DIAGNOSTIC UTILITY OF NUCLEATED RED BLOOD CELLS IN DIAGNOSIS OF EARLY ONSET NEONATAL SEPSIS

By

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Dissertation submitted to the

BLDE (DEEMED TO BE UNIVERSITY),
Vijayapur, Karnataka

In partial fulfillment of the requirements for the award of the degree of

DOCTOR OF MEDICINE

IN

PATHOLOGY

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2019

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ACKNOWLEDGEMENT

It is most appropriate to begin by expressing my gratitude to Almighty for all his blessings.

I thank my parents **Mrs.Latha.N.P and Mr.Shankar.B.P** for their constant support and encouragement.

I would like to express my sincere and deepest gratitude to my teacher and guide **Dr. R M Potekar, Professor, Department of Pathology**, for his encouragement and invaluable guidance throughout the course of my study. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my study.

I am equally grateful to **Dr. B.R. Yelikar, Professor and H.O.D, Department of Pathology** for his valuable suggestions given at all the steps of the study. He has a profound influence on both my personal growth and professional pursuits.

I am also extremely fortunate to have a caring, approachable and supportive department, who have advised and mentored me and made it possible for me to expedite this dissertation.

I am thankful to **Dr. S. S. Kalyanshettar, Professor, Department of Pediatrics** for his valuable suggestions and guidance in all the time of research and thesis writing.

A sincere and heartfelt thanks to **Dr. S.U. Arakeri** Prof**, Dr. S.B. Hippargi** Prof, **Dr. Mahesh H. Karigoudar** Prof, **Dr. Girija Patil** Assoc Prof, **Dr. Prakash M. Patil** Assoc Prof, **Dr. Vijayalaxmi S Patil** Asst. Prof, **Dr. Savitri M. Nerune**

Asst prof, **Dr. Mamatha K.** Asst Prof and **Dr. SnehaJawalkar** Asst Prof for their supervision, assiduous concern and positive feedback at all steps of this work.

I am very grateful to **Dr. Anil Reddy,** Asst Prof for his valuable opinion and support throughout the study.

Special thanks to my dearest friends, my seniors and juniors who have helped and encouraged me during my work.

I also thank **Mr Mohd. Shahnawaz** Lecturer Statistics, for his guidance during my dissertation work.

My heartfelt thanks to my husband **Dr. Anup Banur**, my brother **Mr.Ruthwik.B.S**, and parents in law **Savitri** and **Umapathi**, for their help, constant encouragement and moral support that led me to successfully complete this dissertation work.

I am thankful to the Technicians and non teaching staff of Department of Pathology.

Last but not the least**,** my sincere gratitude to all my study subjects for their contribution to this study.

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LIST OF ABBREVATIONS USED

EOS	: Early onset sepsis
LOS	: Late onset sepsis
nRBC	**:** Nucleated red blood cells
CRP	: C Reactive Protein
SAA	: Serum Amyloid A
NK cells	: Natural killer cells
PCT	: Procalcitonin
LBW	: Low birth weight
VLBW	: Very low birth weight
IL	: Interleukin
HSS	: Hematological Scoring System
PPV	: Positive predictive value
NPV	: Negative predictive value

ABSTRACT

BACKGROUND:

Neonatal sepsis is one of the principal cause of neonatal morbidity and mortality in the developing countries. Early onset neonatal sepsis presents with vague and non specific signs and symptoms. The blood culture which is considered to be standard test, will be available after 48-72 hours. When mother has been treated with intrapartum antibiotics, the test becomes unreliable. The nucleated RBCs are commonly found in neonatal blood. Increased counts are often the result of prematurity, increased erythropoiesis, acute stress mediated release from marrow stores and post natal hypoxia.

OBJECTIVE:

To study the utility of nucleated red blood cells in diagnosis of early onset neonatal sepsis

MATERIALS AND METHODS:

A cross sectional hospital based study was carried out on neonates fulfilling the inclusion and exclusion criteria attending inpatient department of Pediatrics, referred to the Department of Pathology.

Study period: 1st October, 2016 to 30th June, 2018

Blood samples were collected in plain and EDTA anticoagulated vacutainers from umbilical cord blood or arterial or venous blood for blood culture and analysis of hematologic parameters respectively.

On peripheral smear nRBCs were counted per 100 WBCs, immature granulocytes, toxic granules in neutrophils, platelet counts and B:N ratio were noted. Modified

hematological sepsis score was done using six parameters which included nRBCs and scores were assigned accordingly.

RESULTS:

Of the 131 neonates in the study, 78 were males and 53 were females. 109 cases were term neonates and 22 cases were preterm neonates. The nRBC counts were increased in 18 out of 19 culture proven cases of sepsis. The sensitivity, specificity, positive predictive value and negative predictive value were 95%, 46%, 23% and 98% respectively. The sensitivity and specificity of Modified hematological scoring system was 94.7% and 83.9% respectively.

CONCLUSION:

The nucleated red blood cells are an immediate reliable marker in the diagnosis of early onset neonatal sepsis. It is an inexpensive, rapid and easy test. Modified hematological scoring system uses six hematological parameters including nRBC counts which has better sensitivity and specificity. The efficacy of complete blood count is increased with the use of the scoring system.

KEYWORDS:

Nucleated red blood cells, blood culture, early onset sepsis

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INTRODUCTION

"Neonatal sepsis is a systemic response by host to the bacterial infection, is one of the prime causes of neonatal morbidity and mortality in India".[1]According to the data obtained from National Neonatal Perinatal Database (NNPD) 2002-2003, the incidence of neonatal sepsis in India was 30 per 1000 live births.[2,3]

Any newborn with clinical suspicion of sepsis, early diagnosis is of paramount importance and treatment has to be provided early in order to prevent severe and life threatening complications. The clinical manifestations of early onset neonatal sepsis are vague and nonspecific.[2] The mortality related to sepsis can be prevented with the use of appropriate antibiotic therapy and supportive care.

All the neonates with clinical suspicion of sepsis should get a sepsis screen done for the validation of diagnosis. Blood culture is regarded as the gold standard diagnostic test for diagnosis of neonatal sepsis but a minimum of 48-72 hours have to be waited for the culture and sensitivity report.[2,3]

The significance of various factors like C reactive protein, hematological parameters like total neutrophilic count, total leucocyte count, immature to total mature cells ratio, platelet count and toxic granules in the neutrophils have been studied in diagnosing neonatal sepsis.[1-4]

Various studies have been done on the role cell surface antigens, cytokines and chemokines, polymerase chain reaction and molecular markers in early diagnosis of neonatal sepsis. Though these tests are rapid and have a less turnaround time, these cannot be used in routine clinical practice due to scarcity of resources and excess cost.

The nRBCs are found normally in circulation in neonates. The counts can increase as result of premature birth, post natal hypoxia, acute stress mediated release from the bone marrow stores and increased erythropoiesis in chronic conditions [4,6]

There is a persistent search for a test which is rapid, fundamental and cost effective that can be done in the early newborn period, which can bring a significant impact in the neonatal health care.

This study was undertaken to understand the role of nucleated red blood cells in the diagnosis of early onset neonatal sepsis.

AIMS AND OBJECTIVES

To study the diagnostic utility of nucleated red blood cells and modified hematological scoring system in early onset neonatal sepsis

REVIEW OF LITERATURE

Neonatal sepsis is one of the principle cause of neonatal morbidity and mortality in the developing countries accounting for upto 30 – 50% of total neonatal deaths per year.[3]

Klebsiella pnuemoniae (32.5%) was the most common organism isolated followed by Staphylococcus aureus (13.6%) among the hospital births. Among the neonates referred from other hospitals/community, Klebsiella pneumonia (27%) was most frequently isolated pathogen followed by Staphylococcus aureus (15%) and Pseudomonas (13%).[2,3]

"Neonatal sepsis is defined as a clinical syndrome characterized by signs and symptoms of infection with or without accompanying bacteremia in first month of life".[2,3] It includes infection of various systems like pneumonia, osteomyelitis, meningitis and urinary tract infections.

Depending on the onset of symptoms it is classified into two divisions.

Early onset neonatal sepsis (EOS): The neonate presents with signs and symptoms of sepsis within 72 hours of birth. The neonates can present with symptoms at birth in cases of severe maternal infection. The infection is transmitted directly from the mother to the neonate before or at the time of birth.

The incidence of EOS is ten folds higher in very low birth weight neonate. The mortality rate of VLBW neonate with sepsis is higher compared to term neonate with sepsis or VLBW neonate with no sepsis. [2,7,8]

The following factors are associated with increased risk of early onset sepsis [2,9,10]

1. Premature born or Low birth weight neonate (<2500gms)
2. Foul smelling and/or meconium stained amniotic fluid
3. Prolonged rupture of the amniotic membranes
4. Prolonged labor (>24 hrs including 1st and 2nd stage of labor)
5. Any signs of bacterial infection in the mother within 2 weeks prior to the delivery
6. Unsterile vaginal examination or > 3 sterile vaginal examinations during the process of labor
7. Perinatal asphyxia

The neonates with any two of the above mentioned risk factors should be thoroughly investigated for the presence of sepsis and treated appropriately.

Late Onset Sepsis (LOS): The neonate usually presents with signs and symptoms of sepsis after 72 hours of age. The common source of infection in late onset sepsis is either community acquired or hospital acquired (nosocomial). The risk factors associated with nosocomial infection are prematurity, low birth weight, admission to the neonatal intensive care unit, mechanical ventilation, invasive procedures and administration of parenteral fluids. The factors which increase the risk of community acquired LOS are poor care of the cord, poor hygiene, bottle feeding and prelacteal feeds.[2,7,8,10]

Pathogenesis of sepsis:

The fetus is protected from the microbial flora in the intrauterine life by the intact amniotic membrane, certain antimicrobial factors in amniotic fluid and placenta. The organisms can be introduced by certain procedures like chorionic villous sampling, transcutaneous blood sampling, amniocentesis and cervical encirclage.

Few organisms like Listeria monocytogenes and Treponema pallidum can infect the fetus from the maternal blood inspite the protective mechanisms of the placenta. But the infection caused by these organisms cause congenital anomalies or still birth of the fetus.[11,58]

Initially microflora from the birth canal colonizes the neonate after the rupture of the membranes during the process of delivery. If membranes are ruptured for a long period of >24 hours the organisms from the vagina ascend upwards leading to inflammation of placenta, amniotic membranes and the umbilical cord.

The fetus can aspirate the infected amniotic fluid resulting in infection and leading to neonatal sepsis, preterm delivery and intra uterine deaths. Any infection in the mother, specifically genital tract infection is the main pathway of transmission of infection from mother to the fetus. Transplacental spread of infection can also occur. The neonate can also acquire infection from the surroundings in the NICU or at home.[11,58]

Prepartum and intrapartum complications can cause increased risk of sepsis like maternal fever, forceps extraction, prolonged rupture of membranes(PROM) and early onset of labor. The risk increases with the use of equipments for respiratory

support and nutrition of the newborn like ventilators, urinary catheters, intravenous and intraarterial cannulas and central venous cannulas.[11,58]

Complements - The complement levels are lower and have less activity compared to healthy term neonates. Due to deficiency of chemotactic activity derived from the complements, opsonization of organisms is diminished in the absence of antibodies.

Neutrophils – The neutrophils are more in number with peak at 12 hours in preterm and term neonates and return to adult levels after 22 hours.

The band forms of neutrophils are less than 15% in neonates and they increase as result of infection and as a response to other stress. In both preterm and term neonates the chemotaxis of neutrophils is defective and abnormal. They have decreased aggregation, deformability and adhesion which may delay the initiation of host response against the infection.

In preterm neonates the oxidative respiratory burst is abnormal, which increases the risk of sepsis.

Neonates with intrauterine growth retardation and preterm neonates frequently show neutropenia which is factor in increased risk of sepsis.[12,13]

Natural killer cells (NK cells) - NK cells are present in the cord blood and appear in early period of gestation.

They have decreased antibody dependant cell mediated cytotoxicity (ADCC) and cytotoxic activity compared to NK cells present in adults.

Monocytes and macrophages – In preterm infants the function of macrophages is diminished in the reticuloendothelial system, though the number of circulating monocytes are normal in number.

The chemotaxis of monocytes is defective in both term and preterm neonates which affects the host response to infectious agent.

Cytokines - In response to infection various inflammatory mediators are released like IL-6, IL-8 and TNF α.[12,13]

Clinical evaluation: [14]

- History
- Maternal infection during pregnancy or during labor

  Chorioamnionitis

  UTI
- Gestational age and birth weight
- Order of birth
- Duration of rupture of membranes
- Complicated delivery (Use of forceps, vaccum assisted)
- Fetal distress
- Any medical intervention – Endotracheal tubes

  Intravenous and arterial cannulas

  Indwelling catheters

Clinical features of neonatal sepsis:

Non Specific signs and symptoms- The signs of early onset sepsis are nonspecific and subtle. Neonates may present with one or more of the following signs and symptoms.

a) Fever or hypothermia
b) Refusal for feeds, feeble cry, lethargy
c) Poor capillary filling time
d) Absence of reflexes and hypotonia
e) Respiratory distress, gasping respiration
f) Bradycardia/ Tachycardia
g) Hyperglycemia/hypoglycemia
h) Metabolic acidosis

Specific signs and symptoms related to various organ systems-

Central nervous system - Excessive crying, irritability, seizures, neck stiffness.

