CHAPTER I

1. INTRODUCTION

According to Nelson textbook of pediatrics neonatal sepsis (sepsis neonatorum) is a clinical syndrome resulting from the pathophysiologic effects of local or systemic infection in the first month of life. Sepsis neonatorum is the term used to describe any systemic bacterial infection documented by a positive blood culture in the first month of life (Behrman *et al*., 1992). It is an important and common cause of neonatal morbidity and mortality. The incidence of neonatal sepsis in the developed countries is 1-10 per 1000 live births. It is roughly three times more in developing countries like Nepal. This high incidence is mainly due to poor antenatal care and lack of trained staff to conduct deliveries (Waseem *et al.,* 2005).

According to World Health Report 2005, causes of death of neonates in South-East Asia, 75% of total death of neonates is due to severe infection (Pneumonia, Sepsis, and Meningitis). Neonatal sepsis can be classified into two relatively distinct illnesses based on the postnatal age of onset. Early-onset sepsis (EOS) occurs in the first 7 days of life, is usually a fulminate and multi systemic infection and has a higher case fatality rate than late-onset sepsis (LOS). Late-onset sepsis is usually more insidious but may have an acute onset.

Bacterial pathogens of neonatal septicemia may vary from one country to another and within a country from one hospital or region to another. These organisms may even vary at different times within the same place. In developed countries Group B S*treptococcus* (GBS), *E. coli* and *Listeria monocytogenes* are the most common causes of neonatal sepsis. Gram negative organisms had been the most common cause of neonatal sepsis in developed countries in 1960s. In 1970s, group B *Streptococcus* (GBS) was the most common organism, while coagulase negative *Staphylococcus* (CNS) ruled the roast during the 1980s and 1990s. At present, in the developing

countries, gram negative bacilli, coagulase negative *Staphylococcus* organisms remain the major culprits. These organisms have developed multi-drug resistance over the last two decades. The reasons for this resistance are indiscriminate and irrational use of antibiotics, over-the-counter sale of antibiotics and ineffective infection control in maternity centers. Due to multi-drug resistance, management of the patients is becoming a major problem.

Identification of the causative organism is important since it can embitter the management policy. For effective management of neonatal septicemia with appropriate antibiotics, study of bacteriological profile and their antibiotic sensitivity pattern plays a significant role. This would minimize the risk of severe morbidity and mortality besides reducing the emergence of multi-drug resistant organisms by rational antibiotic use, the present study will be carried out to determine the bacteriological profile with antibiotic sensitivity pattern of neonatal sepsis in Paropkar Maternity and Women's hospital, Thapathali. In spite of some rapid indicators of neonatal sepsis like C - reactive protein (CRP), micro ESR, Absolute Neutrophil Counts (ANC) and thrombocytopenia, isolation of the microorganism is the "gold standard" for the definite diagnosis of sepsis (Anwar *et al.*, 2000).

Transplacental, haematogenous transmission of bacteria is an uncommon route of EOS and occurs primarily with *Listeria monocytogenes*. The most common route of EOS in preterm and term infants is via an ascending amniotic infection. Members of the maternal genital flora, such as Group B *Streptococcus* (GBS) and *Escherichia coli*, the organisms responsible for the majority of cases of EOS, may ascend through the birth canal to the amniotic fluid either through intact amniotic membranes or, more commonly, after rupture of membranes. Once infected amniotic fluid is aspirated or swallowed by the fetus, pathogens may penetrate through immature mucosal barriers, resulting in pneumonia or bacteraemia, and may penetrate the blood-brain barrier, leading to meningitis. Bacteria have been implicated as a major cause of premature

rupture of membranes and, consequently, of premature labor and delivery (Kaufman and Fairchild, 2004).

Aggressive management of suspected maternal chorioamnionitis with antibiotics prior to delivery and rapid delivery of the new born infants may decrease the morbidity and mortality of neonatal sepsis. Selective intrapartum chemoprophylaxis has been shown to be effective in preventing early onset infections (Behrman *et al*., 1992).

Paropakar Maternity and Women's Hospital is a government hospital in Kathmandu valley where around 1500 to 2000 deliveries are conducted per month. In our country pre-existing data on both early and late onset sepsis has been shown great diversity in the changing patterns of the organisms and their sensitivity patterns. This study was carried out to determine the bacteriological profile with antibiotic susceptibility patterns and to compare it with various factors associated with neonatal septicaemia. Continued surveillance is mandatory to detect these temporal changes in spectrum and sensitivity of causative organisms, which will help in treating the septic neonates and eventually will reduce the neonatal morbidity and mortality.

CHAPTER II

2. OBJECTIVES

2.1. Objectives of the study

2.1.1. General objectives

The purpose of this hospital based study is to identify the bacteriological agents responsible for Neonatal Septicaemia in various neonates admitted in NICU and PBU.

2.1.2. Specific objectives

1. To describe the spectrum of isolates in case of neonatal septicaemia.
2. To assess the appropriate antibiotic susceptibility pattern for the isolated bacterial pathogens.
3. To associate various factor and growth of the organisms in blood culture.

CHAPTER-III

3. LITERATURE REVIEW

3.1 Bacteraemia and Septicaemia

Bacteria may enter the bloodstream, giving rise to bacteraemia. Bacteria may enter the bloodstream from an existing focus of infection, from a surface site with normal flora, or by direct inoculation of the contaminated materials into the vascular system. These organisms are generally washed out from the blood by immune system of the body within minutes so bacteraemia is often entirely silent and transient. However, it may be persistent if the immune system of the body is suppressed. In such condition, organism persists in blood, they survive and the signs and system of the disease become apparent.

Septicaemia (literally sepsis of blood) implies a more serious clinical condition than bacteraemia (literally bacteria in blood). Septicaemia means the presence and active multiplication of bacteria and release of their toxin in the blood and metastatic infection in the body tissue. Septicaemia is applied specially to the rapid multiplication of highly pathogenic bacteria. Traditionally the term bacteraemia referred to the transitory presence of bacteria in the blood of a patient in the absence of systems; the origin of the bacteria was usually from a site of commensal colonization. The term septicaemia meant the presence of bacteria in the blood with clinical signs and symptoms of infection. There origin was from a focus of infection from which they entered the circulation. (Collier *et al.*, 1999)

3.2 Bacteraemia and Septicaemia in Neonates

According to Nelson Textbook of Pediatrics neonatal sepsis (sepsis neonatorum) is a clinical syndrome resulting from the pathophysiologic effects of local or systemic infection in the first month of life. Because of the lack of specificity of many of the signs associated with this syndrome and the limitation of laboratory criteria, the diagnosis will continue to be difficult to establish (Behrman *et al.*, 1992). Gheibi said that sepsis neonatorum is the term used to describe any systemic bacterial infection documented by a positive blood culture in the first month of life (Gheibi *et al.*, 2008).

