whitney) for difference in median between				
Proven sepsis (Culture positive) X Probable sepsis (culture negative) =0.06[NS]				
Proven sepsis (Culture positive) X Healthy controls =0.003				
Probable sepsis (culture negative) X Healthy controls =0.48[NS]				

* WBC= White Blood Cells, P= P-Value, No=Number, NS=Non Significant

The median blood PCT was significantly higher among both cases groups (3.30 and 1.76 ng/ml among proven and probable sepsis groups respectively) compared to healthy controls (0.23 ng/ml). In addition, the median for proven sepsis group was significantly higher than that of probable sepsis group, **Table(3-7).**

Table (3-7): The Difference between the Study Groups in Median of Procalcitonin.

	Study Group			
	Proven sepsis (Culture positive)	**Probable sepsis (culture negative)**	**Healthy controls**	**P**
Blood PCT (ng/ml)				<0.001
Range	(1.34 to 5.96)	(0.18 to 3.45)	(0.12 to 0.42)	
Median	3.30	1.76	0.23	
Inter-quartile range	(1.95)	(0.72)	(0.21)	
No	30	45	75	
Mean Rank	130	97.8	40.3	
P (Mann-Whitney) for difference in median between				
Proven sepsis (Culture positive) X Probable sepsis (culture negative) <0.001				
Proven sepsis (Culture positive) X Healthy controls <0.001				
Probable sepsis (culture negative) X Healthy controls <0.001				

* PCT=Procalcitonin, P= P-Value, No=Number

The median CD64 neutrophil and CD69 lymphocytes expression was significantly higher among both cases groups compared to healthy controls. In addition, the median for proven sepsis group was significantly higher than that of probable sepsis group**, Table(3-8).**

Table (3-8): The Difference between the Study Groups in Median of CD64 and CD69 Markers.

	Study Group			
	Proven sepsis (Culture positive)	**Probable sepsis (culture negative)**	**Healthy controls**	**P**
CD64 neutrophil expression				<0.001
Range	(18 to 94)	(3 to 92)	(2 to 19)	
Median	72	60	10	
Inter-quartile range	(24)	(23)	(7)	
No	30	45	75	
Mean Rank	121.3	103.1	40.6	
P (Mann-Whitney) for difference in median between				
Proven sepsis (Culture positive) X Probable sepsis (culture negative) =0.005				
Proven sepsis (Culture positive) X Healthy controls <0.001				
Probable sepsis (culture negative) X Healthy controls <0.001				
CD69 lymphocyte expression				<0.001
Range	(2 to 80)	(3 to 68)	(2 to 50)	
Median	47	39	8	
Inter-quartile range	(34)	(33)	(11)	
No	30	45	75	
Mean Rank	110.9	95.1	49.6	
P (Mann-Whitney) for difference in median between				
Proven sepsis (Culture positive) X Probable sepsis (culture negative) =0.023				
Proven sepsis (Culture positive) X Healthy controls <0.001				
Probable sepsis (culture negative) X Healthy controls <0.001				

* P= P-Value, No=Number

Results of flowcytometry were expressed as a percentage of positive cells. Considering the percentage of (R2+R4) ≥ 20% as positive result while the percentage of (R2+R4)< 20% as negative result, **Figure(3-2), Figure(3-3)and Figure(3-4).**

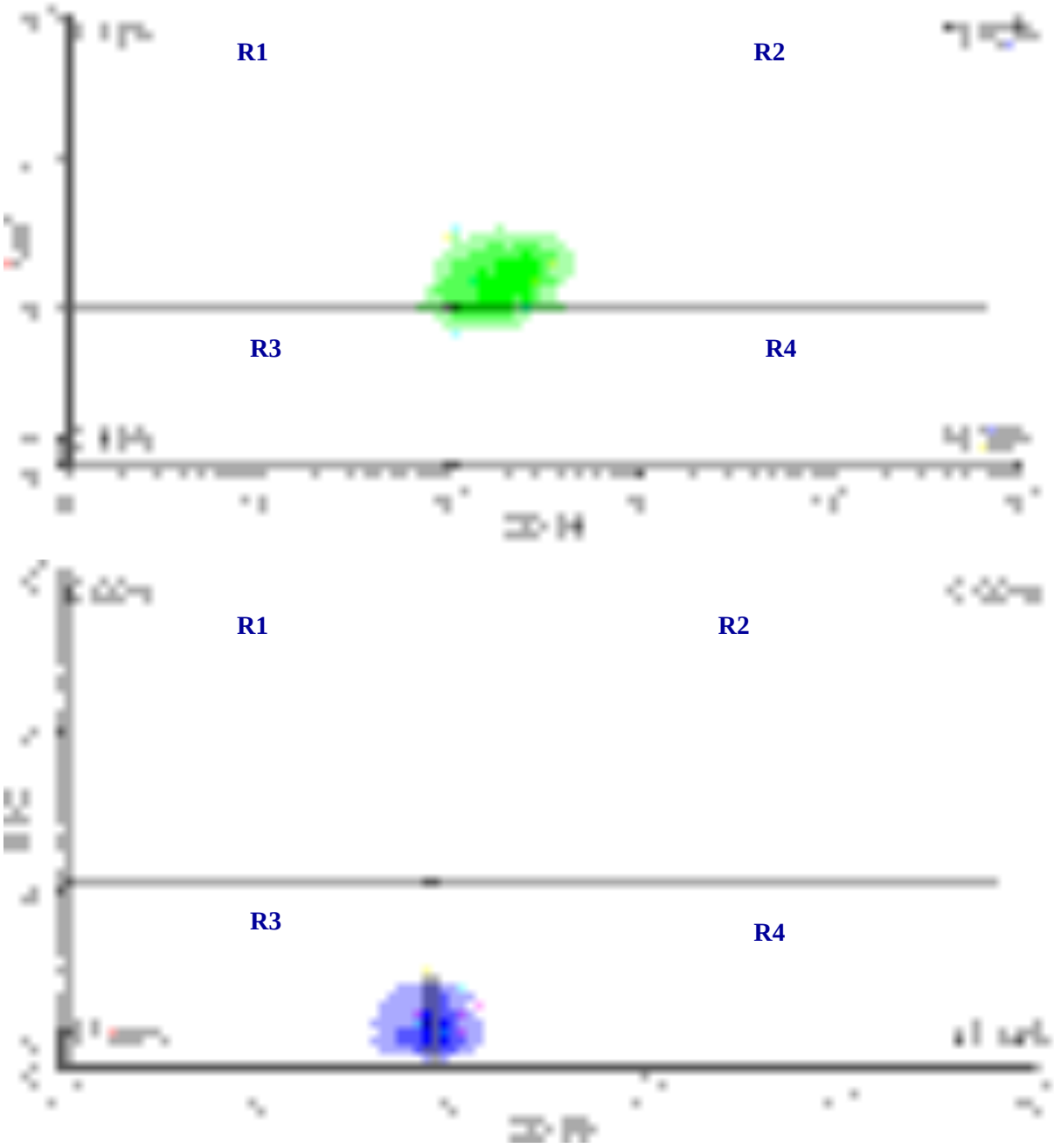

Figure (3-2): Results of Flowcytometric Analysis for CD64 Detection on Neutrophils (90.42%) Compared with CD69 Detection on T-Lymphocytes (42.14%) in a Patient with Neonatal Culture Proven Sepsis.