Cardiovascular system – Shock, hypotension, poor perfusion.

Renal system – Acute renal failure

Gastrointestinal system – Necrotizing enterocolitis, diarrhea, vomiting, intolerance to feeds, paralytic ileus.

Hepatobiliary system – Hyperbilirubinemia

Haematlogical changes – Petechiae, bleeding from various sites

Skin changes – Mottling, pustules, abscess, discharge from the umbilicus.[2,8,15,16]

INVESTIGATIONS-

Many investigations are carried out to rule out septicemia which include blood culture, acute phase reactants like C- Reactive protein, serum procalcitonin, inter-alpha inhibitor protein, serum amyloid A, cytokines and chemokines.

Blood culture - The gold standard test for diagnosis of sepsis is blood culture and it is done in all the cases of clinically suspected sepsis before starting antibiotics. It is a best guide for the clinician to start antibiotics when a positive blood culture is obtained along with sensitivity of the bacterial isolate.

But the turn around time is quiet long for this test based on which the clinical decisions on treatment have to be made. The blood cultures have to be observed for minimum of 72 hours before giving the report as sterile. [8]

When mother has been treated with intrapartum antibiotics, the test becomes unreliable. The blood culture cannot detect bacteraemia in very low birth weight infants because bacteraemia is intermittent and transient. And its also because of insufficient blood sample being inoculated into blood culture bottle. [8] The blood samples taken from the intravenous lines and catheters will be contaminated.

Sepsis screen - The neonates with clinical suspicion of sepsis should have a sepsis screen done to establish a diagnosis. It is comprised of the following – Leucocyte count, immature to total neutrophil ratio, absolute neutrophil count, micro ESR and C reactive protein.

Components of sepsis screen-[2]

Components	Abnormal value
Total leucocyte count	$<5000/mm^3$
Absolute neutrophil count	$>600/mm^3$
Immature/total neutrophil ratio	> 0.2
Micro ESR	>15 mm in 1^{st} hour
C reactive protein	> 1 mg/dl

HEMATOLOGICAL PARAMETERS-

Leucocyte count – Total counts above $30,000X10^9$ and below $4000X10^9$ are considered to be abnormal. In 50% of cases with sepsis leucocyte count will be normal in the initial phase and become abnormal after twelve hours. [8]

Table No 3.1:Leukocyte Reference Ranges in Term and Preterm Neonates during the First 72 Hours of Life (10^3 cells/µL) [17]

Age	Total WBC	Neutrophil	Band form	Lymphocyte	Monocyte	Eosinophil	Basophils
Term							
Birth	10-26	5-13	0.4-1.8	3.5-8.5	0.7-1.5	0.2-2	0-1
12 hrs	13.5-31	9-18	0.4-2	3-7	1-2	0.2-2	0-1
72 hrs	5-14.5	2-7	0.2-0.4	2-5	0.5-1	0.2-1	0-1
Preterm							
Birth	5-19	2-9	0.2-2.4	2.5-6	0.3-1	0.1-0.7	0-1
12hrs	5-21	3-11	0.2-2.4	1.5-5	0.3-1.3	0.1-1.1	0-1
72 hrs	5-14	3-7	0.2-0.6	1.5-4	0.3-1.2	0.2-1.1	0-1

Zawar MP *et al*[18]observed sensitivity and specificity of 82%and 70% respectively. Leucopenia was present in 83% of culture positive cases.

Bhat RY *et al*[19] observed total leucocyte count of < 5000 and > 20000 in 29.7% of cases. It had significantly more positivity among the symptomatic than the asymptomatic neonates.

Buch *et al*[20] considered total count of <5000 cells/cumm or >20,000 cells/cumm as abnormal. They observed 50.77% sensitivity and 63.4% specificity. They concluded that total leucocyte count alone is not significant in the diagnosis of neonatal septicemia.

Table No 3.2 : Reference Ranges for ANC and I:T Neutrophil Indices in the Neonate during the First 72 Hours of Age [21]

	ANC(in cumm)	I:T ratio
AGE		
Birth	1800-5400	<0.16
12 hours	7800-14400	<0.16
24 hours	7200-12600	<0.13
72 hours	1800-7000	<0.13

Immature to total neutrophil ratio-

The normal value for the first 24 hours of life is 0.16, falls by 60 hours to 0.13. From 5 – 28 days the normal value is 0.12.

Christensen RD *et al*[22] observed that in many infants, in spite of a shift to left in the neutrophil count due to exhaustion of marrow reserves, there was no rise in the absolute number of band cells in circulation. Inspite of its less sensitivity, immature neutrophil count is specific for diagnosis of neonatal sepsis and elevated counts of immature cells are unusual in an unaffected neonate. Immature to mature neutrophil ratio of greater than 0.3 was present in 93% cases with sepsis compared to normal neonates.

Philip AG *et al*[23] observed that immature to total neutrophil ratio >0.2 had a better sensitivity in detection of septicemia. They reported an elevated Immature: Total neutrophil (I:T) ratio and Immature : Mature neutrophil (I:M) ratio in 85% and 87% of the cases of neonatal sepsis, respectively. Thus it was concluded that an increased I:T ratio can aid in the early diagnosis of infection.

Platelet count – The platelet count of neonates is same as that of adults and older children. Irrespective of the gestational age the counts less than $150X10^3/\mu l$ are considered as low.

Sepsis is usually associated with thrombocytopenia. At the beginning changes occur in leucocytes later the platelet levels fall. So thrombocytopenia is considered as a late sign of sepsis.

The various mechanisms which are responsible for thrombocytopenia are

1. Decreased production of platelet from the bone marrow
2. Few infections cause disseminated intravascular coagulation.
3. Sepsis causes damage to the endothelium leading platelet adhesion and aggregation , excessive destruction by the reticuloendothelial system.[24]

Few studies have shown that fungal infections or infections by gram negative organisms are associated with thrombocytopenia for prolonged durations compared to infection by gram positive organisms. [25]

Grading of thrombocytopenia:

a) Normal - >1,00,000/μL
b) Mild thrombocytopenia – 50,000-99,000/μL
c) Moderate thrombocytopenia – 30,000-49,000/μL
d) Severe thrombocytopenia - < 30,000/ μL

Nucleated red blood cells

Lippmann in 1924 reported the presence of nucleated red blood cells in circulatory blood of forty one out of forty two neonates in their first day life. Normal

values of nRBCs were about 500 nRBCs/mm^3 or 0.1% of the neonates blood cells in circulation. If it is expressed per 100 WBCs, values above 10 nRBCs are considered to be elevated.

Nucleated red blood cells are produced by the bone marrow of the fetus due to stimulation by erythropoietin and they are stored in the bone marrow.[26]

In the first half of gestational period nRBC s are present in the placental blood vessels, but are reduced in second half of pregnancy. They are absent or only few may be present in the term placenta. The presence of nRBCs in the placenta of term baby is non specific and indicate fetal hypoxia, anemia in fetus and TORCH infections.[26,27]

Studies have been done which showed that nRBCs decrease consistently as the gestational age increases. The pre term neonates have high counts of nRBCs than term neonates.

nRBCs are increased in circulation in many acute and chronic conditions, either due to increased activity of erythropoietin or release from marrow storage pool. Acute chorioamnionitis is one of the important cause of raised levels of erythropoietin and increased levels of nRBCs. [26,27]

Maier *et al*[28] found that neonates whose placentas showed features of chorioamnionitis had increased levels of erythropoietin. Leikin *et al*[29] found that placentas which showed chorioamnionitis on histology without clinical evidence of infection showed increase in nRBCs.

Abhishek MG *et al*[1] carried out a study on 60 neonates and found that nRBCs were reliable parameter in diagnosing neonatal sepsis.

Boskabadi H *et al*[30] conducted a study on 154 neonates and found that mean nRBC count was higher in the case group than the control group. They concluded that nRBC counts are helped in diagnosis of sepsis along with other hematological parameters.

Units of reporting-

The nRBCs are expressed as absolute count of cells per unit volume as nRBC/L or nRBC/mm3. They can be reported as number of nRBCs relative to 100 white blood cells. Most of the research papers and laboratories use nRBCs/100WBCs to report nucleated RBCs.[26, 27]

Morphology of neutrophils

Neutrophils also show degenerative and morphological changes such as dohle bodies, toxic granules and cytoplasmic vacuolations. Toxic granules are primary granules (azurophilic granules) seen within the cytoplasm of neutrophils. They are eosinophilic, peroxidase positive granules and stain more darker than the granules within normal cells.

Buch AC *et al*[20] demonstrated that the engulfed bacteria are digested by the neutrophils which depends upon enzymes released from granules (lysosomes) present within the white blood cells. The cell membrane invaginates along with these products of digestion and they are enclosed within a vacuole. The membrane of the vacuole fuses with the membrane of the lysosome.

Erythrocyte sedimentation rate – It is one of the important constituent of septic screen and has been recognized as valid investigation in diagnosis of sepsis. The normal range for ESR is day of life plus 3mm/hr up to 15mm/hr.[31]

ESR raises only after 24 – 48 hours after the infection. It is more useful for screening and for serial monitoring of the disease.[32]

C reactive protein –

Tillet and Francis first described CRP in 1930. It is named after C-carbohydrate of pneumococcus. It is an acute phase reactant synthesized by the liver in response to any infection and tissue injury. It is an endogenous peptide secreted by liver by the activation of IL-6.

The normal reference range is 0-10μg/ml. The levels above 8μg/ml indicate an inflammatory response. Its levels raise in the blood by six to eight hours to any exposure to injury or infection. It has a half life of nineteen hours and during acute phase response it increases by 1000 folds. [33]

CRP can be elevated in certain conditions like respiratory distress syndrome, fetal hypoxia, meconium aspiration syndrome and intraventricular hemorrhages. These conditions mimic sepsis clinically.[34]

Serial measurements of CRP is more helpful in assessing the prognosis of sepsis. A fall in the CRP levels by 25% compared to the previous day levels is a good indicator of improvement. CRP alone cannot be used as a marker for the primary diagnosis of neonatal sepsis but can be used as a part in a panel of tests conducted to diagnose sepsis.[35,36]

In a study conducted by Khassawneh M *et al*[37] it was concluded that IgM, IL-6 and CRP were useful in the early diagnosis of sepsis caused by gram negative organisms.

Another study conducted by Cetinkaya M *et al*[38] compared the utility of CRP and procalcitonin (PCT) with that of Serum Amyloid A (SAA). It showed that SAA is a reliable and more accurate marker compared to CRP and PCT in early diagnosis and follow up of sepsis. [3, 37]

Russell G.A.B *et al*[39] found that serial estimation of CRP was better than percentage of band forms and immature to total neutrophils ratio in diagnosing sepsis.

Stephan E *et al*[40] found that CRP levels were most important parameter in guiding the duration of treatment with antibiotics of neonates with suspected sepsis.

Serum procalcitonin-

Procalcitonin is a precursor of calcitonin, which is produced by thyroid gland. It contains 116 amino acids. It is thought to be produced by macrophages of the liver in cases of sepsis.[11,41]

In study conducted by Cheisa C *et al*[11] among 28 culture proven cases of sepsis, 24 had raised procalcitonin levels with a sensitivity of 85.7%. After first 48 hours of life, PCT concentration was ideal marker in identification of neonates with sepsis.

In study conducted by Sucilathangam *et al*[41] 22 out of 50 cases showed elevated PCT of >10ng/ml whereas CRP was elevated only in 18 cases. Serum PCT levels were increased in 13 out of 14 culture positive cases.

Kocabas E *et al*[42] studied the role of procalcitonin, C reactive protein, interleukin 6, interleukin 8 and tumor necrosis factor α in 26 neonates who had blood cultures positive and found that IL 6, CRP and IL 8 had lower sensitivity, specificity

and diagnostic accuracy. They concluded that PCT and TNF α were best markers that helped in diagnosis and also to assess the prognosis of the disease.

Hematological scoring system – It is panel of tests including various hematological parameters. Scores are assigned for each parameter for assessment of possibility of sepsis in neonate.

Rodwell *et al*[43] formulated a criteria comprised of complete blood cell count for evaluation of sepsis in neonates. A scoring system was prepared and scores were assigned for each category.

Totally 298 neonates were evaluated for sepsis. Twenty six out of 27 neonates with sepsis and 23 neonates with probable infection had a score of > 3. The cases with score >3 had 31% likelihood of sepsis. Higher the score greater the possibility of sepsis. Thus it was concluded that hematologic scoring system improved the diagnostic accuracy of complete blood count as a screening tool for diagnosis of sepsis.[43]

Table No. 3.3: Criteria of hematological scoring system[43]

Criteria	Abnormality	Score
Total WBC count	<5,000/μl >25,000/μl at birth >30,000/μl 12–24 h >21,000/μl Day 2 onwards	2 1
Total PMN count	No mature PMN seen Increased/decreased	2 1
I:T PMN ratio	Increased	1
I:M PMN ratio	>0.3	1
Degenerative changes in PMN	Toxic granules/cytoplasmic vacuoles	1
Platelet count	<1,50,000/μl	1
Immature PMN	Increased	1

Khair BK *et al*[44] conducted a study on 100 clinically suspected cases of sepsis and evaluated various hematological parameters. They found that a score of >4 had PPV of 26%, NPV of 100%, specificity of 60% and sensitivity of 100%. Due to high sensitivity and negative predictive value they concluded that HSS was a reliable tool for diagnosis of sepsis than considering individual hematological parameters.