According to WHO neonatal sepsis may be defined both clinically and/or microbiologically. Isolation of an organism from a blood culture of a neonate with clinical symptoms of infection constitutes the common definition of sepsis. Gheibi said that neonatal septicaemia remains one of the important causes of mortality and morbidity despite considerable progress in hygiene, introduction of new and potent antimicrobial agents and advanced measures for diagnosis and treatment. Up to 10% of infants have infections in the first month of life which are responsible for 30-50% of total neonatal deaths in developing countries (Gheibi *et al.,* 2008).

Neonatal sepsis may be classified according to the time of onset of the disease: Early Onset (EOS) and Late Onset (LOS). EOS sepsis occurs in the first 7 days of life is usually a fulminate and multi-systemic infection and has a higher case fatality rate than LOS sepsis. LOS sepsis is usually more insidious but may have an acute onset (Shahsanam *et al.,* 2008). EOS disease is mainly due to bacteria acquired before and during delivery, vertical transmission, and LOS disease is mainly due to bacteria acquired after delivery (nosocomial or community sources), horizontal transmission (WHO report, 1999).

Table 3.1 Neonatal Infection by Age of Onset

Characteristic	**Early onset**	**Late onset**
Age at Onset	Birth to 7 days	7 to 30 days
Maternal Obstetric complication	Common	Uncommon
Prematurity	Frequent	Varies
Organism Source	Maternal and genital tract	Maternal and genital tract/environment
Manifestation	Multi System	Multi system or focal
Site	Normal nursery, NICU, community	NICU, Community

Source: Nelson Textbook of Pediatrics

The incidence of neonatal bacterial sepsis varies from 1–4 cases per 1,000 live births in developed countries, with considerable fluctuation over time and with geographic location. The reported incidence of neonatal sepsis varies from 7.1 to 38 per 1000 live births in Asia, From 6.5 to 23 per 1000 live births in Africa, and from 3.5 to 8.9 per 1000 live birth in South America and the Caribbean. By comparison, rates in Unites States and Australia range from 1.5 to 3.5 per 1000 for EOS sepsis and up to 6 per 1000 live births for LOS sepsis, A total of 6 to 9 Per 1000 for Neonatal sepsis (Vergnano *et al.*, 2002).

Bacterial sepsis is considered to be an important cause of neonatal mortality. (WHO, 1999; Dawodu *et al*., 2002; Rubin *et al*., 2002 Motara *et al*., 2005; Movahedian *et al*., 2006). The World Health Organization estimated that there are approximately five million deaths per year of which 98 % occurring in developing countries (WHO 1996). Baqui AH said that every year, there are an estimated four million neonatal deaths, accounting for almost 40% of death in children younger than five years. About a quarter of global neonatal deaths occur in India, which has a neonatal mortality of 43 per 1000 live birth. Therefore, innervations to address neonatal mortality are crucial if child mortality is to be reduced globally and in India. Globally, the main causes of neonatal deaths are thought to be pre term birth (28%), sepsis (26%), and birth asphyxia (23%) (Baqui *et al.*, 2006). Again, In a literature Dr. Rizwan Waseem says that neonatal sepsis has a significant contribution in neonatal mortality death (NNMR). In an elevatation of neonatal deaths from a community based study in and around Lahore, Jailil *et al*. recorded an infectious etiology in almost 75% of all deaths and this was also recognized as an important factor in almost one third of all first week deaths. A similar study in northern Pakistan confirmed that vast majority of neonatal deaths was related to pneumonia or diarrhea. Hospital based data indicate 30-38% overall mortality associated with neonatal sepsis (Waseem *et al.*, 2005).

Table 3.2: Study of neonatal sepsis in developing countries

			Early Onset		Late Onset		
Country	**Duration of study (month)**	**Total no of positive Blood Culture**	**EOS %**	**Mortality %**	**LOS %**	**Mortality %**	**Most common Isolates**
Malaysia	9(1991)	136	26	12	74	18	*Acinetobacter, Klebsiella*
Kenya	6(1997-8)	121	30	4	30	10	*Klebsiella, Citrobacter*
Nigeria	11(1994-5)	62	47	3	53	5	*Staph. aureus, Pseudomonas*
India	15(1996-7)	157	86	49	14	68	*Klebsiella, Pseudomonas*
Panama	216(1975-92)	577	86	49	14	22	*Klebsiella, Staph. aureus*
Saudi Arebia	60(1983-8)	61	39	21	61	24	*Staphylococci, Klebsiella*

Source: Neonatal Sepsis, an international perspective, (Vergnano *et al.,* 2002)

3.3 Routes of Infection

Transplacental, haematogenous transmission of bacteria is an uncommon route of EOS and occurs primarily with *Listeria monocytogenes*. The most common route of EOS in preterm and term infants is via an ascending amniotic infection. Members of the maternal genital flora, such as Group B *Streptococcus* (GBS) and *Escherichia coli*, the organisms responsible for the majority of cases of EOS, may ascend through the birth canal to the amniotic fluid either through intact amniotic membranes or, more commonly, after rupture of membranes. Once infected amniotic fluid is aspirated or swallowed by the fetus, pathogens may penetrate through immature mucosal barriers,

resulting in pneumonia or bacteraemia, and may penetrate the blood-brain barrier, leading to meningitis (Behrman *et al.*, 1992). Bacteria have been implicated as a major cause of premature rupture of membranes and, consequently, of premature labor and delivery (Kaufman and Fairchild, 2004). Thus, prevention and timely treatment of intra-amniotic infection are important steps in preventing preterm delivery and improving neonatal outcome (Kaufman and Fairchild, 2004).

Kaufman and Fairchild (2004) noted that LOS most commonly occurs via horizontal or nosocomial transmission, but it may occur via vertical transmission at birth, leading to colonization and, later, to infection. Skin or mucosal colonization with potential pathogens may be acquired from the hands of health care workers or family members, from water used in incubator or ventilator humidification systems, or from contaminated fomites such as stethoscopes, which may carry organisms directly from one patient to another. Colonizing organisms may enter the bloodstream through breaks in the skin or mucosa or by gastrointestinal translocation or may be introduced through invasive devices such as vascular catheters, endotracheal tubes, or feeding tubes. Alternately, nosocomial infection may result from infusion of contaminated intravenous solutions (especially lipid-based or high-glucose solutions) or from contaminated formula or breast milk (Kaufman and Fairchild, 2004). According to Nelson textbook of pediatrics term male infants have an approximately twofold higher incidence of sepsis than term females do. This sex difference is less clear in preterm low-birth weight (LBW) infants. Attack rates of neonatal sepsis increase significantly in LBW infants in the presence of maternal chorioamnionitis, congenital immune defects, asplenia, galactosemia *(E. coli)*, and malformations leading to high inocula of bacteria (obstructive uropathy) (Behrman *et al.*, 1992).