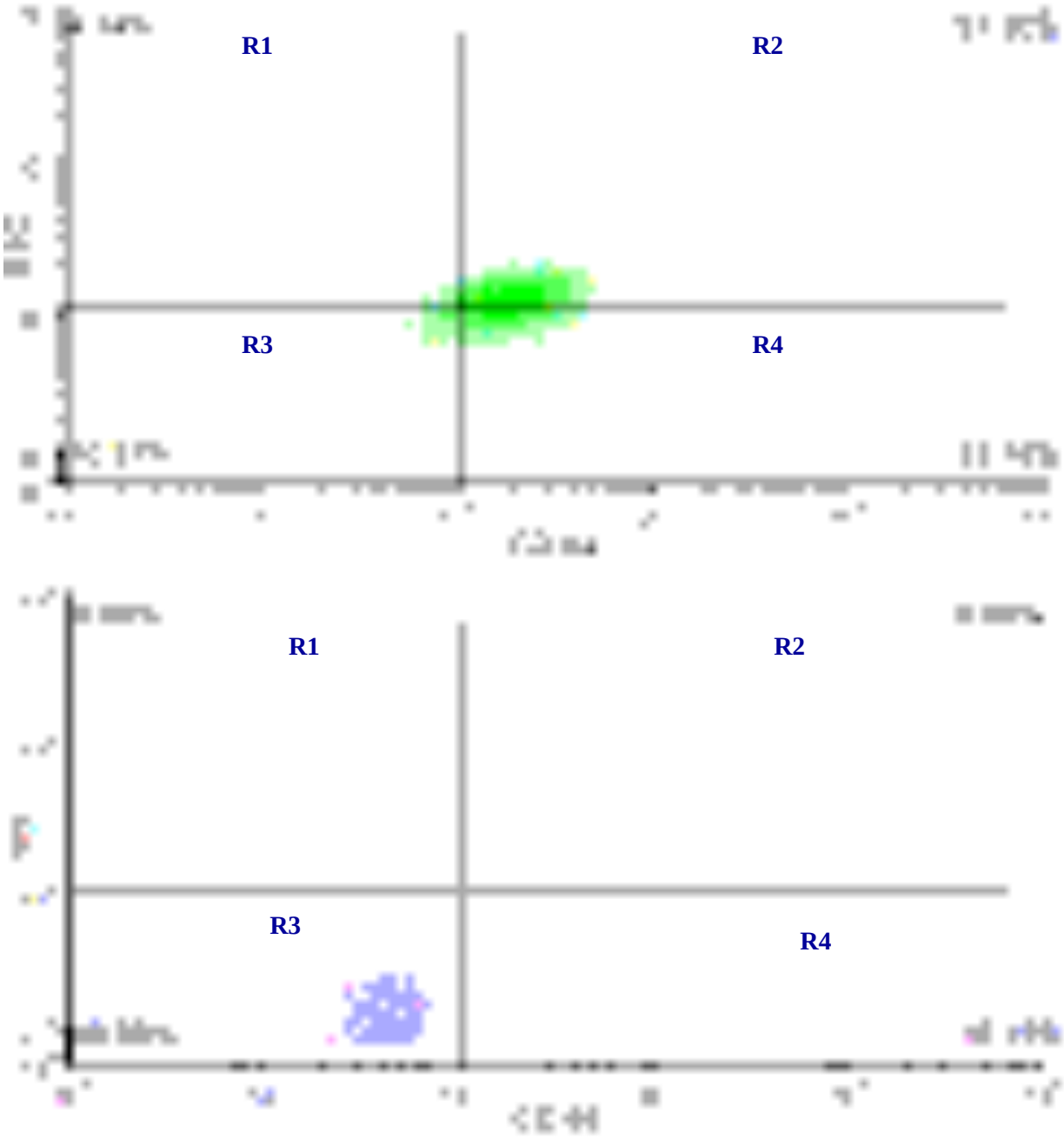

Figure (3-3): Results of Flowcytometric Analysis for CD64 Detection on Neutrophils (84.36%) Compared with CD69 Detection on T-Lymphocytes (10.45%) in a Patient with Probable Neonatal Sepsis.

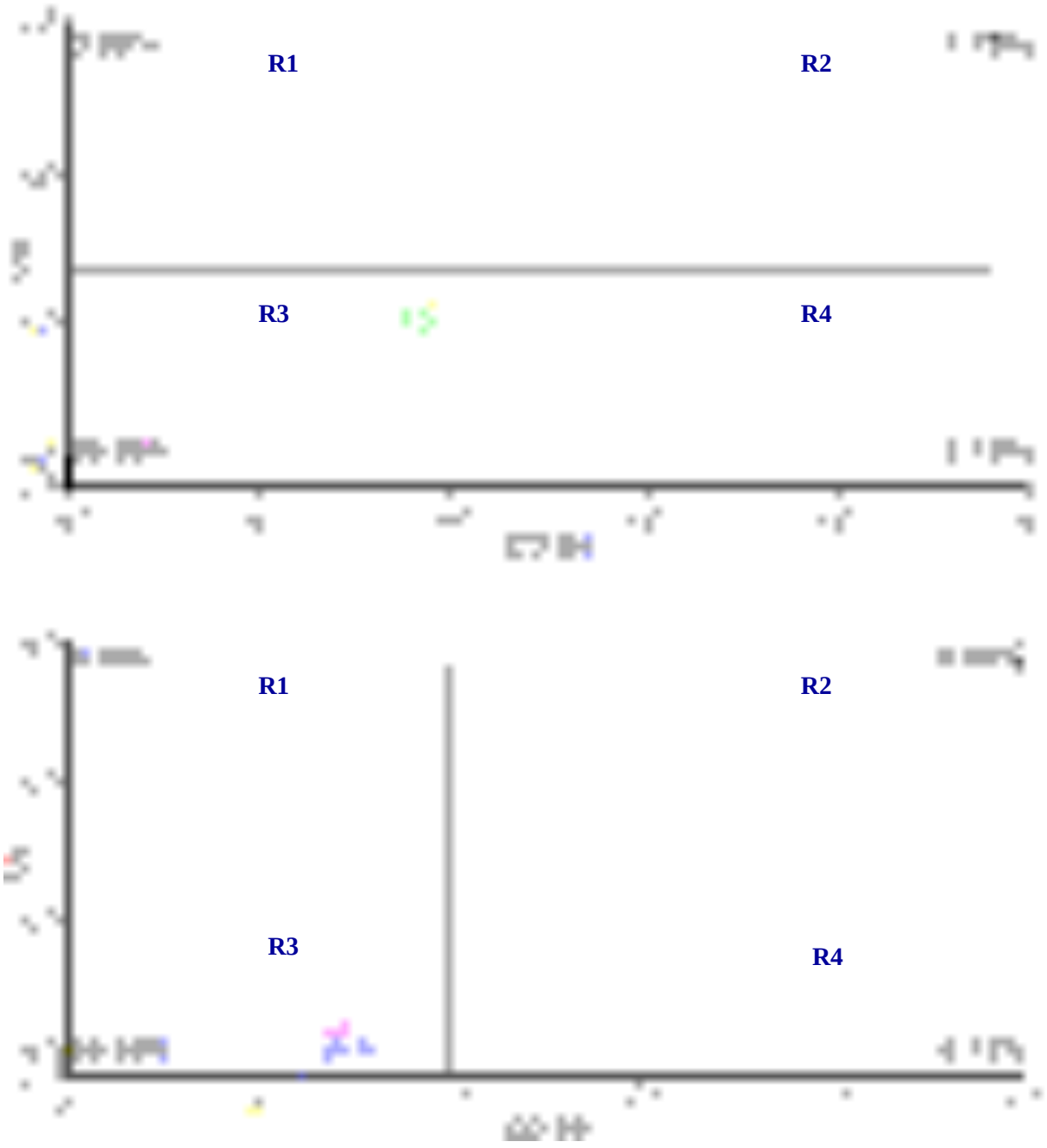

Figure (3-4): Results of Flowcytometric Analysis for CD64 Detection on Neutrophils (9.96%) Compared with CD69 Detection on T-Lymphocytes (0.12%) in Healthy Neonates.

3.6 Diagnosis of Culture Positive (Proven) Neonatal Sepsis

Five outcome measurements were tested for their performance in predicting a proven neonatal sepsis, differentiating it from a healthy neonate. These measurements include: Blood PCT, CD64 neutrophil expression, CD69 lymphocyte expression, Blood neutrophil count and Blood WBC count.

As shown in **Table(3-9)**, blood PCT and CD64 expression were associated with the highest validity in diagnosing neonatal sepsis and provided a perfect test (area under ROC curve being equal to one). The CD69 lymphocytes expression ranked second in its diagnostic validity and qualified as a good test (ROC area = 0.878). The blood neutrophil count was considered as a poor test (ROC area = 0.685), while blood WBC count was regarded as a test failure with an area under ROC curve of < 0.6), **Figure (3-5)**.A blood PCT of 0.88 ng/ml and higher is associated with a perfect test, being 100% accurate for a diagnosis of proven neonatal sepsis.

Similarly, a CD64 neutrophil expression of ≥30% is 99% accurate in diagnosing proven neonatal sepsis. A positive test (CD64 positive of 30% or higher) is 100% specific and can establish a possible diagnosis of sepsis with 100% confidence in any clinical situation. A negative test on the other hand (obtaining CD64 positive neutrophil expression of <30%) can exclude a possible diagnosis of neonatal sepsis with 99.6% confidence in a clinical situation where neonatal sepsis is of very low possibility (pre-test probability = 10% only), **Table(3-10)**.