Ghosh *et al*[45] studied 103 peripheral blood smears of neonates using hematological scoring system of Rodwell et al. for early detection of sepsis. They found that abnormal immature to total neutrophil ratio followed by abnormal immature to mature neutrophil ratio were most sensitive indicators in identifying

neonates with sepsis. They concluded higher the score greater the probability of sepsis.

Narasimha A *et al*[46] studied 50 cases of neonates and evaluated for sepsis using HSS using Rodwell *et al* criteria. The sensitivity for I: M ratio was higher in their study and thrombocytopenia was consistently associated with poor prognosis.

Sriram R *et al*[47] correlated septic screen with sepsis score. They concluded that sepsis screen was more sensitive for excluding the diagnosis of neonatal sepsis, which could be done with two screens 12 to 24 hours apart. In a neonate who is stable or suspected of sepsis because of maternal risk factors, antibiotics should be initiated after the septic screen. Since symptoms suggestive of sepsis may be caused by many other illness, confirmation of sepsis by sepsis screen may help in avoiding unnecessary antibiotic therapy.

Makkar M *et al*[48] did performance evaluation of hematological scoring system in early diagnosis of neonatal sepsis. All smears were analyzed by using Rodwell criteria. Assigned score of 1 for each 7 criteria. Hematological scoring system had higher sensitivity and specificity in preterm than in term neonates. Positive predictive value were higher in preterm than term for HSS. It was also seen that with increasing scores, the likelihood of sepsis also increased.

Namdeo UK *et al*[49] studied the usefulness of a combination of various parameters in the early diagnosis of neonatal septicemia and reported that Band to total neutrophil count ratio of >0.3, micro ESR of > 8mm, neutrophils with toxic granules in more than 40%, leucopenia were particularly predictive of septicemia with a specificity of 98% and positive predictive accuracy of 89%. If more than one of the

four tests were positive, they became more sensitive. They also observed an elevated I:T ratio in 84% of the culture positive cases studied by them.

Misra RN *et al*[50] formulated a sepsis screen using following parameters: Band to neutrophil ratio of >0.2, micro ESR >8mm at the end of one hour, leucopenia < 5000 cells/cumm, thrombocytopenia < 1,50,000 cells/cumm. He found that when two positive test combinations were analyzed for detection of sepsis, it was observed that the best combination was band to total neutrophil count ratio with micro ESR and leucopenia which had highest predictive accuracy of 94% and also high specificity of 96%.

Sharma *et al*[51] evaluated the use of sepsis screen in the early diagnosis of septicemia. The parameters and their cut off values used by them were total leucocyte count < 5000 cells/cumm or >20,000 cells/cumm, band to neutrophil ratio of >0.2, gastric aspiration cytology > 5 polymorphs/hpf, micro ESR >10 mm at the end of one hour and CRP > 6μg/ml. Among the individual tests, CRP had the maximum sensitivity (80%) and specificity (93.8%). Toxic granules were seen in 60% of the cases.

Varsha *et al*[52] evaluated the validity of hematological parameters in identification of early and late onset neonatal sepsis. Criteria used by them were total leucocyte count, TNC, I:T ratio, I:M ratio and CRP. They concluded that CRP elevation, leucopenia, neutropenia and elevated I:T ratio are comparably good diagnostic aids while after 3 days of life, CRP is the best single test.

Shirazi Haider *et al*[53] studied on role of hematological profile in early diagnosis of neonatal sepsis. They found sensitivities of the parameters studied were

below 60%. However the specificities were more than 70%. In their tests, individual parameters had not desired specificities but if put together can be a good tool in ruling out the possibility of the neonatal sepsis.

Ahirrao BM *et al*[54] conducted a prospective study of 303 neonates with clinical suspicion of sepsis and perinatal risk factors. 77 cases showed positive blood culture. I:M ratio and I:T ratio were increased in most of the cases. HSS showed a score of >3 in all 77 culture positive cases.

Chaware AS *et al*[55] carried out a study on 160 neonates and concluded that hematological scoring system was a simple, cost effective, sensitive and readily available test for diagnosis of sepsis. According to them a score of >4 was a reliable than individual hematological parameter.[56]

Krishnamurthy V *et al*[57] carried out a study on 75 neonates. The samples were scored for hematological scoring system and a modified hematological scoring system. Few components of hematologic scoring system were repetitive because of same pathogenesis and were falsely giving higher scores. So in modified HSS, immature neutrophils count and immature : mature neutrophils ratio were removed and nRBC counts were included. Role of nRBC count in neonatal sepsis is recognized recently and it deserved its inclusion in modified HSS. They found that at score of 3, the sensitivity and specificity of modified HSS was 84% and 82% respectively. It was similar to sensitivity and specificity of HSS, but specificity was increased by 10%.[57]

MATERIALS AND METHODS

Source of data :

A cross sectional hospital based study was carried out on neonates fulfilling the inclusion and exclusion criteria attending inpatient department of Pediatrics, referred to the Department of Pathology in BLDE (Deemed to be University) Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapur.

Study period: 1st October, 2016 to 30th June, 2018

Methods of collection of data.

The study included the neonates with clinical suspicion of sepsis at birth, within 72 hours and had any history of infection in the mother.

Data of the neonates with clinical suspicion of sepsis admitted to the NICU were collected. Clinical, perinatal history and demographic data like age, sex, gestational age -preterm/full term, weight and manner of delivery were noted.

Based on clinical and laboratory parameters, neonates were grouped into

1. Proven sepsis
2. Clinical sepsis
3. Suspected sepsis

Neonates with positive culture reports were considered as proven cases of sepsis. Neonates with clinical features of sepsis along with positive septic screen were considered as clinical sepsis. Neonates with clinical features of sepsis were considered as suspected sepsis.

Blood samples were collected in plain and EDTA anticoagulated vacutainers from umbilical cord blood or arterial or venous blood for blood culture and analysis of hematologic parameters respectively.

Procedure for collection of blood sample – A small area of skin was prepared over the vein identified. The area was cleaned with alcohol and with povidone iodine. It was allowed to dry for one minute. Blood sample were drawn and collected in plain and EDTA vacutainers.

Blood sample collected in plain vacutainer was inoculated into glucose broth and incubated for 48 hours and streaked to Mc conkey agar.

Blood samples collected in EDTA anticoagulated vacutainers were run in quantitative haematology analyzer Sysmex XN-1000 to obtain various hematologic parameters. Peripheral smears were prepared, stained using Leishman's stain.

Procedure of Leishman's stain – A thin blood film was prepared and air dried.

The slide was flooded with stain. After 2 minutes double the volume distilled water was added and stained for 5-7 minutes. The slide was washed with buffered water and kept upright for drying.

On peripheral smear nRBCs were counted per 100 WBCs, immature granulocytes, toxic granules in neutrophils, platelet counts and B:N ratio were noted.

Modified hematological septic score was done using the following parameters scores were assigned accordingly.

Table No. 4.1: Modified hematological scoring system

Criteria	Abnormality	Score
Total WBC count	<5,000/µl >25,000/µl at birth >30,000/µl 12–24 h >21,000/µl Day 2 onwards	2 1
Total PMN count	No mature PMN seen Increased/decreased	2 1
I:T PMN ratio	Increased	1
Degenerative changes in PMN	Toxic granules/cytoplasmic vacuoles	1
Platelet count	<1,50,000/µl	1
nRBC count	>10% <10%	1 0

Sample Size :

Using expected incidence as 30 per 1000 live births, expected sensitivity as 35% and expected specificity as 54% and desired precision as 15%, the sample size is 131.

This sample size will give the precision of 15 % or less for both sensitivity and specificity.[1]

Formula used:

$$n = \frac{Z^2 P(1-P)}{D^2}$$

n= sample size

Z = Z statistic for a 15% level of confidence

P = expected incidence

D =desired precision

Statistical analysis:

Data was analyzed using

1. Mean ± S.D
2. Chi square test for association
3. Comparison of means using test
4. ANOVA for comparison between and among groups
5. Diagrammatic presentation
6. Sensitivity and specificity analysis

Inclusion criteria

- Neonates with clinical suspicion of sepsis at birth and within 72 hours of birth
- Neonates with history of infection in mother

Exclusion Criteria

- Neonates with suspected inborn errors of metabolism
- Neonates with any congenital anomalies
- Neonates with hemolytic disease of newborn

RESULTS

A total of 131 neonates/newborns were included in the study group, with an age range of 1- 3 days who presented with signs and symptoms of sepsis to BLDE (Deemed to be University) Shri B.M. Patil Medical College Hospital & Research Center between October 2016 to June 2018.

Among these 131 cases, 109 (83.2%) were 1 day old, 10 (7.6%) were 2 days old and 12 (9.2%) cases were three days old. (Table No.5.1 & Fig No. 5.1).Seventy-eight (59.5%) cases were males and 53 (40.5%) cases were females (Table no. 5.2 & Fig No. 5.2)

TABLE NO 5.1: DISTRIBUTION OF CASES ACCORDING TO AGE

AGE IN DAYS	N	%
1	109	83.2
2	10	7.6
3	12	9.2
Total	131	100.0

FIGURE NO. 5.1: DISTRIBUTION OF CASES ACCORDING TO AGE

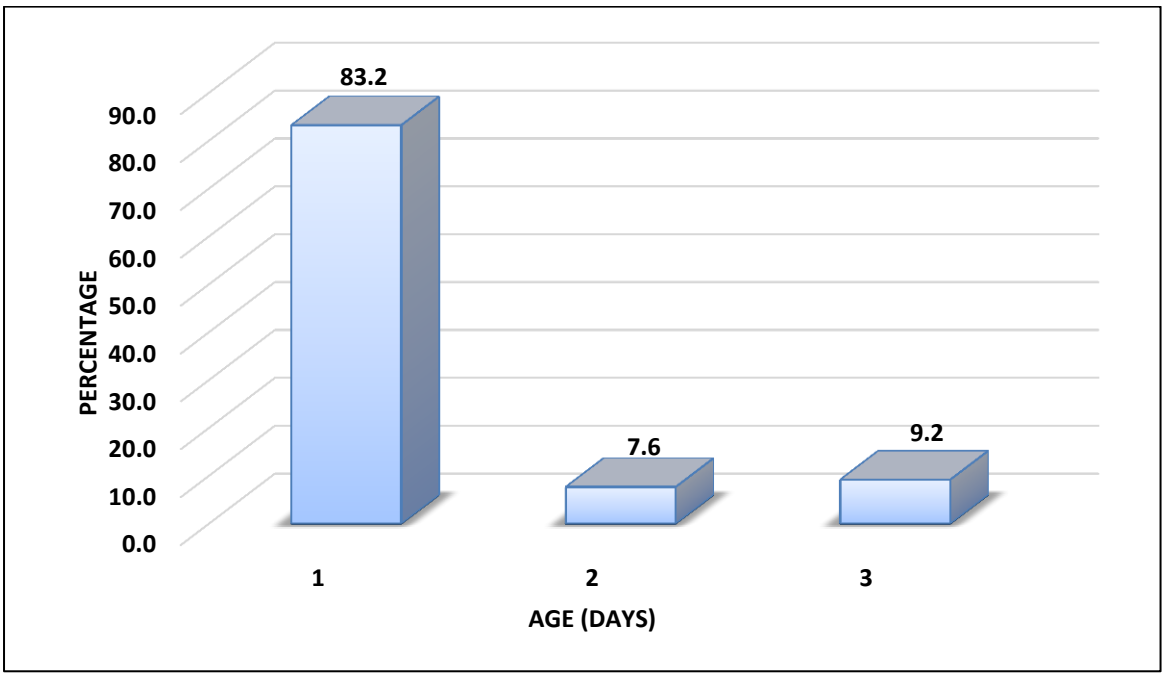
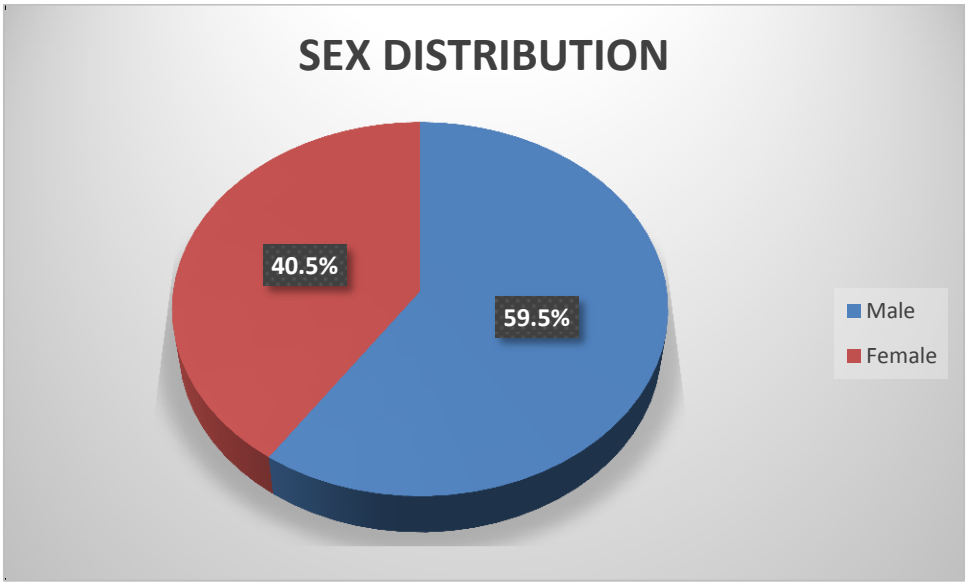