Behrman claims that nosocomial, or hospital-acquired, infections are responsible for significant morbidity and late mortality in hospitalized newborns. Neonatal nosocomial infection is not uniformly defined. Many define nosocomial infections as infections occurring after 3 days of life that are not directly acquired from the mother's genital tract. The Centers for Disease Control and Prevention (CDC) defines a nosocomial

infection as any infection occurring after admission to the NICU that was not transplacentally acquired. Rates of nosocomial infection in healthy term infants who are either rooming in with their mothers or staying in the well baby nursery are low (less than 1%). The majority of nosocomial infections occur in preterm or term infants who require special care. Risk factors for nosocomial infection in these infants include prematurity, LBW, invasive procedures, indwelling vascular catheters, parenteral nutrition with lipid emulsions, endotracheal tubes, ventricular shunts, alterations in the skin and/or mucous membrane barriers, frequent use of broad-spectrum antibiotics, and prolonged hospital stay (Behrman *et al.*, 1992). Most nosocomial infections are bloodstream infections associated with an intravascular device. Other serious infections are pneumonia, meningitis, omphalitis, and necrotizing enterocolitis.

Various bacterial and fungal agents colonize hospitalized infants, health care workers, and visitors. Pathogenic agents can be transmitted by direct contact or indirectly via contaminated equipment, intravenous fluids, medications, blood products, or enteral feedings. Colonization of the infant's skin, umbilicus, and respiratory or gastrointestinal tract with pathogenic agents often precedes the development of infection. Antibiotic use interferes with colonization by normal flora, thereby facilitating colonization with more virulent pathogens (Behrman *et al.*, 1992).

Multiple studies have reported that coagulase-negative staphylococci are the most frequent neonatal nosocomial pathogens. Among a cohort of 6,215 VLBW infants in the NICHD Neonatal Research Network, gram-positive agents were associated with 70%, gram-negative with 18%, and fungi with 12% of cases of late-onset sepsis. Coagulase-negative staphylococcus, the single most common organism, was isolated in 48% of these infections. The emergence of nosocomial bacterial pathogens resistant to multiple antibiotics is a growing concern. Among NICU patients, methicillin-resistant *S. aureus*, vancomycin-resistant enterococci, and multidrug-resistant gram-negative pathogens are particularly alarming (Behrman *et al.*, 1992).

Viral organisms may also cause nosocomial infection in the NICU and include respiratory syncytial virus, varicella, influenza, rotavirus, and enteroviruses. For viral as well as bacterial agents, nursery outbreaks may occur in addition to individual cases. Hospital infection control policies are essential to prevent and/or contain nursery outbreaks (Behrman *et al.*, 1992).

The mean age at onset of the 1st episode of late-onset nosocomial sepsis is 2–3 wk, independent of the infecting pathogen. Nosocomial infections increase the risk of adverse outcomes, including prolonged hospitalization and mortality (Behrman *et al.*, 1992).

3.4 Risk Factors

Behrman notes that attack rates of neonatal sepsis increase significantly in LBW infants and in the presence of maternal (obstetric) risk factors such as prolong rupture of membranes (>18 hours), maternal intrapartum fever (>37.5 ^{O}C), maternal laukocytosis (>18,000), uterine tenderness, fetal tachycardia (>180 beats per min), and chorioamnionitis. Inhalation of infected amniotic fluid may produce pneumonia and sepsis in utero, manifestd by fetal distress or neonatal asphyxia. Exposure to adult pathogenesis is risk factor after birth. Host risk factors include male sex, developmental or congenital immune defects, galactosemia (*E. coli*), congenital anomalies (urinary tract, asplenia, myelomeningocela, sinus tract), omphalitis, twining (especially the second twin of an infected twin), and possibly the administration of high dose of vitamin E. Pre-maturity is a risk factor for early and late onset disease (Behrman *et al.*, 1992).

Presence of the following high-risk factors has been associated with an increased risk of early onset sepsis (Jayan *et al.*, 2009).

a. Low birth weight (<2500 grams) or preterm baby.
b. Febrile illness in the mother within 2 weeks prior to delivery.
c. Foul smelling and/ or meconium stained liquor amnii.
d. Prolonged rupture of membranes >24 hours.
e. More than 3 vaginal examinations during labor.

f. Prolonged and difficult delivery with instrumentation.
g. Perinatal asphyxia or difficult resuscitation.

3.4.1 Perinatal asphyxia

Perinatal asphyxia was found to lower and compromise the immunological profile of the newborn. Both cellular and humoral immunity were found to be affected. The T-cell function was affected more than the B-cell function, i.e. cellular immunity was affected more than the humoral immunity. The CD4/CD8 ratio was reversed in asphyxiated newborns, implying a deficient immune status. Perinatal asphyxia is caused by a decreased supply of oxygen to the fetus or newborn. It can happen in the antepartum period, during labor, or at the time of birth. When it occurs, it results in an inadequate exchange of respiratory gases and impaired tissue perfusion. Various systemic functions of the newborn suffering from birth asphyxia have been evaluated and have been found to be deranged (Jayan *et al.,* 2009).

3.4.2 PROM

Prolonged leaking and premature rupture of membranes is considered as a major risk factor for sepsis because of the danger of ascending infection (Jayan *et al.,* 2009).

3.4.3 Endotracheal Intubation

Endotracheal intubation provides a major portal of entry for colonization and infection with potential pathogens (LOS association) (Jayan *et al.,* 2009).

3.4.4 PCOD

Polycystic ovary syndrome (PCOS) is a common reproductive disorder associated with many characteristic features, including hyperandrogenaemia, insulin resistance and obesity which may have significant implications for pregnancy outcomes and long term health of the woman (Jayan *et al.,* 2009).

3.4.5 Aspiration

Congenital pneumonia is a usual manifestation of neonatal sepsis. This is due to aspiration of infected amniotic fluid. Autopsy findings (Naeye *et al.,* 1971) reveal the

presence of poly morpho nuclear leucocytes in the alveoli, often mixed with squamous cells and vernix, suggesting aspiration of infected amniotic fluid. In a large proportion no bacterial pathogens could be isolated. It has hence been proposed that these pathological changes result from hypoxia and aspiration of maternal inflammatory cells rather than active infection (Jayan *et al.*, 2009).