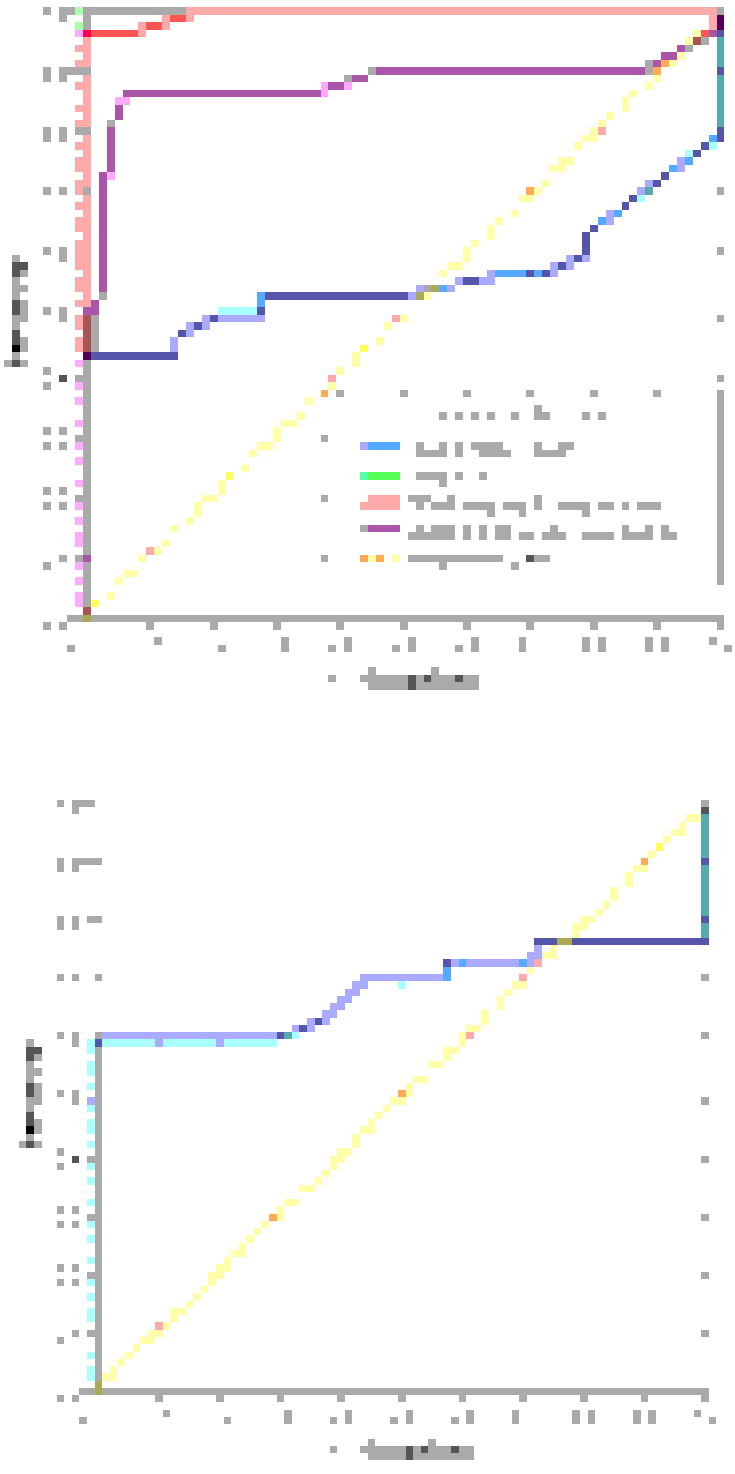

Figure (3-5): ROC Curve Showing the Performance of Selected Quantitative Outcome Measurements when Used as Tests to Diagnose Cases with Proven Sepsis (Culture Positive) Differentiating them from Healthy Control Group.

Table (3-9): Area Uunder ROC Curve Comparing the Validity of Selected Quantitative Outcome Measurements when Used as Tests to Diagnose Cases with Proven Sepsis (Culture Positive) Differentiating them from Healthy Control Group.

	ROC Area	*P*
Blood PCT	1.000	<0.001
CD64 neutrophil expression	0.996	<0.001
CD69 lymphocyte expression	0.878	<0.001
Blood neutrophil count	0.685	0.003
Blood WBC count	0.560	0.34[NS]

*PCT= Procalcitonin , WBC =White Blood Cells , *P*= P-Value

Table (3-10): Validity Parameters of Selected Quantitative Outcome Measurements when Used as Tests to Diagnose Cases with Proven Sepsis (Culture Positive) are Differentiating them from Healthy Control Group. (Higher Values of the Test are More Suggestive of the Diagnosis).

				PPV at Pre-Test Probability =		***NPV at Pre-Test Probability = 10%***
Positive if ≥ cut-off value	**Sensitivity**	**Specificity**	**Accuracy**	**50%**	**90%**	
Blood PCT						
0.88 (Perfect cut-off)	100.0	100.0	100.0	100.0	100.0	100.0
CD64 neutrophil expression						
30 (Highest specificity and optimum cut-off)	96.7	100.0	99.0	100.0	100.0	99.6
CD69 lymphocyte expression						
20 (optimum cut-off)	86.7	93.3	91.4	92.9	99.2	98.4
52 (Highest specificity)	43.3	100.0	83.8	100.0	100.0	94.1

3.7 Diagnosis of culture negative (probable) neonatal sepsis

As shown in **Table(3-11)**, blood PCT and CD64 neutrophil expression were associated with the highest validity in diagnosing neonatal sepsis and provided an excellent test (area under ROC curve being >0.9). The CD69 lymphocytes expression ranked second in its diagnostic validity and qualified as a good test (ROC area =0.823). The blood WBC count was considered as a poor test (ROC area =0.670), while blood neutrophil count was regarded as a test failure with an area under ROC curve of < 0.6), **Figure (3- 6)**.

A blood PCT of 0.43 ng/ml and higher is the optimum cut-off value for this parameter. At this cut-off value the test is 88.9% sensitive and 100% specific (perfect specificity), **Table(3-12)**.

In the present study the CD64 neutrophil expression of ≥30% is the optimum cut-off value for this parameter. At this cut-off value the sensitivity was found to be 96.7%, the specificity was 100% (perfect specificity), positive predictive value was 100% and its negative predictive value was 99.6%.

Although the neutrophil count was found as a failure test, one can use its highest specificity cut-off value of <950 $x10^6/mm^3$. Testing positive at this cut-off value (having a neutrophil count of less than 950) can establish a possible diagnosis of sepsis with 100% confidence (since it has a perfect specificity) in any clinical situation (any pre-test probability), **Table(3-13)**.