TABLE NO 5.2: DISTRIBUTION OF CASES ACCORDING TO SEX

SEX	N	%
Male	78	59.5
Female	53	40.5
Total	131	100

FIGURE NO. 5.2: DISTRIBUTION OF CASES ACCORDING TO SEX



DISTRIBUTION OF CASES ACCORDING TO SEPSIS

Cases were stratified based on the clinical and investigation proved cases of sepsis into the following categories like suspected cases, positive for sepsis screen and positive for blood culture. Thirty-eight cases were positive for sepsis screen and 19 were positive for blood culture. An analysis of sex wise distribution was also done, with male neonates having more number of proven cases of sepsis in comparison to females. The detailed representation of the stratification is tabulated in Table No. 5.3 & 5.4

TABLE NO 5.3: DISTRIBUTION OF CASES ACCORDING TO SEPSIS

SEPSIS	N	%
PROVEN	19	14.5
CLINICAL	38	29.0
SUSPECTED	74	56.5
Total	131	100

FIGURE NO 5.3: DISTRIBUTION OF CASES ACCORDING TO SEPSIS

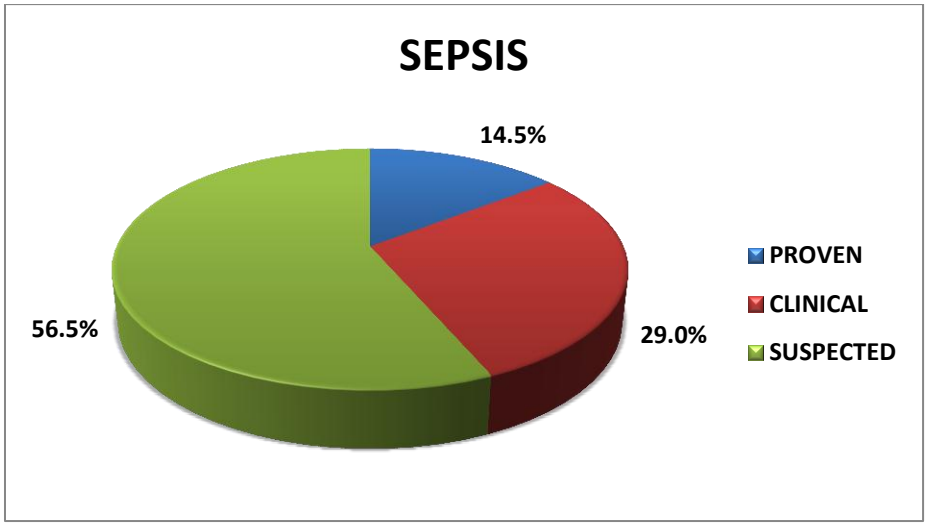
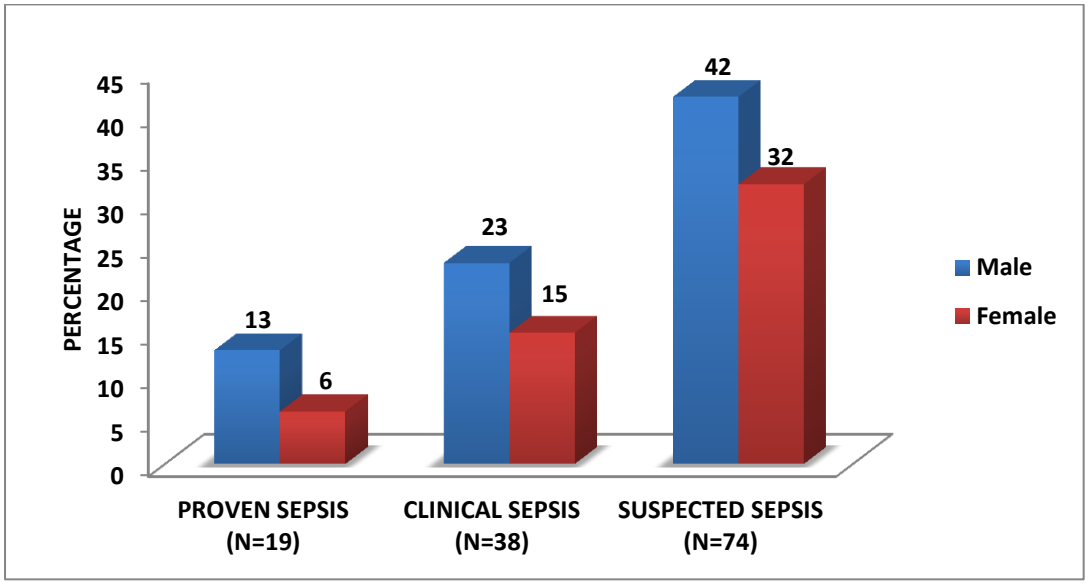


TABLE NO 5.4: DISTRIBUTION OF SEPSIS GROUPS ACCORDING TO SEX

CULTURE	Male		Female		p value
	N	%	N	%	
PROVEN	13	16.7	6	11.3	0.646
CLINICAL	23	29.5	15	28.3	
SUSPECTED	42	53.8	32	60.4	
Total	78	100.0	53	100.0	

FIGURE NO 5.4: DISTRIBUTION OF SEPSIS GROUPS ACCORDING TO SEX



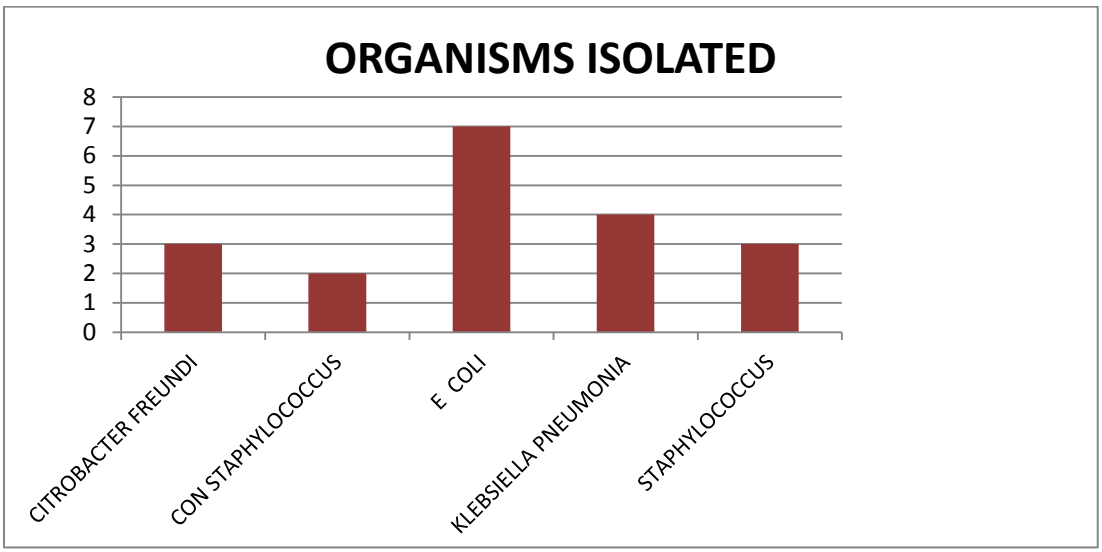
ORGANISMS ISOLATED

Out of 19 cases, Escherichia coli (5.3%) was the most common isolated organism followed by Klebsiella pneumonia(3.1%). Other organisms were Citrobacter freundi (2.3%), Staphylococcus (2.3%) and Coagulase negative Staphylococcus (1.5%).

TABLE NO 5.5: DISTRIBUTION OF CASES ACCORDING TO ORGANISMS ISOLATED

BLOOD CULTURE	N	%
CITROBACTER FREUNDI	3	2.3
CON STAPHYLOCOCCUS	2	1.5
ESCHERICHIA COLI	7	5.3
KLEBSIELLA PNEUMONIA	4	3.1
STAPHYLOCOCCUS	3	2.3

FIGURE NO 5.5: DISTRIBUTION OF CASES ACCORDING TO ORGANISMS ISOLATED



CASES ACCORDING TO GESTATIONAL AGE

Considering the gestational age, 83.2% of the neonates were term and the remaining 16.8% were preterm. Among 19 cases of proven sepsis, 18 were term neonates and a single case belonged to the preterm category. Even in the 38 cases of clinical sepsis, 26 were term neonates and 12 were preterm neonates. The same trend continued in the 74 cases of suspected sepsis as well with 65 term neonates and 9 preterm neonates.

TABLE NO 5.6: DISTRIBUTION OF CASES ACCORDING TO GESTATIONAL AGE

Gestational age	N	%
Preterm	22	16.8
Term	109	83.2
Total	131	100

FIGURE NO 5.6: DISTRIBUTION OF CASES ACCORDING TO GESTATIONAL AGE

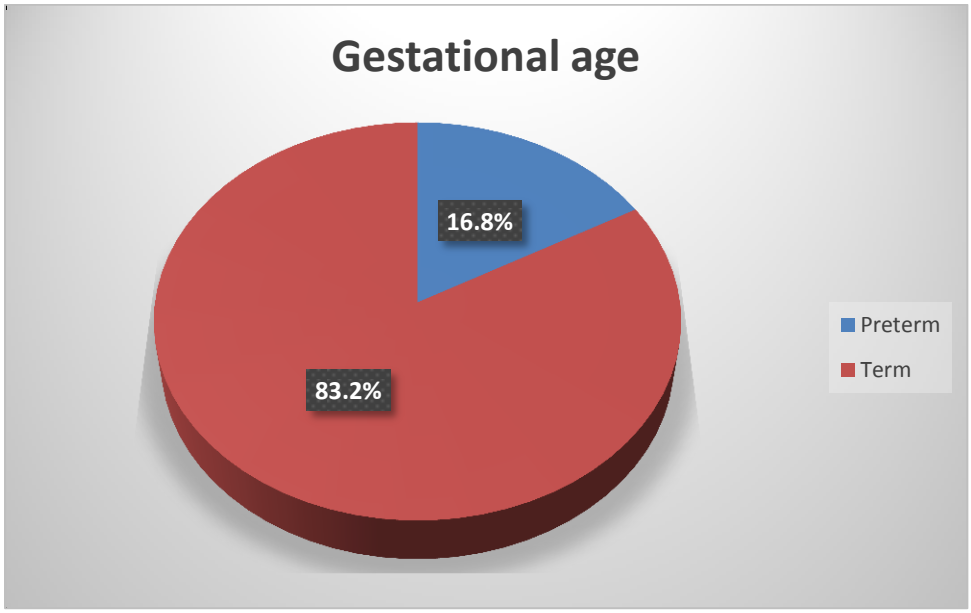
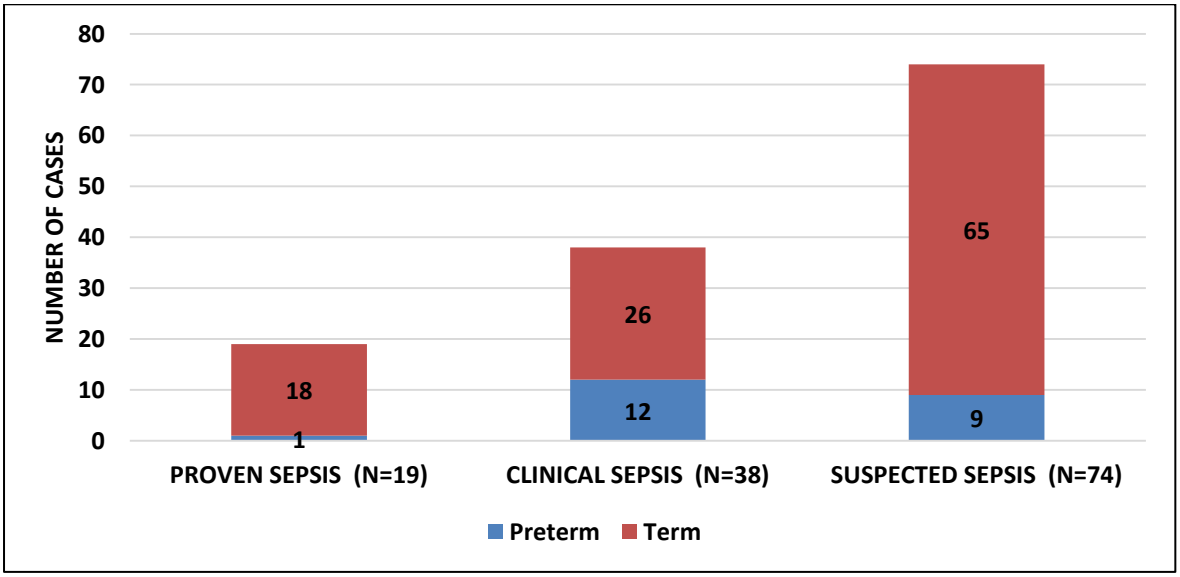


TABLE NO 5.7: DISTRIBUTION OF GESTATIONAL AGE ACCORDING TO SEPSIS

Gestational age	PROVEN SEPSIS (N=19)		CLINICAL SEPSIS (N=38)		SUSPECTED SEPSIS (N=74)		p value
	N	%	N	%	N	%	
Preterm	1	5.3	12	31.6	9	12.2	0.012 *
Term	18	94.7	26	68.4	65	87.8	
Total	19	100.0	38	100.0	74	100.0	

Note: * significant at 5% level of significance ($p<0.05$)

FIGURE NO 5.7: DISTRIBUTION OF GESTATIONAL AGE ACCORDING TO SEPSIS



For establishing the diagnosis and further evaluation of the cases the following parameters as mentioned in Table No. 5.8 were analyzed. Amongst the 131 cases, elevated CRP and ANC were noted in 125 and 98 cases respectively, followed by elevated nucleated red blood cells seen in 79. Considering these parameters along with the nature of the sepsis diagnosis, elevated CRP levels were found in 100% of the cases of proven sepsis, whereas, elevated nRBCs were noted in 94.7% of the cases followed by toxic granules and B:N ratio with 78.9% each. However, elevated nRBCs, toxic granules in neutrophils and B:N ratio were having a statistically significant difference with a p-value of <0.005.