3.4.6 Age at admission

Eighty-five percent of newborns with early-onset infection present within 24 hours, 5% present at 24-48 hours, and a smaller percentage of patients present between 48 hours and 6 days of life. Early onset sepsis syndrome is associated with acquisition of microorganisms from the mother (Jayan *et al.*, 2009).

8.4.7 Low Birth Weight

Neonates are deficient in humoral and cellular immunity; they produce immunoglobulins at a lower rate than adults. Transplacental maternal antibodies mediate humoral immunity primarily, hence very low birthweight (VLBW) premature infants are less likely to receive as any immunoglobulins as term infants. T−cell function is also less efficient in neonates. Complement function and phagocytic function inclusive of phagocytosis, phagocyte migration and toxin production are also deficient The incidence of sepsis and its complications are therefore greater in VLBW infants and extremely premature babies Onset of infection within the first six days of life is thought to be primarily due to vertical transmission from mother-to-infant, while onset of infection at seven days of life or greater is more likely to be acquired through horizontal transmission. By virtue of the length of time VLBW infants may spend in the hospital setting, they are at prolonged risk for acquiring infection, particularly nosocomial infections. The identification of strategies to reduce infection in these infants will result in decreased mortality and morbidity. It can also be hypothesized that by virtue of the

length of time VLBW infants spend on the neo-natal unit the organisms causing infection will be predominantly nosocomial in origin (Jayan *et al.,* 2009).

3.4.8 Abortion

It was recently suggested that a previous abortion increases the risk of intrapartum infection in a following pregnancy. A case-control study of neonatal sepsis was conducted using the Washington State Birth Registry. Cases of sepsis were selected among singleton live births during the period 1984-90, and compared with a control group for the occurrence of spontaneous or induced abortion in previous pregnancies. According to this study induced abortion is associated with an increased risk of neonatal sepsis in a subsequent pregnancy, but the association between spontaneous abortion and sepsis is small and non-significant. The authors suggest that the procedures involved in a therapeutic abortion might produce a latent, sub-clinical infection that persists until the next pregnancy, and is then transmitted to the newborn (Jayan *et al.,* 2009)..

3.4.9 PIH

Utero placental insufficiency is the underlying pathology of Pregnancy Induced Hypertension (PIH) which usually leads to low birth weight babies. Low birth weight is a condition that predisposes to neonatal sepsis (Jayan *et al.,* 2009).

3.4.10 UTI

Transplacental infection or an ascending infection from the cervix may be caused by organisms that colonize in the mother's genitourinary tract, with acquisition of the microbe by passage through a colonized birth canal at delivery (Jayan *et al.,* 2009).

3.4.11 Prematurity

The most important neonatal factor predisposing to infection is prematurity or LBW. Preterm infants have a 3- to 10-fold higher incidence of infection than full-term, normal-birth weight infants do. A number of possible explanations have been proposed for the increased incidence of infection in preterm infants. Maternal genital tract infection is considered to be an important cause of preterm labor, with an increased risk of vertical transmission to the newborn. The frequency of intra-amniotic infection is

inversely related to gestational age. Premature infants have documented immune dysfunction (Behrman *et al.,* 1992).

Premature infants often require prolonged intravenous access, endotracheal intubation, or other invasive procedures that provide a portal of entry or impair clearance mechanisms. (Behrman *et al.,* 1992).

Premature infants have an increased susceptibility to sepsis and subtle nonspecific initial presentations; therefore, they require much vigilance so that sepsis can be effectively identified and treated (Jayan *et al.,* 2009).

3.4.12 Abruptioplacenta

Early amnion rupture may cause abortion or stillbirth, craniofacial clefts, and cerebral, body wall and limb/skeletal defects. The risk of chorioamnionitis is also increased, with serious consequences to the fetus and neonate (Jayan *et al.,* 2009).

3.4.13 Chorioamnionitis

Attack rates of neonatal infection increase significantly in the presence of chorioamnionitis, which is diagnosed by amniotic fluid analysis or histologically. Clinical signs of chorioamnionitis include intrapartum fever (>38°C), maternal leukocytosis white blood cell [WBC] count >18,000), and uterine tenderness. Rates of histologic chorioamnionitis are inversely related to gestational age and directly related to the duration of membrane rupture (Behrman *et al*., 1992).

3.5 Pathogenesis (Behrman *et al.,* 1992*)*

According to Nelson Textbook of Pediatrics the fetus or neonate in most of the cases is not exposed to potentially pathogenic bacteria until the membranes rupture and the infant passes through the birth canal and/or enters the extrauterine environment. The human birth canal is colonized with aerobic and anaerobic organisms that may result in ascending amniotic infection and/or colonization of the neonate at birth. Vertical transmission of bacterial agents that infect the amniotic fluid and/or vaginal canal may

occur in utero or more commonly during labor and/or delivery. Chorioamnionitis results from microbial invasion of amniotic fluid, usually as a result of prolonged rupture of the chorioamniotic membrane. On occasion, amniotic infection occurs with apparently intact membranes or with a relatively brief duration of membrane rupture. Amniotic fluid infection may be asymptomatic or may produce maternal fever, with or without local or systemic signs of chorioamnionitis. The duration of membrane rupture is directly correlated with the development of chorioamnionitis. Previously, longer than 24 hours was considered prolonged rupture of membranes because microscopic evidence of inflammation of the membranes is uniformly present when the duration of rupture exceeds 24 hours. However, at 18 hours of membrane rupture, the incidence of early-onset disease with group B *streptococcus* (GBS) increases significantly. Therefore, longer than 18 hours is the current cutoff for increased risk of neonatal infection. Difficult or traumatic delivery and premature delivery are also associated with an increased frequency of neonatal infection.

In most cases, bacterial colonization does not result in disease. Factors influencing which colonized infant will develop disease are not well understood but include prematurity, underlying illness, invasive procedures, inoculum size, virulence of the infecting organism, and transplacental maternal antibodies. Aspiration or ingestion of bacteria in amniotic fluid may lead to congenital pneumonia or systemic infection, with manifestations becoming apparent before delivery (fetal distress, tachycardia), at delivery (perinatal asphyxia), or after a latent period of a few hours (respiratory distress, shock). Aspiration or ingestion of bacteria during the birth process may lead to infection after an interval of 1–2 days.