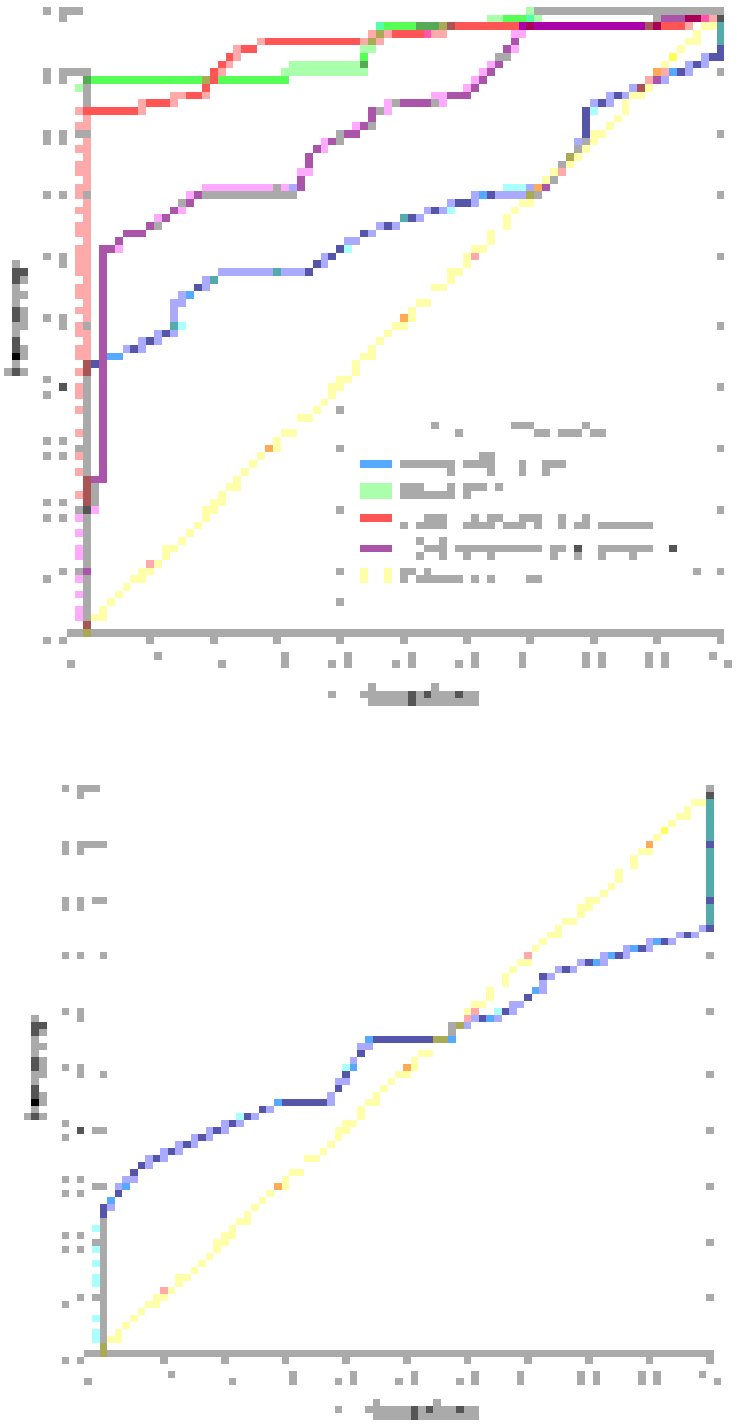

Figure (3-6): ROC Curve Showing the Performance of Selected Quantitative Outcome Measurements when Used as Tests to Diagnose Cases with Probable Sepsis (Culture Negative) Differentiating them from Healthy Control Subjects.

Table (3-11): Area Under ROC Curve Comparing the Validity of Selected

Quantitative Outcome Measurements when Used as Tests to Diagnose Cases with Probable Sepsis (Culture Negative) Differentiating them from Healthy Control Subjects.

	ROC Area	*P*
Blood PCT	0.948	<0.001
CD64 neutrophil expression	0.944	<0.001
CD69 lymphocyte expression	0.823	<0.001
Blood WBC count	0.670	0.002
Blood neutrophil count	0.538	0.49[NS]

				PPV at pre-test probability =		*NPV at pre-test probability = 10%*
Positive if ≥ cut-off value	**Sensitivity**	**Specificity**	**Accuracy**	**50%**	**90%**	
Blood WBC count						
5250 (Highest sensitivity)	84.4	21.3	45.0	51.8	90.6	92.5
7800 (optimum cut-off)	57.8	78.7	70.8	73.0	96.1	94.4
9250 (Highest specificity)	42.2	100.0	78.3	100.0	100.0	94.0
Blood PCT						
0.18 (Highest sensitivity)	100.0	26.7	54.2	57.7	92.5	100.0
0.43 (Highest specificity and	88.9	100.0	95.8	100.0	100.0	98.8

optimum cut-off)						
CD64 neutrophil expression						
3 (Highest sensitivity)	100.0	1.3	38.3	50.3	90.1	100.0
30 (Highest specificity and optimum cut-off)	84.4	100.0	94.2	100.0	100.0	98.3
CD69 lymphocyte expression						
3 (Highest sensitivity)	100.0	1.3	38.3	50.3	90.1	100.0
17 (optimum cut-off)	71.1	81.3	77.5	79.2	97.2	96.2
51 (Highest specificity)	20.0	100.0	70.0	100.0	100.0	91.8

Table (3-12): Validity Parameters of Selected Quantitative Outcome Measurements when Used as Tests to Diagnose Cases with Probable Sepsis (Culture Negative) are Differentiating them from Healthy Control Subjects. (Higher Values of the Test are More Suggestive of the Diagnosis).

Table (3-13): Validity Parameters of Selected Quantitative Outcome Measurements when Used as Tests to Diagnose Cases with Probable Sepsis (Culture Negative) are Differentiating them from Healthy Control Subjects. (Smaller Values of the Test are More Suggestive of the Diagnosis).

				PPV at pre-test probability =		***NPV at pre-test probability = 10%***
Positive if < cut-off value	**Sensitivity**	**Specificity**	**Accuracy**	**50%**	**90%**	
Blood neutrophil count						

950 (Highest specificity)	24.4	100.0	71.7	100.0	100.0	92.3
1225 (optimum cut-off)	55.6	56.0	55.8	55.8	91.9	91.9
1475 (Highest sensitivity)	66.7	28.0	42.5	48.1	89.3	88.3

3.8 Genetic Study:

3.8.1 PCR Amplification

The products of successful binding between the extracted DNA and specific primers for CD14 gene were detected by gel electrophoresis analysis using DNA marker (100 bp DNA ladder) and the products size was 497 bp for both patients and control groups **Figure (3-7).**

Figure (3-7): Agarose Gel Electrophoresis Image that Shown the PCR Product Analysis of CD14 Gene (P1) from Neonatal Sepsis Patient and Healthy Control Blood Sample. Where M: Marker (2000-100bp), Lane (1) Healthy Control Sample and Lane (2-10) Positive PCR Product at 497bp Product Size.

3.8.2 Detection of CD14/ C-159T Gene Polymorphism:

The distribution of *CD14* /C-159T gene polymorphism was detected by PCR-RFLP technique, at this locus there're three genotype; homozygote (TT) at 353bp and 144bp, heterozygous (CT) at 497bp, 353bp and 144bp, and wild type (CC) which still undigested 497bp, **Figure (3-8)** the genotype distribution had no deviation from Hardy-Weinberg equilibrium in all study groups and agree with the reports of **Sharaf et al., (2010).**

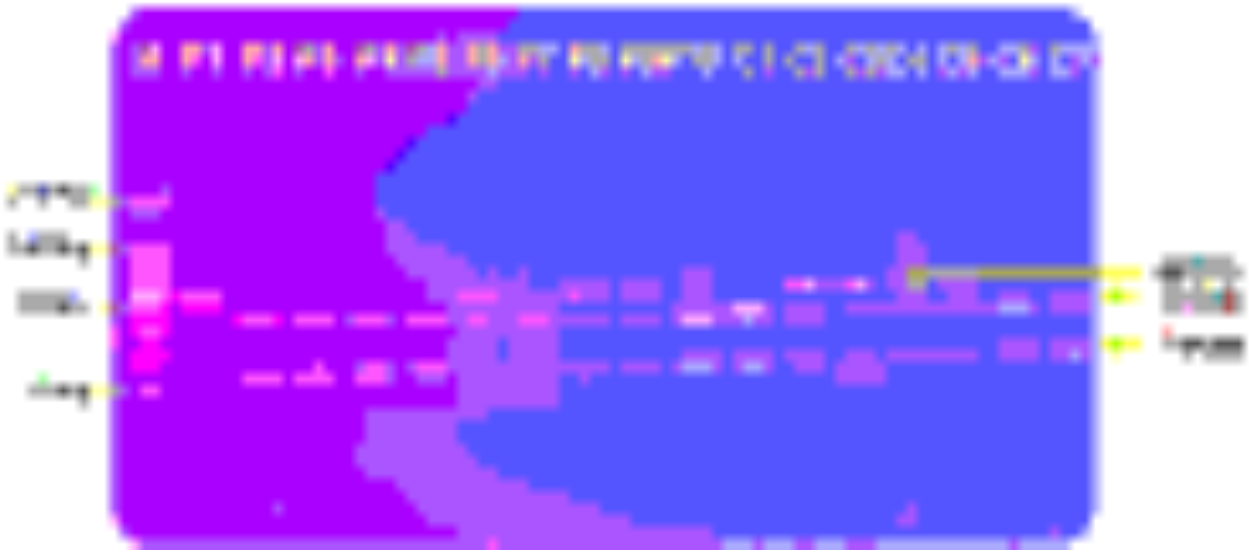

Figure (3-8): Agarose Gel Electrophoresis Image that Shown the RFLP-PCR Product Analysis of 497bp PCR Product CD14/C-159T gene in Neonatal Sepsis Patient and Healthy Control Blood Sample that Digestion by (*AVAII*) Restriction Endonuclease Where M: Marker (2000-100bp), Lane (P1and C3) Patient and Control Sample Show (CC) Homozygote as Undigested 497bp Product Size, Lane (P2, P3,P4, P5, P7,P10, C1,C4,C5,C6, and C7)Patient and Control Sample Show (TT) Homozygote as 353bp and 144bp Product Size, Lane (P6, P8, P9, and C2) Patient and Control Sample Show (CT) Heterozygous as 497bp, 353bp and 144bp Product Size.