TABLE NO 5.8: DISTRIBUTION OF VARIOUS PARAMETERS

PARAMETERS	**N**	**%**
Elevated nRBC s	79	60.3
Total WBC count	14	10.7
Elevated ESR	7	5.3
Elevated B:N ratio	60	45.8
Toxic granules in neutrophils	39	29.8
Elevated CRP(mg/dl)	125	95.4
Elevated ANC	98	74.8

FIGURE NO 5.8: DISTRIBUTION OF VARIOUS PARAMETERS

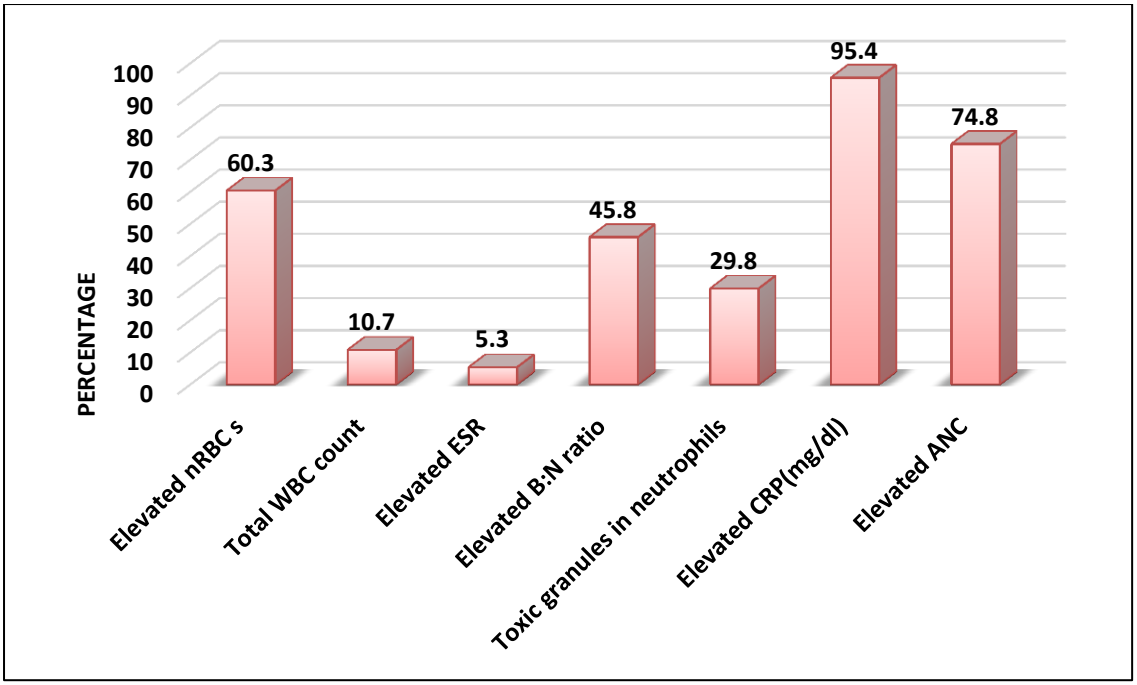


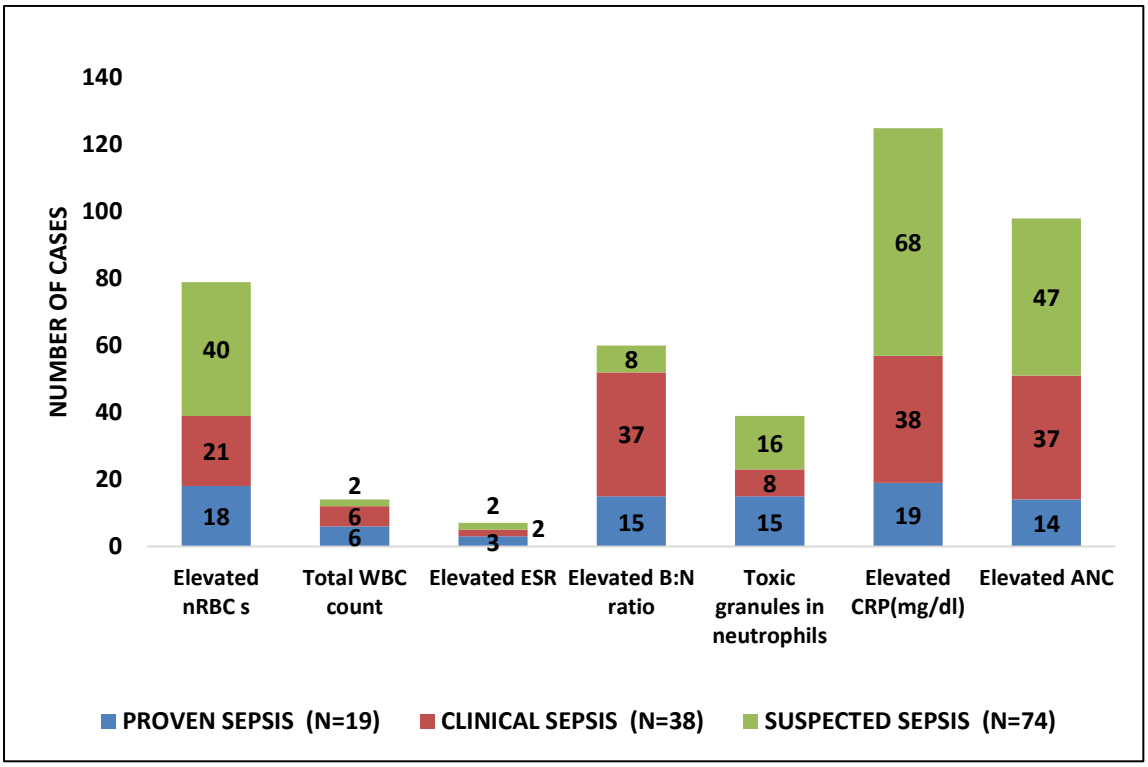
TABLE NO 5.9: DISTRIBUTION OF PARAMETERS ACCORDING TO SEPSIS GROUPS

PARAMETERS	PROVEN SEPSIS (N=19)		CLINICAL SEPSIS (N=38)		SUSPECTED SEPSIS (N=74)		p value
	N	%	N	%	N	%	
Elevated nRBC s	18	94.7	21	55.3	40	54.1	0.004*
Total WBC count	6	31.6	6	15.8	2	2.7	0.001*
Elevated ESR	3	15.8	2	5.3	2	2.7	0.073
Elevated B:N ratio	15	78.9	37	97.4	8	10.8	<0.001*
Toxic granules in neutrophils	15	78.9	8	21.1	16	21.6	<0.001*
Elevated CRP(mg/dl)	19	100.0	38	100.0	68	91.9	0.089
Elevated ANC	14	73.7	37	97.4	47	63.5	<0.001*

Note: * significant at 5% level of significance ($p<0.05$)

Elevated nRBC s was seen in 18 (94.7%) cases out of 19 cases of proven sepsis, 21 (55.3%) cases out of 38 cases clinical sepsis and 40(54.1%) cases out of 74 cases of suspected sepsis. Elevated ESR was seen in 3 (15.8%) cases out of 19 cases of proven sepsis, 2 (5.3%) cases out of 38 cases of clinical sepsis and 2 (2.7%) cases of suspected sepsis. B:N ratio was elevated in 15 (78.9%) cases out of 19 cases of proven sepsis, 37 (97.4%) cases out of 38 cases of clinical sepsis, 8 (10.8%) cases out of 74 cases of suspected sepsis. 15 (78.9%) cases of proven sepsis showed toxic granules in neutrophils. C Reactive protein was elevated all 19 culture proven cases of sepsis and in all 38 cases of clinical sepsis. Absolute neutrophil count was increased in 14 (73.7%) cases out of 19 cases of proven sepsis, 37(97.3%) cases out of 38 cases of clinical sepsis and in 47 (63.5%) cases out of 74 of suspected sepsis.

FIGURE NO 5.9: DISTRIBUTION OF PARAMETERS ACCORDING TO SEPSIS



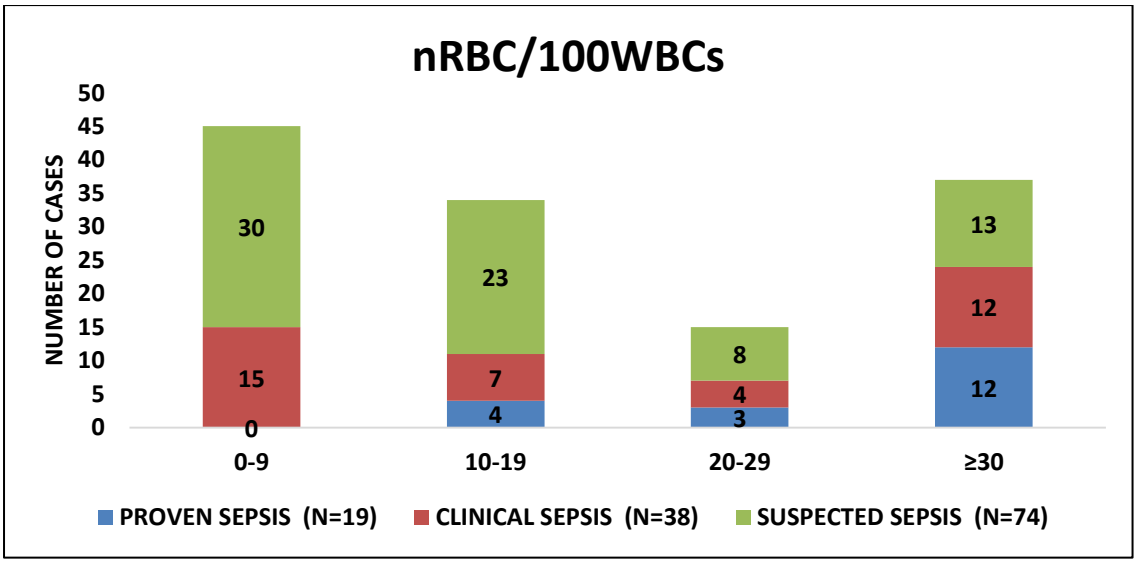
DISTRIBUTION OF nRBC/100WBCS AMONG GROUPS

With respect to the number of nRBCs per 100 WBC analysis, >30 nRBCs were seen in 63.2% of proven sepsis cases and the number of nRBCs were more than 10 per 100WBCs in all the remaining 7 cases. Whereas in case of clinical sepsis and suspected sepsis 31.6% and 17.6% of the cases were having more than 30 nRBCs. These results are found to be statistically significant with a p-value of 0.020.