Resuscitation at birth, particularly if it involves endotracheal intubation, insertion of an umbilical vessel catheter, or both, is associated with an increased risk of bacterial infection. Explanations include the presence of infection at the time of birth or acquisition of infection during the invasive procedures associated with resuscitation.

After birth, neonates are exposed to infectious agents in the nursery or in the community. Postnatal infections may be transmitted by direct contact with hospital personnel, the mother, or other family members; from breast milk (HIV, CMV); or from inanimate sources such as contaminated equipment. The most common source of postnatal infections in hospitalized newborns is hand contamination of health care personnel.

Most cases of meningitis result from hematogenous dissemination. Less often, meningitis results from contiguous spread as a result of contamination of open neural tube defects, congenital sinus tracts, or penetrating wounds from fetal scalp sampling or internal fetal electrocardiographic monitors. Abscess formation, ventriculitis, septic infracts, and subdural effusions are complication of meningitis that occurs more often in new born infants than in older children.

An infant's immunologic status, the virulence of microorganism, the inoculums size, and a verity of other factors determine which infections results in the clinical syndrome of neonatal sepsis. The portals of entry for infectious agent include the skin, mucus membranes, umbilical cord, nasopharynx, lungs, gastrointestinal tract, and urinary tract. These barriers may be breached by congenital anomalies or trauma, including instrumentation and procedure (umbilical catheterization, endotracheal intubation). In addition, the newborn infant, particularly the premature infant, has quantitative and qualitative host immune deficits that predispose to infection or adversely affect the outcome of infection. The neonate may have an antibody deficiency owing to absence of maternal antibody (IgG) that can be transferred across the placenta. Alternatively, the very low birth weight infants may have very low IgG level (<100 mg/dL) owing to diminished passive transfer early in gestation. Levels of opsonic C3, Chemotactic C5a, the factor B may further impair opsonophygocytosis and complement-mediated bacterial killing. Decreased neutrophil mobility and phagocytic activity may be due to in part to decrease expression of membrane adherence protein/C3bi receptors. Neutrophil function may be further impaired by the stress response during sepsis. The frequent occurrence of granulocytopenia during neonatal sepsis, owing to neutrophil

storage pool (bone marrow) depletion, predisposes the infants to profound quantitative defect in host defenses.

3.6 Clinical features

Behrman claims that the initial signs of infection are often subtle or minimum. The mother or nurse may simply observe that the infant does not look well or feeds poorly. There may be temperature instability (hypothermia or hyperthermia) or sign related to one or more organ system. Fever in full term infants does not always signify infection. A temperature of greater than 37.8 C (axillary), as an isolated single measurement, lasting less than 1 hour, and without other manifestation of infection, is usually not due to sepsis and may be caused by increased ambient temperature, dehydration, central nervous system disorder, hyperthyroidism, familial dysautonomia, or ectodermal dysplasia. Late manifestation of sepsis may include apnea, cyanosis, hypotention and disseminated intravascular coagulation with bleeding from multiple sites. Manifestations of sepsis may be present at birth or may appear at any time during the neonatal period. In the delivery room, sepsis with congenital consolidated pneumonia may interfere with the onset of spontaneous respirations and may be a cause of neonatal asphyxia, or it may be a separate coexisting problem. In premature infants, pneumonia may coexist with hyaline membrane disease. The presenting clinical and radiologic finding of sepsis may be difficult to differentiate from those of hyaline membrane disease. The clinical signs of infection in the new borne are often nonspecific, vary greatly in intensity and severity, and are seen in many non-infectious disorders as well (Berhman *et al.*, 1992*).*

Jayan *et al*. (2009) divides the clinical sign of sepsis into two categories.

Non-specific features

The earliest signs of sepsis are often subtle and non specific and need a high index of suspicion for early diagnosis. Babies with sepsis may present with one or more of the following symptoms and signs

(a) Hypothermia or fever (former is more common in low birth weight babies)

(b) Lethargy, poor cry, refusal to suck

(c) Poor perfusion, prolonged capillary refill time

(d) Hypotonia, absent neonatal reflexes

(e) Bradycardia; tachycardia

(f) Respiratory distress, apnea and gasping respiration

(g) Hypoglycemia, hyperglycemia

(h) Metabolic acidosis

Specific features related to various systems

(a) Central nervous system (CNS): Bulging anterior fontanelle, blank look, high-pitched cry, excess irritability, not arousable, comatose, seizures, neck retraction. Presence of these features should raise a clinical suspicion of meningitis

(b) Cardiac: Hypotension, poor perfusion, shock

(c) Gastrointestinal: Feed intolerance, vomiting, diarrhea, abdominal distension, paralytic ileus, necrotizing enterocolitis (NEC).

Hepatic: Hepatomegaly, direct hyperbilirubinemia (especially with UTI)

(d) Renal: Acute renal failure

(e) Hematological: Bleeding, petechiae, purpura,

(f) Skin changes: Multiple pustules, abscess, sclerema, mottling, umbilical redness and discharge.

3.7 Bacteria Causing Neonatal Septicemia

The agents causing neonatal sepsis vary with the time and geographical area. The most common organisms associated with neonatal sepsis vary according to the timing of infection and geographical location (Behrman *et al*., 1992). The pathogens most often implicated in neonatal sepsis in developing countries differ from those seen in developed countries. Overall, Gram negative organisms are more common and are mainly represented by *Klebsiella*, *Escherichia coli*, *Pseudomonas*, and *Salmonella*. Of the gram positive organisms, *Staphylococcus aureus*, coagulase negative staphylococci (CoNS), *Streptococcus pneumoniae*, and *Streptococcus pyogenes* are more commonly

isolated (Vergnano *et al.,* 2005). According to Behrman (1992) all serotypes of GBS and *E. coli* account for about 75% of EOS. Community acquired LOS is largely due to virulent strain of GBS serotype III and *E. coli* serotype K1. Nosocominal infections in NICU have a different pathogenesis and are often related to the use of ventilators, vascular catheter, and other risk factor. They are most often due to CoNS, *S. aureus*, gram negative enteric other than *E. coli, Pseudomonas spp.* etc. Among the anaerobic bacteria, *Bacteroids* and *Clostridium* are established neonatal pathogens (Behrman *et al.*, 1992).