3.8.2.1. CD14 /C-159T Gene Polymorphism and Risk of Culture Positive (Proven) Neonatal Sepsis

As shown in **Table (3-14),** the risk of having proven sepsis, compared to healthy controls was significantly increased by 13.1 times for neonates with TT genotype. Conversely having a CC genotype significantly decreases the risk of proven sepsis by 5.1 times. The CT genotype marginally decreased the risk of sepsis by 50%. This last effect was too weak to be statistically significant.

Presence of T allele significantly increased the risk of having culture positive neonatal sepsis by 5.1 times. Conversely, the presence of C allele significantly reduced the risk of having culture positive neonatal sepsis by 13.1 times. The absence of the protective role of C allele increases the risk of having sepsis by 13.1 times, **Table (3-14)**.

Table (3-14): The Risk of Proven Sepsis (Culture Positive) Compared to Healthy Controls by CD14 (C-159t) Gene Polymorphism.

	Healthy controls		*Proven sepsis (Culture positive)*			*Inverse OR*				
	N	**%**	**N**	**%**	**OR**		**95% CI OR**	**P**	**EF**	**PF**
CD14 (C-159t) polymorphism										
TT	6	8.0	16	53.3	13.1	**	(4.37 - 39.48)	<0.001	0.493	**
CC	42	56.0	6	20.0	0.2	5.1	(0.07 - 0.54)	0.001	**	0.450
CT	27	36.0	8	26.7	0.65	1.5	(0.25 - 1.65)	NS	**	0.127
Total	75	100.0	30	100.0						
CD14 (C-159t) alleles frequency										
T allele	**39**	26.0	40	66.7	7.18	**	2.74 - 18.79	0.0001	0.574	**
C allele	111	74.0	20	33.3	0.068	6.2	0.02 - 0.19	< 0.0001	**	0.82
Total	150		60							

* EF= etiologic fraction, PF= protective fraction, P=P-Value, OR=Odds Ratio, NS=Non significant ,CI= confidence interval,

3.8.2.2. CD14 /C-159T Gene Polymorphism and Risk of Culture Negative (Probable) Neonatal Sepsis

As shown in **Table (3-15),** the risk of having probable sepsis compared to healthy controls was significantly increased by 14.4 times for neonates with TT genotype. Conversely having a CC genotype significantly decreases the risk of proven sepsis by 5.9 times. The CT genotype marginally decreased the risk of sepsis by 50%. This last effect was too weak to be statistically significant.

Presence of T allele significantly increased the risk of having culture positive neonatal sepsis by 5.9 times. Conversely, the presence of C allele significantly reduced the risk of having culture positive neonatal sepsis by 14.4 times. The absence of the protective role of C allele increases the risk of having sepsis by 14.4 times, **Table (3-15).**

Table (3-15): The Risk of Probable Sepsis (Culture Negative) Compared to Healthy Control Group by CD14 (C-159t) Gene Polymorphism.

	Healthy Controls		*Probable Sepsis (Culture Negative)*			*Inverse OR*				
	N	**%**	**N**	**%**	**OR**		**95% CI OR**	**P**	**EF**	**PF**
CD14 (C-159t) polymorphism										
TT	6	8.0	25	55.6	14.4	**	(5.18 - 39.9)	<0.001	0.517	**
CC	42	56.0	8	17.8	0.17	5.9	(0.07 - 0.41)	<0.001	**	0.465
CT	27	36.0	12	26.7	0.65	1.5	(0.29 - 1.45)	NS	**	0.127
Total	75	100.0	45	100.0						
CD14 (C-159t) alleles frequency										
T	39	26.0	62	68.9	1.19	**	0.38 - 3.698	NS	0.11	**
C	111	74.0	28	31.1	0.16	5.01	0.09 - 0.282	< 0.001	**	0.62
Total	150		90							

* EF= etiologic fraction, PF= protective fraction, P=P-Value, OR=Odds Ratio, NS=Non significant, CI= confidence interval

4. Discussion

Neonatal sepsis with its high mortality rate still remains a diagnostic and treatment challenge for the neonatal health care providers. An early diagnosis of neonatal septicemia helps the clinician in instituting antibiotic therapy at the earliest, thereby reducing the mortality rates in the neonates. An early identification of an infected neonate also helps in avoiding the unnecessary treatment of a non- infected neonate.

4.1 Demographic Distribution of the Selected Groups Included in Study

In present study, sex distribution among neonates with proven and probable sepsis was 47/75(62.7%) males and 28/75(37.3%) females, **Table (3-1).**These results confirm other studies which have shown that males have been reported to be 2-6 times more likely than females to develop sepsis **(Ziba et al., 2003).** Nearly 3:2 ratio in this study is constant with this data. The male preponderance has been attributed to the deficiency of an X linked immuno-regulatory gene. A predominance of male infant is apparent in almost all studies of sepsis in newborn **(Cordero *et al*., 2004).**

There were no important or statistically significant differences in median birth weight, gestational age and age of neonate between the 3 study groups, **Table (3-2).**

4.2.Distribution of Proven Sepsis Neonates Accordingto the Onset of Disease

Maximum culture positive cases were seen in neonates more than three days old (LOS) as compared to neonate aged less than three days (EOS) in the present study, **Table (3-3).**

Although blood culture is the gold standard for the diagnosis of sepsis, culture reports would be available only after 48-72 hours **(Tripathi and Malik, 2010).**

In present study, the rate of proven sepsis was (40%) as in **Table (3-3) and Table (3-3)** which is higher than that observed by **Kadhim (2010)** in which the isolating rate was (28.9%)**,** but comparable with studies conducted by **Ananthakrishnan and Gunasekaran (2009)** in which the positivity rate was (40.3%)**,** and lower than that observed by **Mahmoud *et al*.,(2014)** in which the isolating rate was (60%)**.**

The varying results may be due to different study population different defining of proven sepsis and as the culture positivity depend on time of sampling and the extent of bacteremia in neonate **(Borna and Borna, 2005).** Sometimes the blood cultures are positive in only 5%–10% of suspected sepsis cases, even at highly resourced facilities **(Darmstadt *et al.,* 2009).** Blood culture also fails to detect bacteremia in 27%-92% of preterm VLBW infants. This is often due to the volume of blood inoculated into the blood culture bottle being insufficient or suboptimal processing of the specimen, but perhaps the most important reason is that bacteremia is often transitory or intermittent **(Sarkar *et al*., 2006).**

Isolates of EOS cases were ***Klebsiella*** 4/7 (57.1%), ***E. coli*** 2/7(28.6) and ***S.aureus*** 1/7(14.3) while that of LOS cases were ***S.aureus*** 14/23(60.9) and ***S.epidermidis*** (CoNS) 9/23(39.1%)**, Table (3-4)**.

The our study is similar to findings of **Marchant *et al.,* (2013)** who found that gram-positive organisms accounted for the majority of neonatal sepsis cases (up to 70%) while sepsis due to gram-negative organisms accounted for (15 to 20%).

Other study by **Aletayeb *et al.,* (2011) and Shah *et al.,* (2012)** reported that the percentage of Gram -ve isolates from sepsis cases were (92.8%) and (52%) respectively. **Ballot *et al.,* (2012)** found that Gram-positive infections predominated in EOS, with ***Streptococcus agalactiae*** being the most common pathogen.

Li *et al., (*2013*)* also reported that Coagulase-negative ***Staphylococcus*** (CoNS) was the major Gram-positive bacteria in late onset sepsis (LOS) (54.4%). On the other hand, **Muhammad *et al.*, (2010)** found that ***Klebisella and Moraxella*** were the most common isolates of LOS.

This variability in the etiology between EOS and LOS can be explained by the fact that EOS is conventionally regarded as maternally-acquired, with causative organisms, such as Gram negative organisms which are usually found in the maternal genital tract, whereas LOS is considered environmental in origin-either hospital or community acquired **(Aly *et al.*, 2012)**.