TABLE NO 5.10: DISTRIBUTION OF nRBC/100WBCs

NRbc/100wbc	PROVEN SEPSIS (N=19)		CLINICAL SEPSIS (N=38)		SUSPECTED SEPSIS (N=74)		p value
	N	%	N	%	N	%	
0-9	0	0.0	15	39.5	30	40.5	0.020*
10-19	4	21.1	7	18.4	23	31.1	
20-29	3	15.8	4	10.5	8	10.8	
≥30	12	63.2	12	31.6	13	17.6	
Total	19	100.0	38	100.0	74	100.0	

FIGURE NO 5.10: DISTRIBUTION OF nRBC/100WBCs



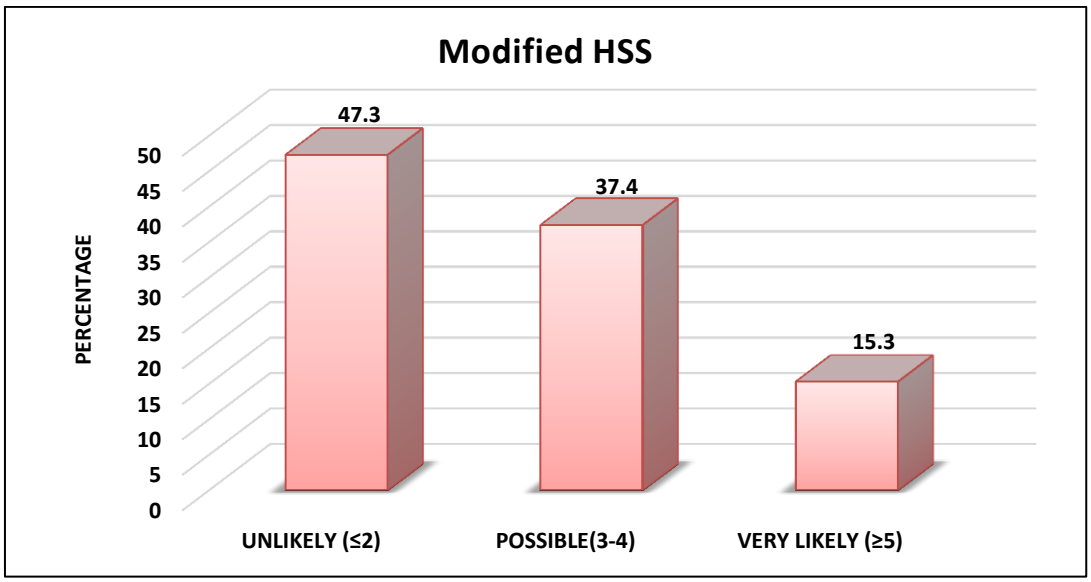
DISTRIBUTION OF SCORES ACCORDING TO MODIFIED HEMATOLOGICAL SCORING SYSTEM

Out of 131 cases, sepsis was unlikely in 62 (47.3%) cases having hematological score of ≤2, in 49 (37.4%) cases there was possibility of sepsis with hematological score of 3-4 and sepsis was very likely in 20 (15.3%) cases with hematological score of ≥5.

TABLE NO 5.11: DISTRIBUTION OF SCORES ACCORDING TO MODIFIED HEMATOLOGICAL SCORING SYSTEM

HSS	N	%
UNLIKELY (≤2)	62	47.3
POSSIBLE(3-4)	49	37.4
VERY LIKELY (≥5)	20	15.3
Total	131	100

FIGURE NO 5.11: DISTRIBUTION OF SCORES ACCORDING TO MODIFIED HEMATOLOGICAL SCORING SYSTEM



DISTRIBUTION OF MODIFIED HSS ACCORDING TO SEPSIS GROUPS

Among proven cases of sepsis, 13 cases showed a score of ≥5 indicating sepsis was very likely. Among clinical sepsis 22 cases showed a score of 3-4 indicating possibility of sepsis. Among suspected cases of sepsis 51 cases showed a score of ≤2 indicating sepsis was unlikely and 21 cases showed score of 3-4 indicating possibility of sepsis.

TABLE NO 5.12: DISTRIBUTION OF MODIFIED HSS ACCORDING TO SEPSIS GROUPS

HSS	PROVEN SEPSIS (N=19)		CLINICAL SEPSIS (N=38)		SUSPECTED SEPSIS (N=74)		p value
	N	%	N	%	N	%	
UNLIKELY (≤2)	0	0.0	11	28.9	51	68.9	<0.001 *
POSSIBLE(3-4)	6	31.6	22	57.9	21	28.4	
VERY LIKELY (≥5)	13	68.4	5	13.2	2	2.7	
Total	19	100.0	38	100.0	74	100.0	

Note: * significant at 5% level of significance (p<0.05)

FIGURE NO 5.12: DISTRIBUTION OF MODIFIED HSS ACCORDING TO SEPSIS GROUPS

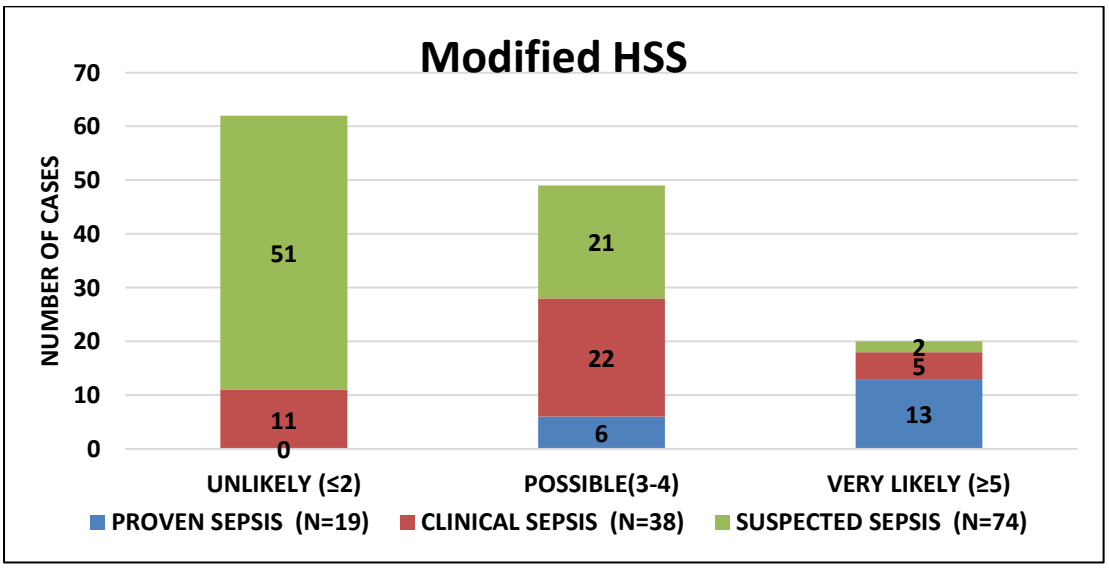


TABLE NO 5.13: SENSITIVITY ANALYSIS OF PARAMETERS IN DETECTING PROVEN CASES OF SEPSIS

The Table no. 5.13 shows sensitivity, specificity, positive predictive value and negative predictive value of various hematologic parameters.

PARAMETERS	Sensitivity	Specificity	PPV	NPV
Elevated nRBC s	95%	46%	23%	98%
Total WBC count	32%	93%	43%	89%
Elevated ESR	38%	91%	43%	89%
Elevated B:N ratio	79%	69%	25%	94%
Toxic granules in neutrophils	72%	60%	58%	76%
Elevated CRP(mg/dl)	100%	5%	15%	100%
Elevated ANC	83%	25%	14%	85%
HSS	100%	55%	28%	100%

TABLE NO 5.14: ROC ANALYSIS CUT OFF VALUE OF PARAMETERS IN DETECTING PROVEN CASES OF SEPSIS

At a cut off value of ≥21 for nRBCs, the sensitivity and specificity was 73.7% and 69.6% respectively and at cut off score of ≥3.5 the sensitivity and specificity of HSS was 94.7% and 83.9% respectively.

Test Variable(s)	Cutoff	Sensitivity	Specificity
nRBC s	≥21.5	73.7%	69.6%
Total WBC count	≥13480	52.6%	48.2%
B:N ratio	≥0.125	78.9%	59.8%
CRP(mg/dl)	≥4.5	84.2%	50.0%
ANC	≥8678	52.6%	50.9%
HSS	≥3.5	94.7%	83.9%

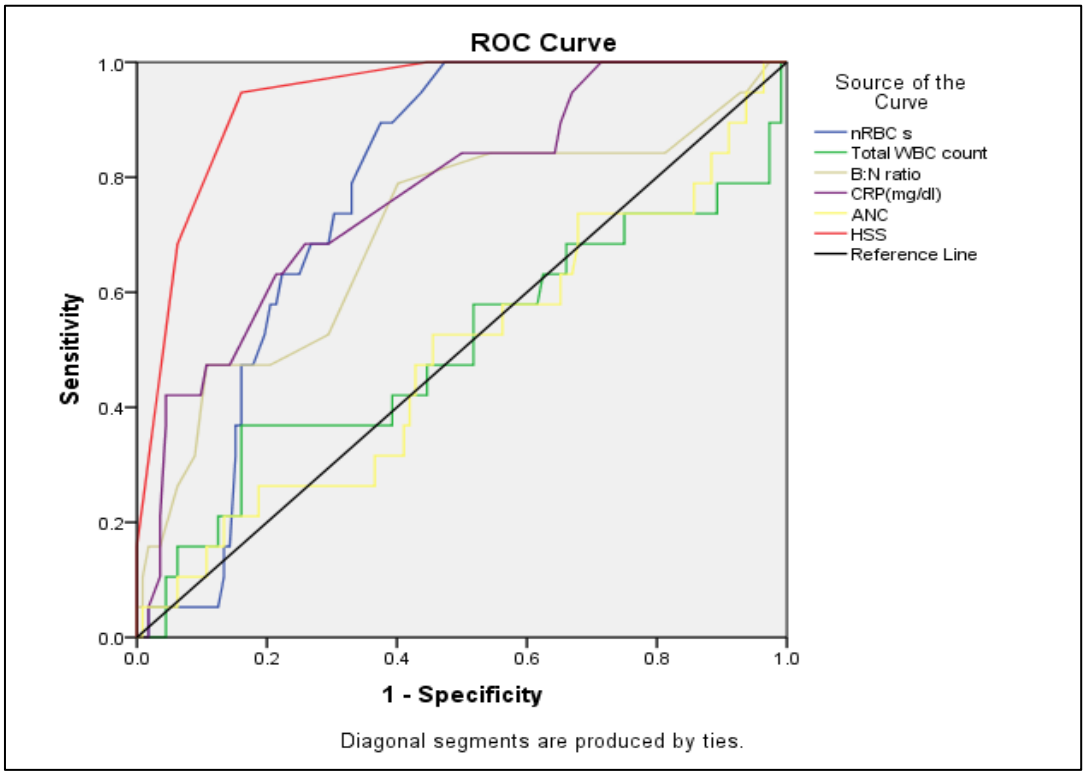
According to the ROC analysis the nRBCs, modified HSS and CRP have maximum area under the curve and have a p value of <0.001 which is statistically significant.

TABLE NO 5.15: ROC ANALYSIS OF PARAMETERS IN DETECTING PROVEN CASES OF SEPSIS

Test Variable(s)	Area Under the Curve	Std. Error	p value
nRBC s	0.775	0.041	<0.001*
Total WBC count	0.501	0.083	0.992
B:N ratio	0.703	0.072	0.005*
CRP(mg/dl)	0.771	0.057	<0.001*
ANC	0.489	0.075	0.883
Modified HSS	0.938	0.023	<0.001*

Note: * significant at 5% level of significance (p<0.05)

FIGURE NO 5.13: ROC CURVE



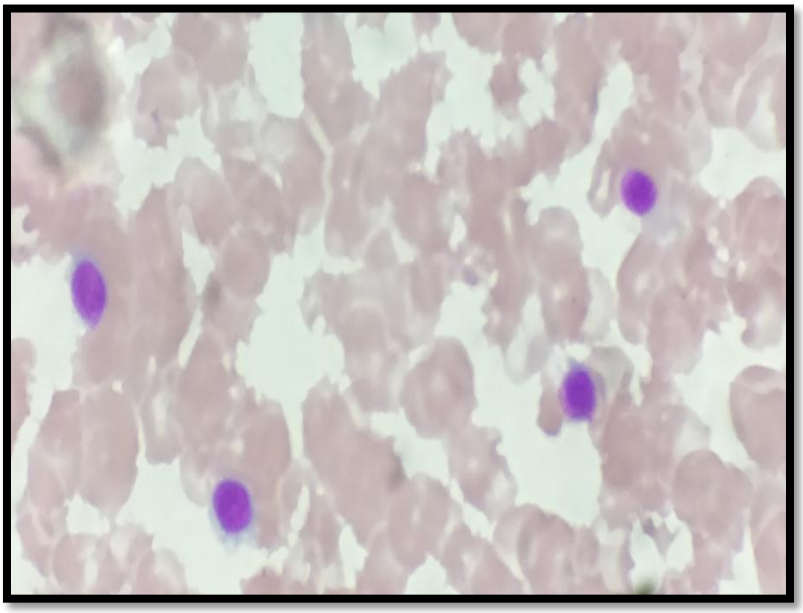


Fig No.5.14: nRBCs in the peripheral smear (1000X)

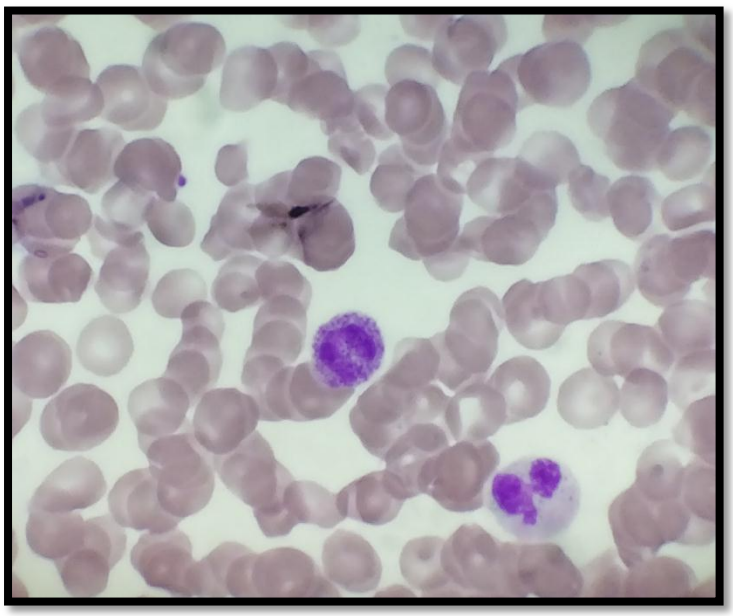


Fig No. 5.15: Neutrophil showing toxic granules (1000X)

DISCUSSION

Sepsis is one of the leading causes of neonatal morbidity and mortality.[1,59,60] In early onset neonatal sepsis, infection can be acquired in intrauterine life or immediately after birth. There are many factors associated with increased risk of acquiring neonatal sepsis. Any neonate suspected to have sepsis, should be investigated appropriately with the help of laboratory investigations and blood culture. Establishing the diagnosis of sepsis is very important for early institution of the treatment so as to prevent further complications. Several other conditions like birth asphyxia, prematurity, hypoglycemia, hypothermia, and intracranial hemorrhage have clinical features similar to septicemia. [2,3,61,62]

Blood culture is considered to be the gold standard diagnostic test, but, it takes around 48-72 hours for the availability of blood culture reports which has low positivity and is time consuming. [1,63,68]

The nRBCs are commonly found in circulation in neonates . The counts can increase as result of various factors like premature birth, post natal hypoxia, acute stress mediated release from the bone marrow stores and increased erythropoiesis in chronic conditions.[4,6,64,65]

The current study was undertaken to evaluate the efficacy of nucleated red blood cells in the diagnosis of early onset neonatal sepsis along with other hematological parameters.