3.7.1 Gram-Negative Organisms

Gram-negative organisms are responsible for a large fraction of neonatal sepsis than are gram-positive organisms, they are also associated with the highest mortality. Considerable geographic differences exist in the distribution of gram-negative organisms causing neonatal sepsis. In the United States, *E. coli* is the most frequent cause of both EOS and LOS due to gram-negative bacteria, including in neonates born prior to term, while in India, Africa, and Israel, *Klebsiella pneumoniae* accounts for the major proportion of cases (kaufman and Fairchild; 2004). Gram-negative bacteria, particularly members of the *Enterobacteriaceae*, are normal inhabitants of the intestinal tract. Neonates may acquire early infection from the maternal gram-negative flora or may develop intestinal colonization after birth with organisms that may subsequently translocate across immature or injured intestinal mucosa, resulting in LOS. Other gram negative organisms such as *Pseudomonas* may be acquired through the respiratory tract, particularly in patients requiring mechanical ventilation.

3.7.1.1 *Enterobacteriaceae*

***Escherichia coli*:** - Since *E. coli* is the most common cause of neonatal sepsis by gram-negative bacteria. A number of *E. coli* virulence factors have been identified and linked to neonatal sepsis, including the K1 capsule, fimbriae, hemolysin, rough lipopolysaccharide, Ibe (invasion of brain endothelium) proteins, and cytotoxic necrotizing factor 1. Recently, a pathogenicity island, or cluster of genes present in

pathogenic but not in avirulent strains, was found in *E. coli* C5, a strain commonly implicated in neonatal meningitis. Mutant strains lacking this pathogenicity island were less able to induce high-level bacteremia in a neonatal-rat model. In a study by Friesen and Cho, *E. coli* isolates from term infants with sepsis were more likely to express multiple virulence factors than were those from preterm infants with sepsis implying that bacterial factors contribute to the infectivity of *E. coli* in term infants while host factors contribute to disease susceptibility in preterm neonates.

EOS with *E. coli* often presents at delivery and is characterized by bacteraemia with or without meningitis. Septic shock due to endotoxemia may be a presenting sign. Alternatively, neonates may become colonized with *E. coli* at birth or through contact with colonized caregivers while in the NICU and may develop infection later. Environmental sources include ventilation systems and storage shelves. Outbreaks of both enteropathogenic and nonenteropathogenic strains of *E. coli* have been described in the NICU.

***Enterobacter, Klebsiella,* and *Serratia* species.** Gram-negative enteric organisms of the e*nterobacteriaceae* family, notably *Enterobacter* , *Klebsiella* , and *Serratia* species, are common inhabitants of the neonatal intestine which may cause nosocomial sepsis. Like the other well-known member of the family, *E. coli*, these organisms are surrounded by a capsule and fimbriae that contribute to their virulence in neonates. This capsular polysaccharide prevents activation of the alternative complement system protecting the bacteria from opsonization, phagocytosis, and bacteriolysis . In a 1999 Centers for Disease Control and Prevention-sponsored point prevalence survey of nosocomial infection in NICUs (including both term and preterm neonates), *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Serratia marcescens* each accounted for 5 to 6% of the total pathogens causing various types of infection. These three organisms together accounted for 5.1% of bloodstream infections in this survey and 8.7% of LOS in the NICHD Neonatal Network survey. These organisms spread rapidly in the NICU, and outbreaks with each of these pathogens have been reported in the literature. Epidemiologic studies utilizing techniques such as pulsed-field gel electrophoresis and *Enterobacteriaceae*

repetitive intergenic consensus PCR, have shown that most nursery outbreaks are due to a limited number of clones transmitted from patient to patient on the hands of health care workers. Intensive efforts at reducing nosocomial transmission of members of the *Enterobacteriaceae* have successfully eradicated colonization and disease with virulent enteric strains.

***Citrobacter* species.** Another uncommon but notable gram-negative pathogen found in neonates is *Citrobacter*. Invasive infections with *Citrobacter koseri*, formerly *C. diversus*, are much more common in neonates than in other patient groups, while *C. freundii* rarely causes disease in neonates. *C. koseri* is responsible for less than 5% of cases of sepsis in preterm LBW infants but is well known because of its propensity for CNS invasion and its association with epidemic outbreaks in both well-baby and intensive care nurseries. *C. koseri* is not a normal inhabitant of the maternal urogenital tract, but it is occasionally present, causing maternal UTI and chorioamnionitis. Vertical transmission may lead to a neonate with severe sepsis at birth or in the first days of life.

3.7.1.2 Members of other families

***Pseudomonas* species.** *Pseudomonas* infection is rarely perinatally acquired, however, it is among the more common gram-negative organisms causing nosocomial sepsis in NICU patients. Most *Pseudomonas* infections are due to *P. aeruginosa*, although cases of *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) infection have been reported. *P. aeruginosa* is commonly found in moist environments such as humidified incubators , sinks , and ventilator circuits. Hands of health care workers have also been identified as an important reservoir. Foca et al. reported that 10% of nurses and 5% of clinicians in a NICU had hand cultures positive for *P. aeruginosa*. Both this group and another group investigating an outbreak of *Pseudomonas* infection in NICU described the possible role of long and artificial fingernails of health care workers in transmission of the organism. *P. aeruginosa* is a particularly virulent pathogen for VLBW infants. Focal *P. aeruginosa* infections include pneumonia, conjunctivitis, and, rarely, a disease entity termed noma neonatorum, a gangrenous process affecting the nose, mouth, eyelids, and

perineal area and often associated with bacteremia. *P. aeruginosa* conjunctivitis should be rapidly diagnosed and treated since it may lead to rapidly progressive invasive eye infection, as well as to systemic complications including bacteremia, meningitis, brain abscess, and death. In several studies, *Pseudomonas* had the highest mortality of all organisms causing LOS, with 50 to 75% of patients dying.

***Haemophilus influenza*: -** *Haemophilus influenzae* may be vertically transferred from mother to infant at the time of delivery and occasionally causes EOS in preterm infants. *H. influenzae* accounted for 8.3% of EOS cases in the most recent NICHD survey, and the incidence of *H. influenza* sepsis in preterm neonates appears to be increasing in some centers. Most cases involve non-typable strains, with fewer than 10% caused by *H. influenzae* type b, presumably due to maternal immunity to the latter. The presentation of *H. influenza* sepsis in preterm neonates is generally quite fulminate and often includes pneumonia simulating severe respiratory distress syndrome. Mortality has been reported as high as 90%.

3.7.1.3 Anaerobic Bacteremia

In most recent surveys, anaerobes accounted for less than 5% of cases of neonatal bacteraemia, with premature infants representing a substantial proportion of these cases. In a review of 178 cases of neonatal anaerobic bacteraemia reported in the literature, 73 isolates were *Bacteroides* species (69 were *B. fragilis*), 57 were *Clostridium* species (mostly *C. perfringens*), 35 were *Peptostreptococcus* species, 5 were *Propionibacterium acnes*, 3 were *Veillonella* species, 3 were *Fusobacterium* species, and 2 were *Eubacterium* species (Noei *et al.*, 1988). Other reports have confirmed this predominance of *B. fragilis* and *C. perfringens* among anaerobic isolates from cultures of blood samples from neonates.