Intervention procedures may affect the distribution of microorganisms. As regard EOS, after introduction of intrapartum GBS prophylaxis, the rate of this infection decreased and allowed Gram negative predominance to occur, while predominance of CoNS in LOS is attributed to the poor infection control attitude in hospitals especially in developing countries **(Stefanovic, 2011)**.

4.3. Positivity Rate of Common Signs and Symptoms between Proven and Probable Sepsis Neonates.

The most frequent clinical presentation of neonates with proven sepsis and Probable sepsis groups were fever (73.3% ,66.7%), tachypnea (46.7%, 17.8%), poor feeding (46.7%,35.6), and tachycardia (40%, 22.2%) respectively ,while the less frequent clinical presentation were vomiting (33.3% ,24.4%) , lethargy (33.3%, 15.6%) and PROM (30%, 15.6%) respectively, **Figure(3-1).**

In a study done by **Borna and Borna (2005**), found that the hyperthermia and poor feeding were the most common clinical signs among 200 infants, but some clinical signs such as tachypnea ,apnea ,cyanosis and tachycardia were more common in proven sepsis group. Another study revealed that the most common presenting clinical features were respiratory distress

(32%), poor feeding (23%), vomiting (14%), abdominal distension (14%), lethargy (9%) and irritability (9%), **(Trotman and Bell, 2006).**

The clinical presentation is similar to those in the previous reports, which illustrated the subtle and non-specific signs and symptoms of sepsis in infants. Thus, the physician must have a high index of suspicion for making the diagnosis of sepsis based on clinical signs in these infants **(Jia-Horing *et al.*, 2004**).

4.4. Effect of Sepsis on Selected Measurements

Hematological profiles reveal that the rate of neonates with low neutrophil count was significantly different between the three study groups. A significantly higher percentage of culture positive neonates had low neutrophil count (60%) compared to both culture negative neonates (24.4%) and healthy controls (0%). In the same way high percentage of leukocytosis was observed in culture positive neonates and culture negative neonates (36.7% and 24.4%) respectively, and healthy controls (0%)**, Table (3-5).**

These results were in agreement with **Bhandari *et al.* (2008) and Mondal *et al.* (2012)** who found that the hematologic profiles of neonates with septicemia were characterized by higher white blood cell count, high immature / total neutrophil ratio, lower platelet count and hemoglobin level. On the other hand, **Sucilathangam *et al.* (2012)** reported that total WBCs count and Hb level were normal in (85%) of cases.

The I\T neutrophil ratio believed by many as a single most helpful test available for diagnosing neonatal sepsis **(Polin *et al*., 2005; Bhandari *et al*., 2008)**. Also **Buch *et al.*, (2011)** concluded that the most useful individual test for confirming and excluding neonatal sepsis was (I\T) ratio in combination with other signs. This is attributed to the release of neutrophils from bone narrow in response to infection, with increasing number of immature cells entering the blood stream and producing a differential cell count with a shift to the left.

On the other hand, **Aly *et al.*, (2012)** reported that abnormal neutrophil counts taken at the time of symptoms onset are only observed in two thirds of infants, so the neutrophil count does not provide an adequate confirmation of sepsis.

In neonates, an elevated PCT level may help in predicting septicaemia; furthermore, low PCT levels were helpful in ruling out septicaemia as a diagnosis. Therefore, the PCT assessment could help the physicians in limiting the number of prescriptions for the antibiotics.

In this study, the PCT levels were remarkably high in the neonates with proven sepsis and also in the suspected sepsis cases, **Table (3-7)**. This finding was comparable with that of the study which was conducted by **Park *et al.*, (2014)** who found PCT levels were remarkable high in neonates with proven sepsis. Similar elevated values for PCT in neonatal sepsis have been reported by other workers **(Zahedpasha *et al*., 2009 & Naher *et al*., 2011).**

Although it exists in extremely low concentrations in healthy persons, its levels increase in the presence of bacterial infections, such as sepsis, meningitis, and urethritis, and it rises rapidly in severe sepsis or septic shock **,**at that time, high serum PCT levels are found because macrophages and monocytes, and the C cells of the thyroid produce it through induction by bacterial endotoxin**(Joram *et al.*, 2006).**

Boo *et al.*, (2008) found that PCT is first detected 4 hours after injection of a small quantity of endotoxin in healthy people due to inflammation; its levels increase rapidly after 6–8 hours, reach a plateau, and then return to normal levels after 24 hours, and its levels show a physiologic increase 24–48 hours after birth, and it is normalized after 3 days **(Joram *et al.*, 2006).**

Other than infection, PCT levels increase in premature infants, hypoxia, RDS, and hemodynamic instability, decreasing its specificity in early-onset sepsis **(Ballot *et al*., 2004).**

The results of this study revealed significant elevated levels of CD64 in neonates with sepsis when compared with healthy controls, **Table (3-8)**. These findings are also in agreement with other studies **(Ng *et al*.2004 & Shao et al., 2005),** that have reported the same results in neonates with early-onset sepsis. Another study reported similar results in very low-birth-weight neonates with late-onset neonatal sepsis **(Ng *et al.,* 2002).**

Table (3-8) showed high statistically significant difference (P<0.001) between patients and controls regarding mean percentage of expression of CD64 on neutrophils, Similar results by **Khalifa *et al.,(*2007)** , indicated that quantitation of the neutrophil CD64 is a specific indicator of sepsis than the other available diagnostics tests. Also Similarly **Genel *et al.*, (2011),** founded that there was a highly significant difference between patients group and control group regarding the percentage of CD64 positive neutrophils.

In the study of **Elawady *et al.*, 2014,** neutrophil CD64 expression was increased significantly which confirmed the clinical sepsis groups as compared with the control group. A study of **Saiful Islam *et al*., (2014),** showed high significant difference (P=<.001) between patients and controls regarding mean percentage of expression of CD64 on neutrophils. Similar results by **Labib *et al*., (2013); Young *et al*., (2012) and Dhlamini *et al.,* (2011**.

4.5. The Validity of selected parameter in Diagnosis of culture positive (proven) neonatal sepsis

As the diagnostic utilities (sensitivity, specificity, PPV, NPV) ultimately determine the usefulness of a clinical test, the one with a high sensitivity and NPV (approaching 100%) would be the most desirable in this situation because all genuinely infected cases are required to be identified and treated. The test should also have an acceptable specificity (>80%) so that misclassification of no

infected cases and unnecessary use of antibiotics could be kept to a minimum **(Faix, 2013).**

ROC curve analysis to determine the diagnostic usefulness of PCT, CD64 neutrophil expression, CD69 lymphocyte expression, blood neutrophil count and blood WBC count for detecting neonatal sepsis was performed. In the ROC curve, the area under ROC curve (AUC) for PCT and CD64 was equal to one thus, both markers had a great diagnostic value, and that for CD69 was (ROC area = 0.878) ranked second in its diagnostic validity and qualified as a good test; The blood neutrophil count was considered as a poor test (ROC area = 0.685), while blood WBC count was regarded as a test failure with an area under ROC curve of < 0.6), however, PCT and CD64 levels showed more usefulness **,Table(3-9)**.

To check diagnosis accuracy, we used the following guidelines based on the AUC level: noninformative (0.5), less accurate (0.5<AUC≤0.7), moderately accurate (0.7<AUC≤0.9), and highly accurate (0.9<AUC<1), **(Greiner *et al*.,2000).** Thus, based on our results, PCT, CD64 are a highly accurate marker, whereas CD69 is moderately accurate for the diagnosis of neonatal sepsis. The sensitivity of PCT for detecting sepsis (cut-off value of 0.88 ng/ml and higher) and its specificity was in maximum (100%), its positive predictive value was 100% and its negative predictive value was 100 %. A positive test can establish a possible diagnosis of probable sepsis with 100% confidence in any clinical situation. A negative test on the other hand (obtaining a blood PCT of < 0.43 ng/ml) can exclude a possible diagnosis of neonatal sepsis with 98.8% confidence in a clinical situation where neonatal sepsis is of very low possibility (pre-test probability = 10% only), **Table(3-10)**.

Other study done by **Sucilathangam *et al.*, (2012),** found that the sensitivity of PCT for detecting sepsis (more than 0.5 ng /ml) was 92.8%, its specificity 75.0%, its positive predictive value was 59.0% and its negative predictive value was 96.0 %.