Sex distribution in neonatal sepsis:

The age of the neonates ranged from birth to 72 hours. Of the 131 neonates in the study, 78 were males and 53 were females. Among the 19 proven cases of sepsis, 13 were males. Several other workers such as Abhishek M G *et al*[1], Chandna *et al*[68], and Krishnamurthy V *et al*[54] have reported similar findings. [Table No: 6.1]

Table No 6.1: Comparison of cases according to sex in various studies

STUDIES BY	NO. OF CASES	MALE:FEMALE
Abhishek MG *et al*[1]	60	1.7:1
Chandna *et al*[68]	60	1.3:1
Krishnamurthy V *et al*[54]	75	1.7:1
Present study	131	1.4:1

Septicemia is more common in male neonates than female neonates. The probable reason to this is the fact that, the factors that regulate the synthesis of Y globulins are situated on X chromosome. Males have less immunological protection than females since they have a single X chromosome. [68]

In present study 109 cases were term neonates and 22 cases were preterm neonates. These findings were similar to studies done by Abhishek MG *et al*[1] and Krishnamurthy V *et al*[57].

Organisms isolated in neonatal sepsis:

In present study the most common organism isolated was E coli followed by Klebsiella pneumonia. Similar findings were seen in Cortese F *et al*[16], Chandna A *et al*[68] and Renolder B *et al*[9]. [Table No 6.2]

Table No 6.2: Comparison of most common organisms isolated among various studies

	Most common organism isolated
Cortese F *et al*[16]	E.coli
Chandna A *et al*[68]	E.coli
Renolder B *et al*[9]	E.coli
Hasan Sobaih B *et al*[7]	Staphylococcus epidermidis
Present study	E.coli

In study done by Renolder *et al*[9] they found E. coli was associated with maternal fever and chorioamnionitis. Neonates who had E. coli infection had lower birth weight and were born preterm.

Various parameters in neonatal sepsis:

Among total of 131 cases, 79 cases showed elevated nRBCs, and 18 cases out of 19 culture proven sepsis showed increased nRBCs. The result of this study was comparable with the result of the study done by Abhishek MG *et al* [1](17 out of 60 cases showed increase in nRBCs and 6 cases out of 14 culture positive cases). [Table No 6.3]

Table No 6.3: Comparison of ESR, Total WBC count, Toxic granules in neutrophils and nRBCs among positive blood culture cases

Parameters	Present study (Out of 19 cases)	Abhishek MG *et al*[1] (Out of 14 cases)
Increased nRBCs	94% (18)	42.8% (6)
Elevated ESR	15.7% (3)	14.2% (2)
Total WBC count	31% (6)	28.5% (4)
Toxic granules in neutrophils	78% (15)	28.5% (4)

Association of nRBC counts with neonatal sepsis:

In present study out of 19 cases of proven sepsis, maximum number of cases (12 cases) showed a nRBC count of >30/ 100WBCs and in study done by Abhishek MG *et al*[1], 3 cases showed a nRBC count of >30/ 100WBCs.[Table No. 6.4]

Table No 6.4: Relationship between nRBC count and proven sepsis

nRBCs/100	Present study	Abhishek MG *et al*[1]
10-19	4	1
20-29	3	2
>30	12	3

In study conducted by Boskabadi H *et al*[30] there was a significant association between the nRBC counts of the case group and infant mortality with a p value of < 0.0001, which was statistically significant. The diagnostic cut-off point for nRBC count to predict infant mortality was more than 7 per 100 WBCs. At this cut-off point

they obtained a sensitivity of 65% and a specificity of 85%, while positive and negative predictive values were 44% and 93%, respectively.

Table No 6.5: Comparison of sensitivity and specificity of nRBC parameter

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	p value
Abhishek MG *et al*[1]	35%	53.48%	23.07%	67.64%	-
Dulay *et al*[6]	-	-	-	-	0.009
Present study	95%	46%	23%	98%	<0.001

In our study, the sensitivity of nRBC parameter was 95%, and its specificity was 46%, positive predictive value was 23%, and negative predictive value was 98%.The present study showed a better sensitivity and positive predictive value of nRBC parameter for identifying neonatal sepsis.

The test showed a higher sensitivity because 18 out of 19 culture proven cases of sepsis showed increased nRBCs. Increase in the nRBC count can be attributed to the factor that most of the neonates in this study were one day old, where the nRBC counts will be on higher side and E coli was the most common organism isolated among these cases. It is most commonly associated with early onset sepsis. They have several virulence factors which might result in increased IL 6 production, which causes increased production of nRBC[16,69]

The present study showed a p value of <0.001 which was significant at 5% level of significance. This was concordant with the studies conducted by Dulay *et al*[6], Szwajcowska *et al*[70], and Boskabadi H *et al*[30].

According to ROC analysis of cut off values for detecting proven cases of sepsis, nRBC at a cut off value of >21 showed a sensitivity and specificity of 73.7% and 69.6%.

According to the study conducted by Dulay *et al*[6], the elevated nRBC counts directly correlated with Interleukin 6 present in the cord blood. They also demonstrated that, in the absence of stress or hypoxia, the production and release of nRBCs into circulation is an adaptive response to an inflammation in the intrauterine period. The nRBCs and other hematological indices are good predictors of short term neonatal outcome.

According to the study conducted by Boskabadi H *et al* [30], the mean nRBC count was ten times higher in the infection group than in the control group. Sepsis is an inflammatory response of the body to pathogens. This causes the release of many inflammatory mediators including cytokines which cause production of nRBCs.

According to the study conducted by Szwajcowska M *et al*[70], the mean nRBC count in the study group was 16.9 (0-65) compared to control group in which the mean nRBC count was 4.01(1-20). In the study group, out of total 31 cases, 15 cases showed nRBC count of 0-5 and 16 cases showed nRBC count of > 6.

Hematological scoring system in neonatal sepsis:

Hematological scoring system is a score devised using various hematological parameters like total WBC count, total PMN count, immature PMN count, I:M PMN ratio, degenerative changes in PMN, and platelet count. In present study, a modified hematological scoring system was used including nRBC parameter.

Table No 6.6: Distribution of hematologic scores among various studies

	Total no of cases	Score ≤2	Score 3-4	Score ≥5
Narasimha A *et al*[46]	50	12	26	12
Ahirrao BM *et al*[54]	303	58	153	92
Makkar M *et al*[48]	110	27	32	51
Present study	131	62	49	20

In our study, among 19 culture positive cases, 13 cases showed a score ≥5 and 6 cases showed a score between 3 -4. Score of more than ≥5 shows that sepsis is very likely among these.

A study conducted by Narasimha *et al*[46] on hematological scoring system, 26 cases showed score of 3-4 and 12 cases showed score of ≤2. All the 12 proven cases of sepsis showed a score ≥5.

In a study conducted by Ahirrao *et al*[54], 202 cases out of 238 cases of culture proven and probable sepsis, showed a score of >3.

In a study done by Makkar M *et al*[48], the Out of 42 cases with culture proven sepsis, 35 (83.33%) infants had score ≥5.

In a study conducted by Rodwell *et al*[43], 26 out of 27 neonates in the sepsis group showed a score of >3. A score of >3 was a reliable indicator and predictive of sepsis. Hematological scoring system was more diagnostic than any single parameter for diagnosis of sepsis.

According to these studies a score of >4 was more diagnostic of sepsis. As the score increases the probability of presence of sepsis increases.

Comparison of various components of Hematological Scoring System:

Total WBC count in neonatal sepsis:

The present study shows a sensitivity and specificity of 32% and 93% respectively for Total WBC count which is almost similar to sensitivity and specificity obtained in study done by Ahirrao BM *et al*[54]. Total WBC count has lower sensitivity but higher specificity in detecting neonatal sepsis. It is because of wide normal range of WBC count between 5000- 34000cells/cumm and depends on the age of the neonate.[54]

Table No 6.7: Comparison of sensitivity and specificity of Total WBC count

	Sensitivity (%)	Specificity (%)	PPV(%)	NPV(%)
Narasimha A *et al*[46]	10.52	91.66	80	24.44
Khair KB *et al*[44]	50	91	43	93
Ahirrao BM *et al*[54]	21.8	98.5	98.1	25.6
Makkar M *et al*[48]	56.2	91.6	85.7	70.2
Present study	32	93	43	89

Total PMN count in neonatal sepsis:

The present study shows a sensitivity and specificity of 83% and 25% respectively for Total PMN count which is almost similar to sensitivity and specificity obtained in study done by Ahirrao BM *et al*[54]. Infections can be caused by viral, fungal or bacterial organisms, but bacterial infections are by the far most common cause of neonatal sepsis. Increased Total PMN count is most commonly associated with bacterial infections. So this parameter has a good sensitivity in diagnosing sepsis.

Table No 6.8: Comparison of sensitivity and specificity of total PMN count

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Narasimha A *et al*[46]	89.47	8.33	75.55	20
Khair KB *et al*[44]	92	38	17	97
Ahirrao BM *et al*[54]	85.7	23.1	80.3	30.6
Makkar M *et al*[48]	90.6	72.2	74.3	89.6
Present study	83%	25%	14%	85%

Immature: Total neutrophil ratio in neonatal sepsis:

The present study shows a sensitivity and specificity of 79% and 69% respectively for I:T ratio which is almost similar to sensitivity and specificity obtained in study done by Narasimha *et al*[46]. The immature neutrophils are released into circulation during infection because more number of neutrophils are recruited to site of infection. The number of immature neutrophils depends on the gestational age and age of the neonate. The counts are more in preterm neonates than the term neonates.

Table No 6.9: Comparison of sensitivity and specificity of I:T ratio

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Narasimha A *et al*[46]	63.15	75	88.88	39.13
Khair KB *et al*[44]	100	04	13	100
Ahirrao BM *et al*[54]	92.4	15.4	80	35.7
Makkar M *et al*[48]	93.7	94.4	93.7	94.4
Present study	79%	69%	25%	94%

Degenerative changes in neutrophils in neonatal sepsis:

The present study shows a sensitivity and specificity of 72% and 60% respectively for Degenerative changes in neutrophils. The present study was almost similar to Narasimha A *et al*[46]. These toxic granules in the neutrophils indicate the production of unusual neutrophils during the periods of stress and any infection.[48]

Table No 6.10: Comparison of sensitivity and specificity of Degenerative changes in neutrophils

	Sensitivity (%)	Specificity (%)	PPV(%)	NPV(%)
Narasimha A *et al*[46]	68.42	66.66	66.66	40
Ahirrao BM *et al*[54]	49.6	92.3	95.9	33.3
Makkar M *et al*[48]	78.1	94.4	92.5	82.9
Present study	72%	60%	58%	76%

Platelet count in neonatal sepsis:

The present study shows a sensitivity and specificity of 56.8 and 80.1% respectively for platelet count. It is similar to Narasimha *et al*[46] and Khair KB *et al*[44]. Most of these studies are showing low sensitivity and higher specificity. Throbocytopenia has high specificity but less sensitivity. Thrombocytopenia occurs due to bone marrow suppression because of infection and increased peripheral destruction of platelets. According to Ahirrao *et al*[54] it was more commonly associated with sepsis and carried a poor prognosis.

Table No 6.11: Comparison of sensitivity and specificity of Platelet count

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Narasimha A *et al*[46]	47.3	75	85.7	31
Khair KB *et al*[44]	60	82	31	94
Ahirrao BM *et al*[54]	39.9	92.3	95	29.6
Makkar M *et al*[48]	81.2	94.4	92.8	85
Present study	56.8%	80%	78.2%	29.6%

In present study nRBCs, I:T PMN ratio and degenerative changes in the neutrophils were better in diagnosing neonatal sepsis.