3.7.2 Gram-Positive Organisms

***Staphylococcus aureus*:** *Staphylococcus aureus* is a much less common cause of neonatal sepsis in recent decades than at its peak incidence in the 1950s through the

1970s. However, it can be a highly virulent pathogen in immunocompromised patients such as premature neonates. Extensive research has focused on the pathogenesis of *S. aureus* infection and is the subject of a recent review. Although *S. aureus* is more commonly associated with nosocomial sepsis, maternal-fetal infections have been reported. In a case series spanning 3 years from a single institution, seven preterm infants with congenital *S. aureus* infection were identified, including one with methicillinresistant *S. aureus* (MRSA). In all seven cases, amniotic fluid culture as well as initial blood culture of a sample from the infant yielded *S. aureus*, and in three cases, antenatal invasive procedures (amniocentesis or amnioinfusion) performed within a day of delivery was presumed to have contributed to infection of the fetus (Andre *et al.*, 2000) Late-onset *S. aureus* infections in neonates include skin and deep-seated tissue abscesses, bacteremia/sepsis, endocarditis, septic arthritis, osteomyelitis, pneumonia, and meningitis. In addition, *S. aureus* is one of the most common etiologic agents of ventriculoperitoneal shunt infections in preterm infants with posthemorrhagic hydrocephalus . Compared with other neonatal pathogens, *S. aureus* is associated with a relatively high incidence of deep-seated infection and suppurative complications. Of the pathogens responsible for pneumonia in preterm infants, *S. aureus* is the most likely to cause pneumatoceles and empyema, sometimes requiring chest tube drainage. *S. aureus* meningitis may be associated with brain abscesses, and neuroimaging is recommended to determine the duration of therapy. *S. aureus* endocarditis may occur in the absence of clinical signs such as a heart murmur, and some researchers have advocated routine echocardiography in preterm infants with *S. aureus* bacteremia, particularly those with a central venous catheter in or near the heart and those with two or more positive blood cultures.

S. aureus toxin-associated diseases have been reported in preterm neonates. Staphylococcal scalded skin syndrome (SSSS), in which *S. aureus* exfoliative toxins A or B split the granular layer of the skin, resulting in sloughing and erythema, has been found in NICU patients. Although SSSS is not associated with severe systemic illness or bacteremia, nosocomial spread among NICU patients has been reported, and strict

infection control measures should be implemented when a case is suspected. In contrast to the relatively benign nature of SSSS, toxic shock syndrome (TSS) due to *S. aureus* enterotoxins presents a more fulminant clinical picture.

Criteria for diagnosing TSS include fever, hypotension, multi organ system dysfunction, a diffuse macular rash leading to desquamation, and evidence against an alternative diagnosis.

The vast majorities of S. aureus clinical isolates produce β-lactamases and are resistant to penicillin. Most *S. aureus* strains causing colonization and infection in NICUs have remained sensitive to extended-spectrum penicillins, and treatment with oxacillin or nafcillin usually eradicates infection. Persistent or deep-seated infections may require the addition of an aminoglycoside or rifampin for effective clearance. Methicillin resistance among *S. aureus* strains in NICUs has been reported and is commonly associated with episodic outbreaks from a single clone. Epidemics of MRSA infection have been associated with understaffing, overcrowding, improper cleaning of equipment and hands, and mixing of patients in the NICU. Successful eradication of MRSA outbreaks has been accomplished by scrupulous attention to infection control measures as well as by intranasal mupiricin treatment of colonized patients and health care workers. Hexacholoraphene hand washing has also been used to control an MRSA outbreak in a NICU.

Vancomycin- or glycopeptide-intermediate *S. aureus* isolates with drug MICs in the 8 µg/ml ranges have been detected since the late 1980s in adults; since 2002, vancomycin- resistant *S. aureus* strains have also been found. To date, there are no published reports of vancomycin-intermediate or vancomycin-resistant *S. aureus* isolated in neonates. With increasing use of vancomycin for treating documented MRSA and CoNS infection and as empiric therapy for suspected LONS, it is possible that vancomycin resistance among *S. aureus* strains will evolve in the NICU. One approach to preventing the emergence of resistant *S. aureus* strains is prevention of infection through active and passive immunization of susceptible hosts. Vaccination

with *S. aureus* capsular polysaccharides 5 and 8, which account for up to 90% of infections, has proven efficacious in adults, and hyperimmune intravenous immune globulin (IVIG) preparations against staphylococcal surface proteins are under investigation for use in VLBW infants.

Coagulase-negative staphylococci (CoNS): CoNS are the etiologic agents of the majority of nosocomial infections in premature neonates. Although CoNS are common commensal organisms with little pathogenicity in immunocompetent hosts, premature neonates are particularly susceptible to invasive infection. The first step in the pathogenicity of CoNS involves adherence of the bacteria to skin, mucosal surfaces, or indwelling artificial devices, such as intravascular catheters and CNS shunts, which are commonly used in preterm infants. Adherence of CoNS is facilitated by a capsular polysaccharide adhesin consisting of poly- *N*-succinyl glucosamine. Once adherence and colonization have been established, some CoNS produce an exopolysaccharide "slime," which allows the organisms to form a biofilm and evade host defense mechanisms and antibiotic activity. The major component of slime is polysaccharide intercellular adhesin (PIA), a linear homoglycan composed of *N*-acetylglucosamine residues. In one study of 179 strains of *S. epidermidis*, 51% produced PIA and most of these strains formed a biofilm (Mack *et al.* 1996). Genes encoding PIA are located in a gene cluster termed the *ica* (for "InterCellular Adhesion") operon.

Although slime and other virulence factors are important to the pathogenicity of CoNS, several studies did not find evidence of hypervirulent clones of CoNS causing disease in neonates. However, in a study of 97 blood isolates of CoNS (29 considered sepsis isolates and 68 considered contaminants) from a single center over a 15-year period, sepsis isolates were phenotypically and genotypically more homogeneous than contaminating isolates, suggesting that disease causing strains of CoNS may have a higher invasive capacity (Bjorkqvist *et al.*, 2002).