Also **Vazzalwar *et al.,(*2005),** assessed PCT for the diagnosis of late-onset sepsis in 67 neonates. At a PCT cutoff value of 1.0 ng mL, the sensitivity was found to be 97% and the specificity was 80%.While **Boo *et al*.,(2008**) showed at a PCT cut-off level of greater than or equal to 2 ng mL, the sensitivity and specificity, PPV and NPV were 88.9, 65.2, 40 and 95.7% respectively. **Park *et al*., (2014)** conclude that PCT had a sensitivity of 88.79% , specificity of 58.17% and negative predictive values of PCT were 98.6% with a cutoff value 0.5 mg/L, whereas **Bodkhe *et al*.,(2014)** in his study, found the sensitivity and specificity at 2 ng/ml concentration was 97% and 88.8 % respectively.

Despite the fact that a majority of the cytokine markers have high NPVs (ie, good for ruling out sepsis), these have not been adopted for general medical use. Recently, attention has been directed to the leukocyte cell surface antigens as diagnostic markers of sepsis **(Bhandari *et al*.,2008).**

The goal in this prospective study was to enroll infants undergoing sepsis evaluation from the NICU and to measure the neutrophil CD64 and lymphocytes CD69 expression in blood as an adjunct to previously validated hematologic scoring system for detecting neonatal sepsis.

This was in accordance with the results of previous studies that suggested that neutrophil CD64 from both infected term and preterm infants was significantly up regulated compared with healthy newborns **(Bhandari *et al*., 2008).**

In the study of **Saiful Islam *et al*., (2014)**, neutrophil CD64 showed high sensitivity 100%, specificity 54.9%, PPV, 28.13% and also high NPV 100%. Specificity and PPV were low in this study because of large number of false positive result. This may be due to small sample size and blood culture was found positive only in 22.5% cases of neonatal sepsis. The results of present study clearly indicated that measurement of neutrophilCD64 can be useful for diagnosis of neonatal sepsis in early diagnosis of neonatal sepsis.

The present study was in agreement with those in the study of **Elawady *et al.*,*(*2014**), who found that CD64 had the highest sensitivity (96%), specificity (100%), PPV (96.2%), and NPV (100%), with cutoff values of 45.8%. Also other study of **Ng *et al.*;(2004**) that reached high sensitivity (97%) and a moderate specificity (80%), respectively. On the other hand, **Streimish *et al.*, (2014)** demonstrated that sensitivity was 78% and specificity was 59%.

Mahmoud *et al.*,(2014) results showed that neutrophil CD64 has sensitivity, specificity, PPV and NPV of 100%, 30%, 68.2% and 100% respectively. These results of CD64 expression on neutrophils go with others, as **Choo *et al.*, (2012)** who reported sensitivity of 91% and specificity of 83%. **Faix, (2013)** also demonstrated that sensitivity and specificity of CD64 expression were 75% and 77% respectively. **Jia *et al.*, (2013)** reported sensitivity of 78%, specificity of 81%, and CD64 index has some general favorable characteristics as it does not differ with age, because its expression only occurs upon cell activation and is stable for more than 30 hours at room temperature. In addition to accuracy, the laboratory test for CD64 is rapid (<60 minutes) with the use of flow cytometry and requires minimal blood volume (<100 μL) which is a real advantage in neonates **(Choo *et al.*, 2012)**.

CD69 is increased in septic patients **(Nolan *et al.*, 2008; Giamarellos-Bourboulis, 2010).** The optimum cut-off value for CD69 lymphocytes expression is set at ≥20%, which is the most accurate cut-off value (91.4%) for this parameter. A positive test at this optimum cut-off value (having a CD69 of 20% or higher) can establish a possible diagnosis of neonatal sepsis with 92.9% confidence in a clinical situation where sepsis is of equal odds (50% pretest probability).

The present study found that the Sensitivity Specificity Positive predictive value Negative predictive value of CD69 lymphocyte expression at 20 (optimum cut-off) were, 86.7, 93.3, 99.2 and 98.4, respectively**, Table (3-10)**.

The confidence in a positive diagnosis is further increased to 99.2% when the differential diagnosis of sepsis is highly suspected (90% pre-test probability). A negative test on the other hand (obtaining CD69 positive lymphocytes expression of <20%) can exclude a possible diagnosis of neonatal sepsis with 98.4% confidence in a clinical situation where neonatal sepsis is of very low possibility (pre-test probability = 10% only). Raising the cut-off value further to ≥52% will make the test 100% specific and by extension 100% diagnostic, being able to establish a possible diagnosis of sepsis with 100% confidence in any clinical situation (any pre-test probability)**, Table (3-10)**.

Labib *et al.*, (2013), results showed that lymphocytes CD69 has Sensitivity Specificity Positive predictive value and Negative predictive value as , 80.8% 66.7% 87.5% 75.0% respectively. Also **Hamdi *et al*.,(2014),**study revealed high significant difference between patients group (80.477±8.4151), and control group (74.3±5.1412), regarding mean percentage±SD expressions of CD69 respectively .

A study by **Chirico and Loda, (2011)** which observed that hematological counts and ratios showed a limited accuracy with wide range of sensitivity (17-90%) and specificity (31-100%), due to the relatively long period necessary to give positive results and the significant influence of non-specific factors. However, the (I/T) ratio of > 0.2 may reach a sensitivity of 90% and negative predictive value of 98%. Variations in the results shown by different studies may be due to differences in blood sampling time, severity of infection, the age of neonates and reduced sensitivity of these tests in the first week of life **(Aly *et al*., 2012)**.

4.6. Diagnosis of culture negative (probable) neonatal sepsis

In present study a blood PCT of 0.43 ng/ml and higher is the optimum cut-off value for this parameter. At this cut-off value the sensitivity, specificity, Accuracy, PPV and NPV, were 88.9%,100%, 95.8% 100% and 98.8%,

respectively, this is aperfect specificity. A positive test can establish a possible diagnosis of probable sepsis with 100% confidence in any clinical situation. A negative test on the other hand (obtaining a blood PCT of < 0.43 ng/ml) can exclude a possible diagnosis of neonatal sepsis with 98.8% confidence in a clinical situation where neonatal sepsis is of very low possibility (pre-test probability = 10% only), **Table(3-13)**. Lowering the cut-off value further to 0.18 ng/ml will result in a perfect sensitivity of 100%. A negative test (PCT <0.18 ng/ml) can exclude a possible diagnosis of neonatal sepsis with 100% confidence in any clinical situation.

The present study supports use of PCT as a more sensitive and specific test than CRP in cases of neonatal sepsis. PCT may be used not just as a marker of infection but more importantly as a marker of severity of infection. PCT helps in an early diagnosis of the sepsis on the day of the admission itself, before the blood culture report is ready (usually after 3-5 days). PCT helps in avoiding antibiotic therapy where it is not required and thereby reducing the cost and the occurrence of bacterial resistance. PCT can also be employed for the prognosis of sepsis.

Procalcitonin has been intensively investigated for its diagnostic role in neonatal sepsis. It has been reported that high concentration of plasma PCT was found in infants with severe infection, while PCT levels were very low in those with no infections **(Khoshdel *et al.*, 2008). Sakha *et al.*, (2008),** in their study, showed that the sensitivity and specificity of the serum PCT level were 92.6 and 97.5%, respectively, in the diagnosis of suspected sepsis neonates.

The neutrophil CD64 expression of ≥30% is the optimum cut-off value for this parameter. At this cut-off value the sensitivity, specificity, Accuracy, PPV and NPV, were 84.4%, 100%,94.2%,100% and 98.3 resppectively, (perfect specificity), **Table(3-13)**.

Elawady *et al.*, (2014) in their study, showed that the best cutoff value of CD64 from the ROC curve for assessment; the cutoff value of 46% for probable sepsis, were considered optimal as they exhibited high sensitivity 96%, specificity 100%. PPV (96.2%), and NPV (100%), compared with other hematologic parameters.

The lymphocytes CD69 expression of ≥17% is the optimum cut-off value for this parameter. At this cut-off value the sensitivity, specificity, Accuracy, PPV and NPV, were, 71.1%, 81.3%, 77.5%, 97.2%, and 96.2% respectively. Raising the cut-off value further to ≥51% will make the test 100% specific and by extension 100% diagnostic, being able to establish a possible diagnosis of sepsis with 100% confidence in any clinical situation (any pre-test probability)**,** Lowering the cut-off value further to ≥3% will result in a perfect sensitivity of 100%. A negative test (CD69 lymphocytes expression<3%) can exclude a possible diagnosis of neonatal sepsis with 100% confidence in any clinical situation**, Table (3-12)**.