According to Narasimha A *et al*[46] the I:T ratio and degenerative changes in the neutrophils were most reliable parameters for diagnosing sepsis and modified HSS improved the diagnostic accuracy of complete blood count.

According to Khair KB *et al*[44] a combination of hematological parameters was recommended for diagnosing sepsis because no single parameter was superior compared to other parameter in predicting sepsis.

According to Ahirrao *et al*[54] I:T ratio and I:M ratio had high specificity and sensitivity, high PPV and NPV.

Table No 6.12: Comparison of sensitivity and specificity of Modified HSS

	Krishnamurthy *et al*[57]	**Present study**
Sensitivity (%)	84	94.7
Specificity (%)	82	83.9
PPV (%)	70	28
NPV (%)	91	100

In present study at a cut off score of ≥3.5 the sensitivity and specificity of Modified hematological scoring system were 94.7% and 83.9% respectively. The positive predictive value and negative predictive value were 28% and 100% respectively. It was concordant with Krishnamurthy *et al*[57] with sensitivity and specificity were 84% and 82% respectively.

Table No 6.13: Comparison of sensitivity and specificity of HSS and Modified HSS

	Khair KB *et al*[44]	**Chaware SA *et al*[55]**	**Present study**
Sensitivity (%)	100	90	94.7
Specificity (%)	60	74.5	83.9
PPV (%)	26	65.9	28
NPV (%)	100	93.2	100

The sensitivity, specificity and negative predictive value of modified hematological scoring system was better than hematological scoring system. In modified HSS immature neutrophil count and I:M neutrophil ratio were excluded

since they were repetitive and falsely increasing the scores. The nRBC count was included considering its role in neonatal sepsis.[57] Considering high sensitivity, specificity and negative predictive value, it implies that a score of >4 was more reliable for diagnosing sepsis than individual hematological parameter.

Modified hematological scoring system is a simple, cost effective and rapid method. But standardization and simplification of this test is required. It improves the diagnostic efficacy of the complete blood count.[46]

CONCLUSION

- The present study concluded that nucleated red blood cell count is an immediate reliable marker in the diagnosis of early onset neonatal sepsis. They are normally found in neonates and excess are released into circulation during any infection and stress due to the release of cytokines.
- It is an inexpensive, rapid and easy test.
- It can be used along with other hematological parameters in diagnosing neonatal sepsis which brings a significant impact in the neonatal health care.
- Modified hematological scoring system uses six hematological parameters including nRBC counts, which has better sensitivity and specificity. The efficacy of complete blood count is increased with the use of the scoring system.
- Combining the values of biochemical markers along with modified HSS can improve the diagnostic efficacy in neonatal sepsis.
- It can help the clinicians to start the treatment as early as possible thus preventing further complications and thereby reducing neonatal mortality.

SUMMARY

In the present study 131 neonates were evaluated with an age range of 1-3 days who presented with signs and symptoms of sepsis from 1st October, 2016 to 30th June, 2018 in Pathology department in BLDE (Deemed to be university), Shri. B. M. Patil Medical College, Hospital and Research Centre, Vijayapur.

- Among a total of 131 cases, 109 (83.2%) were 1 day old, 10 (7.6%) were 2 days old and 12 (9.2%) cases were three days old.
- Seventy-eight (59.5%) were male and 53 (40.5%) were females. Male:Female ratio was 1.4:1
- Among 131 cases, 19(14.5%) were culture proven cases of sepsis, 38(29%) cases were having sepsis screen positive and 74(56.5%) were suspected cases of sepsis.
- E. coli was the most common organism isolated followed by Klebsiella pneumonia.
- Out of 131 cases, 109(83.2%) were term neonates and 22(16.8%) were preterm neonates.
- Elevated nRBCs, toxic granules in neutrophils and I:T ratio were statistically significant parameters with a p-value of <0.001
- Among the proven sepsis group, 63.2% cases showed >30 nRBCs/100WBC. 31.6% cases of clinical sepsis and 17.6% cases of suspected sepsis showed >30 nRBCs/100WBC.
- According to hematological scoring system, 13 cases of proven sepsis showed a score ≥5 indicating sepsis was very likely in those. 22 cases of clinical sepsis showed a score of 3-4, indicating possibility of sepsis.

- At a cut off value of $\geq$ 21 for nRBCs, the sensitivity and specificity was 73.7% and 69.6% respectively and at cut off score of $\geq$3.5, the sensitivity and specificity of modified HSS was 94.7% and 83.9%.

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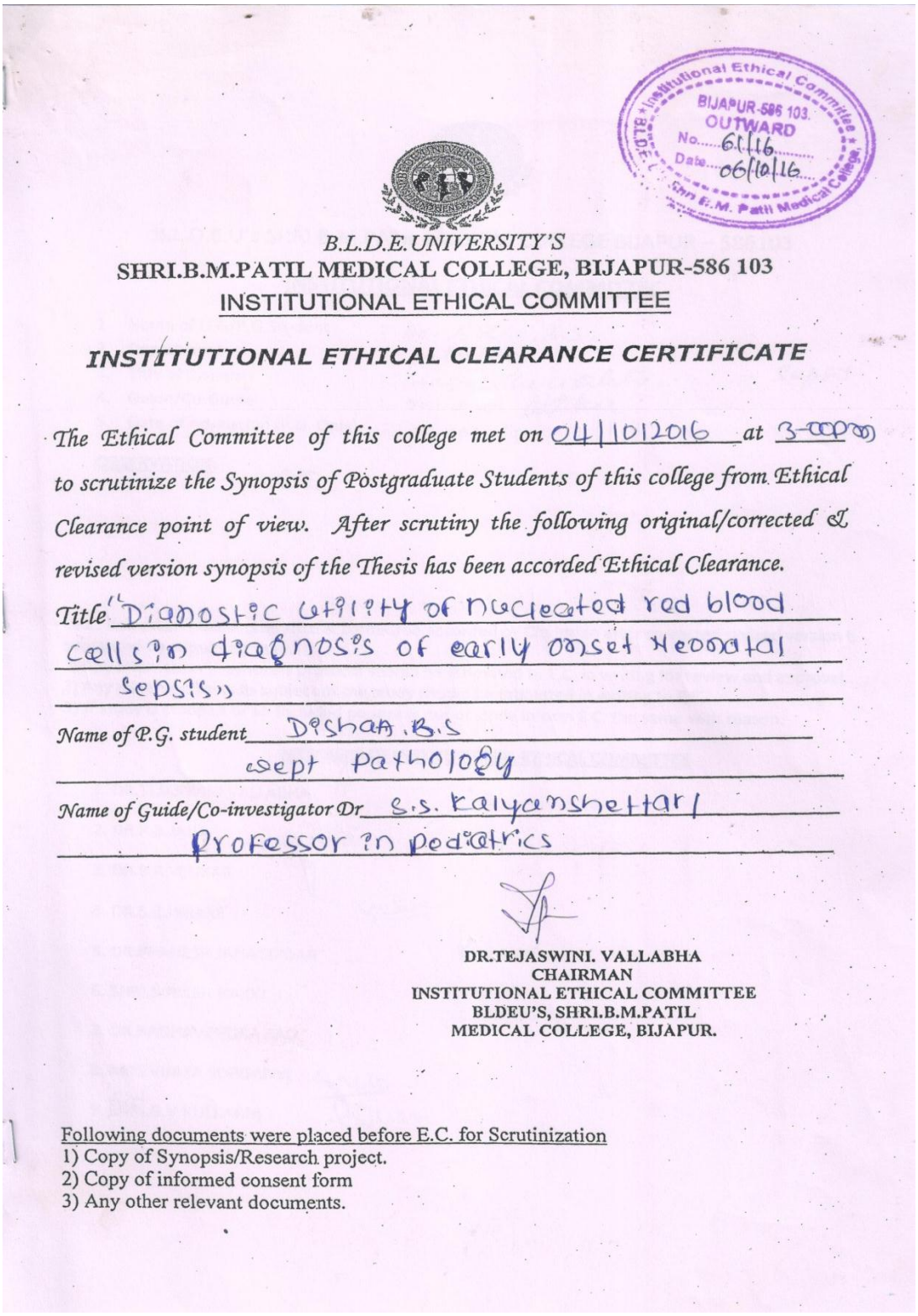
ANNEXURES

ETHICAL CLEARANCE CERTIFICATE



Institutional Ethical Committee
BIJAPUR-586 103
OUTWARD
No. 61/16
Date 06/10/16
Shri B.M. Patil Medical College

B.L.D.E. UNIVERSITY'S
SHRI.B.M.PATIL MEDICAL COLLEGE, BIJAPUR-586 103
INSTITUTIONAL ETHICAL COMMITTEE

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this college met on 04/10/2016 *at* 3-00pm *to scrutinize the Synopsis of Postgraduate Students of this college from Ethical Clearance point of view. After scrutiny the following original/corrected & revised version synopsis of the Thesis has been accorded Ethical Clearance.*

Title "Diagnostic utility of nucleated red blood cells in diagnosis of early onset Neonatal Sepsis"

Name of P.G. student Dishan.B.S
Dept Pathology

Name of Guide/Co-investigator Dr S.S. Kalyanshettar
Professor in Pediatrics

DR.TEJASWINI. VALLABHA
CHAIRMAN
INSTITUTIONAL ETHICAL COMMITTEE
BLDEU'S, SHRI.B.M.PATIL
MEDICAL COLLEGE, BIJAPUR.

Following documents were placed before E.C. for Scrutinization
1) Copy of Synopsis/Research project.
2) Copy of informed consent form
3) Any other relevant documents.

BLDE (DEEMED TO BE UNIVERSITY)
SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL & R.C
VIJAYAPUR, KARNATAKA

INFORMED CONSENT FOR PARTICIPATION IN DISSERTATION/RESEARCH

I, the undersigned, S/O D/O W/O, _____ aged years, ordinarily resident of do hereby state/declare that Dr Disha. B.S of Shri B M Patil Medical College, Hospital has examined me thoroughly on at (place) and it has been explained to me in my own language that I am suffering from _____ _____ disease (condition) and this disease/condition mimic following diseases . Further Doctor informed me that he/she is conducting dissertation/research titled Diagnostic utility of nucleated red blood cells in diagnosis of early onset neonatal sepsis under the guidance of Dr R M Potekar requesting my participation in the study. Apart from routine treatment procedure the pre-operative, operative, post-operative and follow-up observations will be utilized for the study as reference data.

Doctor Disha.B.S has also informed me that during conduct of this procedure _____ like adverse results may be encountered. Among the above complications most of them are treatable but are not anticipated hence there is chance of aggravation of my condition and in rare circumstances it may prove fatal in spite of anticipated diagnosis and best treatment made available. Further doctor has informed me that my participation in this study help in evaluation of the results of the study which is useful reference to treatment of other similar cases in near future, and also I may be benefited in getting relieved of suffering or cure of the disease I am suffering.

The Doctor has also informed me that information given by me, observations made/photographs/ video graphs taken upon me by the investigator will be kept secret and not assessed by the person other than me or my legal hirer except for academic purposes.

The Doctor did inform me that though my participation is purely voluntary, based on information given by me, I can ask any clarification during the course of treatment / study related to diagnosis, procedure of treatment, result of treatment or prognosis. At the same time I have been informed that I can withdraw from my participation in this study at any time if I want or the investigator can terminate me from the study at any time from the study but not the procedure of treatment and follow-up unless I request to be discharged.

After understanding the nature of dissertation or research, diagnosis made, mode of treatment, I the undersigned Shri/Smt _____ under my full conscious state of mind agree to participate in the said research/dissertation.

Signature of patient:

Signature of doctor:

Witness: 1.

2.

Date:

Place:

PROFORMA

NAME **:** OP/IP No. **:**

AGE **:**

SEX **:** D.O.A **:**

D.O.D **:**

RESIDENCE **:**

Presenting Complaints :

Past history :

Perinatal history : term/ preterm

Birth weight-

Maternal history :

Family history :

Treatment history :

General physical examination:

Head to toe examination-

VITALS: PR: RR:

BP: TEMPERATURE:

WEIGHT:

SYSTEMIC EXAMINATION:

Cardiovascular system

Respiratory system:

Per Abdomen:

Central nervous system:

Clinical Diagnosis:

INVESTIGATIONS:

Haematological investigations:

Parameters	
WBC	
RBC	
HGB	
HCT	
MCV	
MCH	
MCHC	
PLATELETS	
LYMPHOCYTES(%)	
NEUTROPHILS(%)	
EOSINOPHILS(%)	
MONOCYTES(%)	
BASOPHILS(%)	
RDW	
PDW	
MPV	
nRBC	

Peripheral Smear Examination:

RBC:

nRBC:

WBC:

B:N ratio-

PLATELETS:

IMPRESSION:

BLOOD CULTURE REPORTS:

DIAGNOSIS:

KEYS TO MASTER CHART

Sex : M- Male, F- Female

Gestational age: 1- Term, 2 – Preterm

Blood culture : SA – Staphylococcus aureus

CON SA- Coagulase negative Staphylococcus aureus

KP – Klebsiella pneumonia

CF – Citrobacter freundii

E coli- Escherischia coli

Toxic granules in neutrophil: 1- Not seen, 2 - Seen

MHSS : Modified hematological scoring system