Labib *et al.*, (2013), results showed that lymphocytes CD69 has Sensitivity Specificity Positive predictive value and Negative predictive value as , 100% , 44.4%, 83.9% and 100% ,respectively.

Regarding the WBC parameters, The confidence in a positive diagnosis is further increased to 96.1% when the differential diagnosis of sepsis is highly suspected (90% pre-test probability). A negative test on the other hand (obtaining a WBC count of <7800 $x10^6/mm^3$) can exclude a possible diagnosis of neonatal sepsis with 94.4% confidence in a clinical situation where neonatal sepsis is of very low possibility (pre-test probability = 10% only). Raising the cut-off value further to ≥9250 $x10^6/mm^3$ will make the test 100% specific and by extension 100% diagnostic, being able to establish a possible diagnosis of sepsis with 100% confidence in any clinical situation (any pre-test probability), **Table(3-12)**.

WBC count was considered as a poor test, one can explore the performance of the optimum cut-off value being set at ≥7800 $x10^6/mm^3$. At this cut-off value the test is 57.8% sensitive and 78.7% specific. A positive test at this optimum cut-off value (having a WBC count of 7800 $x10^6/mm^3$ or higher) can establish a possible diagnosis of probable neonatal sepsis with 73% confidence in a clinical situation where sepsis is of equal odds (50% pretest probability) ,**table (3-12).**

The confidence in a positive diagnosis is further increased to 96.1% when the differential diagnosis of sepsis is highly suspected (90% pre-test probability). A negative test on the other hand (obtaining a WBC count of <7800 $x10^6/mm^3$) can exclude a possible diagnosis of neonatal sepsis with
94.4% confidence in a clinical situation where neonatal sepsis is of very low possibility (pre-test probability = 10% only). Raising the cut-off value further to ≥9250 $x10^6/mm^3$ will make the test 100% specific and by extension 100% diagnostic, being able to establish a possible diagnosis of sepsis with 100% confidence in any clinical situation (any pre-test probability), **Table(3-13)**.

4.7. Genetic study

The CD14 is a component of the LPS receptor complex, and LPS fails to elicit a response in CD14– null mice **(Moore *et al*., 2000).** A polymorphic site with a C to T change has been identified 159 nucleotides upstream of the transcription start site (260 base pairs upstream of the start of translation),**(LeVan *et al*.; 2001).**

This polymorphic site is in the promoter region of the *CD14* gene, and the T allele shows increased transcriptional activity when assayed in reporter assays (**LeVan *et al*.; 2001**). As would be predicted, individuals homozygous for the _159T allele have increased levels of CD14 **(Koenig *et al*., 2002).** A

number of studies have been performed to examine whether the _159 polymorphic site is associated with infection or sepsis, with conflicting results **(Heesen *et al.*, 2002).**

4.7.1. CD14 /C-159T gene polymorphism and risk of culture positive (proven) neonatal sepsis.

Genotype analysis revealed 3 genotypes TT, CC, TC, when genotype distribution of the C-159T polymorphism in septic neonate, TT genotype was the most frequent in both proven and probable sepsis groups (53.3% and 55.6%) respectively. On the other hand, CC genotype was the predominant in control group (56%).There was a statistical significant higher rate of TT and lower CC genotypes in proven septic neonates as compared to control group ($p<0.001$), OR = 13.14 and 95% CI 4.37 – 39.48) ,**Table (3-14).**

Also there was a statistical significant higher rate of TT and lower CC genotypes in probable septic neonates as compared to control group ($p<0.001$), OR = 14.38 and 95% CI 5.18 – 39.9), **Table (3-15).**As there was a significant result with TT genotypes in relation to sepsis, it was verified that TT genotype represents a risk factor for sepsis. SNP of CD14 / -159 as regards CC and TT genotypes there was a statistical significant higher rate of TT genotype and lower CC in septic neonates as compared to control.The homozygous mutant genotype (TT) is uncommon in the control population (8.0%) but has an increased frequency in sepsis neonates (53.3%). This genotype confers an odds ratio (OR) of (13.1). While the heterozygous genotype (CT) is found in (36%) of the control subjects and (26.7%) of the patients and confers an OR of (0.65), in contrast the wild-type homozygous genotype (CC) had a higher frequency in the control subjects (56%) compared with sepsis neonates (20%) (OR) of (0.2), Moreover, the TT genotype has obviously suggests as an etiology for sepsis, as it has etiologic fraction (EF) of (0.493), In contrast, the CC genotype had rather preventive role as it had Protective Fraction (PF) of (0.450), **Table (3-14)**.

4.7.2. CD14 /C-159T alleles frequency

The T allele is higher frequency (66.7%) in proven septic neonates than C allele (33.3%) but in healthy neonates T allele (26 %), C allele (74 %), the T allele has obviously suggests as an etiological factor for sepsis, as it has an etiologic fraction (EF) of (0.574), In contrast, the C allele has rather preventive role as it had protective fraction (PF) of (0.82), **Table (3-14)**.

Similarly the T allele with higher frequency (68.9%) in probable septic neonates than C allele (31.1%) but in healthy neonates T allele (26 %), C allele (74 %), the T allele has etiologic fraction (EF) of (0.11), In contrast, the C allele has rather preventive role as it had Protective Fraction (PF) of (0.62), **Table (3-15)**.

The distribution of genotypes (TT, CC and CT) in patients and controls is in agreement with result of **Sharaf *et al*.,(2010),** It was found that TT genotype was 47.5% among septic neonates, while TT genotype was 25% in control group .

Similarly **Hartel *et al.*, (2008),** stated that CD14 were higher in the CD14 /C-159T homozygotes as CD14/-159T was transcriptionally more active than the allelic variant in monocytic cell. Also, **Martin *et al.,(*2005),** revealed that the homozygotic-159 T mutation of CD14 gene (CD14 /C-159T) is associated a high mortality rate in sepsis due to reduced response of the innate immune system in carriers of these mutation and might be associated with a higher rate of bacterial infections. Other study by **An-qiang *et al*., (2013),** has reported the CD14 /C-159T to be associated with sepsis with inconsistent results. **De Faria *et al.*, (2008),** reported that the CD14 /C-159T gene polymorphism has been associated with increased in vitro CD14 expression in the serum of children with altered IgE serum levels and positive allergic test in different population. These

data provide insight into-the pathogenic role of the CD14/ C-159T polymorphism in pathogenesis of sepsis as 159 TT may favor increased risk of acquiring infection. Larger population studies may clarify the actual role of genetic polymorphisms in a susceptibility to infection and may provide a clue to open the possibility for a new therapeutic intervention.

Conclusion

1. The serum levels of PCT are a more reliable marker than the WBC counts in the early diagnosis of neonatal sepsis.
2. Flowcytometric assessment of neutrophil CD64 and lymphocytes CD69 was able to differentiate between neonates with sepsis from neonates free from sepsis.
3. Neutrophil CD64 is a highly sensitive and specific marker for neonatal sepsis. Prospective studies incorporating CD64 into a sepsis scoring system are warranted. However, the important issues of cost and availability are required to be evaluated in routine clinical setting.
4. Flowcytometric assessment of neutrophil CD64 may be considered as a rapid and reliable marker for the diagnosis of bacterial neonatal sepsis in comparison to other conventional and routine diagnostics markers. Further large scale study may be conducted to get more precise result.
5. CD69 as T-cell marker activity in the first week of neonatal septicemia. And they may help in diagnosis and follow up of patients. Further research is needed to use these markers as early diagnostic onset markers of neonatal sepsis and as prognostic markers.
6. There is significantly higher prevalence of detection of SNP of *CD14 (C-159*t) gene polymorphism among patients with proven septic and probable septic neonates as compared to control group. This provides strong evidence that *CD14 (C-159*t) gene may play a major role in the susbtiplity for sepsis.

RECOMMENDA

1. Further studies are needed to measure the effectiveness of each leucocyte surface marker as a diagnostic tool and highlight more clearly the role of these markers in neonatal sepsis.

2. The benefit of measuring serum PCT routinely in the diagnosis and follow-up of neonatal sepsis, is that it reduces the hospital costs. Such a benefit might support a wider acceptance of the test in the routine practice.

3. In the future, neutrophil CD64 should be further evaluated and considered as a potential neonatal sepsis biomarker in routine clinical settings.

4. With the evidence that genetic factors play a role in the susbtiplity for sepsis as confirmed by other studies, the evaluation of other genetic such as HLA genotyping and polymorphisms properly
addresses the need for a more rigorous and objective studies.