Of the 31 species of CoNS and the 13 known to colonize human skin, species reported to cause disease in infants include *S. epidermidis, S. haemolyticus, S. hominis, S.*

warneri, *S. saprophyticus*, *S. cohnii*, and *S. capitis*. The major species involved in neonatal infection is *S. epidermidis*, which accounts for approximately 50 to 80% of CoNS colonization and 60 to 93% of CoNS bloodstream infection. *S. epidermidis* colonization rates of 86 to 100% have been reported among NICU patients. Occasionally CoNS is acquired from the mother at birth. Hall et al. showed that 51% of pregnant women were colonized with CoNS, and the proportion of slime-positive strains increased from 40 to 68% from the first to the third trimester of pregnancy. In this study, although 30% of neonates were colonized with CoNS at birth, in only three of these cases were the maternal and infant species identical based on biotype, antibiotic sensitivity pattern, slime production, phage type, and plasmid pattern profile. The majority of CoNS colonization is acquired nosocomially, predominantly from the hands of health care workers. In a survey using multiple molecular typing techniques, 62% of NICU nurses were colonized with methicillin- resistant CoNS, with similar species distribution to that of bacteremic strains in the unit.

While reports of *S. epidermidis* bacteremia on the first day of life suggest that the organism may be perinatally acquired, it is more commonly a nosocomial pathogen. Neonates with intravascular catheters, particularly those with central vascular catheters which remain in place for prolonged periods, are at high risk for CoNS bacteremia. Another significant risk factor for CoNS septicemia is the administration of intravenous lipid infusions, which provide a growth medium for the organism. Sepsis with CoNS is often indolent rather than fulminant, although fatalities have been reported.

Neonates with CoNS bacteremia generally present with nonspecific symptoms such as decreased activity, increased apnea, and feeding intolerance. Since these symptoms are common even in nonseptic preterm neonates, a positive blood culture for CoNS may represent either contamination or true bacteremia, and for this reason, many studies require more than one positive blood culture or other laboratory evidence of infection, such as an elevated CRP level, to distinguish CoNS bacteremia from contamination.

In addition to being the most common cause of nosocomial bacteremia in NICU patients, CoNS may cause focal infections. The organism has been isolated from the CSF of septic preterm neonates, sometimes in absence of CSF pleocytosis. CoNS is among the common causes of ventriculoperitoneal shunt infection in VLBW infants. Another nidus of infection with CoNS is the endocardium. Right-sided endocarditis due to *S. epidermidis* may be associated with placement of an umbilical venous catheter in the right atrium.

Resistance to antibiotics such as penicillin, semisynthetic penicillins, and gentamicin is common among clinical isolates of CoNS. One NICU study found an increase in multiple antibiotic resistance among colonizing CoNS strains from 32% at birth to 82% after the first week of life. Similarly high rates were reported by another group. Of particular concern is the emergence of strains of CoNS with decreased sensitivity to vancomycin. While this has not yet beendescribed for neonates, heteroresistance to vancomycin has been reported.

Streptococcus spp: - Species of *streptococci* other than GBS are infrequent agents in EOS in premature neonates and even less common in LOS. In the recent NICHD survey, viridans streptococci accounted for 3.6% of cases of EOS, and other studies have also reported pathogenicity of these organisms, particularly in premature neonates. In one study from France, viridans streptococci were associated with neonatal septicemia more frequently than were all other types of streptococci, including GBS. Group A *Streptococcus*, historically a major agent in puerperal sepsis, has only infrequently been implicated in neonatal sepsis in the last decade. Cases of neonatal sepsis caused by group C, D, and G streptococci are also occasionally reported. Early-onset *S. pneumoniae* sepsis is uncommon but is more likely to occur in preterm than in term infants. In a review of 50 cases of neonatal pneumococcal sepsis, 60% of infants were born prematurely, 91% of infants developed symptoms within 48 h of birth, pneumonia and meningitis were present in 64 and 38% of patients, respectively, and mortality was 50%.

Group B *streptococci*: As a result of intensive efforts at chemoprophylaxis, *Streptococcus agalactiae*, or GBS, is declining in incidence, but it still remains a major cause of sepsis in preterm and term infants in the United States. The pathogenicity of GBS has been attributed to a number of virulence factors, including Lipoteichoic acid, a thick polysaccharide capsule, capsular sialic acid, and the enzyme C5a-ase, which inhibits neutrophil accumulation at the site of infection. In the United States, GBS colonizes the genital and lower gastrointestinal tracts of 15 to 40% of pregnant women. Factors that increase the risk of maternal GBS carriage include diabetes, age younger than 20 years, and African American race, and these factors also increase the risk of preterm delivery. Approximately half of all neonates born to GBS-colonized women acquire surface colonization at delivery, and without intrapartum antibiotic therapy, about 1% of colonized full-term infants develop EONS. Compared to term infants, preterm infants are much more susceptible to invasive GBS disease, in particular LONS and VLONS. In a recent case-control study of 122 infants with GBS LONS, 84% of patients were born at 34 weeks of gestation, and the risk for GBS LONS increased by a factor of 1.34 (95% CI, 1.15 to 1.56) for each week of decreasing gestation. This is probably due in part to low levels of maternal antibodies, which cross the placenta in the third trimester of pregnancy.

A number of studies have shown low levels of GBS type-specific antibodies in infants with GBS sepsis and in their mothers. Other risk factors for early-onset GBS disease, which are common in preterm deliveries, include prolonged rupture of the amniotic membranes (>18 h before delivery), maternal intrapartum fever greater than 38°C, and maternal GBS UTI during the pregnancy or at delivery, which may reflect a high level of colonization or the presence of a particularly virulent strain. GBS sepsis was the first neonatal infection to be defined as EOS, LOS, and LLOS. The majority of LLOS GBS sepsis occurs in preterm infants at an age when the immune system is more mature; thus, mortality due to LLOS GBS sepsis is much lower than that presenting at earlier ages. Infants may have persistent colonization from birth or may acquire the organism through nosocomial routes. Transmission of GBS from breast milk, patient-to-patient

spread, and colonized nursery personnel has been reported. Increased adherence of GBS to buccal epithelial cells from preterm compared to term infants may also be a contributing factor.

Recurrent GBS sepsis after appropriate antibiotic therapy has also been documented, since treatment fails to eradicate colonization in up to 50% of infants, infants can be repeatedly exposed through breast milk or horizontal contact, and systemic infection does not stimulate the production of protective levels of type-specific antibodies, particularly in preterm infants.

***Enterococcus* species: -** Although accounting for only a small proportion of neonatal sepsis, *Enterococcus* species deserve special mention because of the increasing incidence of neonatal enterococcal sepsis in several studies and the emergence of vancomycin resistance among enterococci. Both *Enterococcus fecalis* and *E. faecium* cause sepsis in preterm neonates, with *E. fecalis* accounting for over 80% of cases. McNeely et al. reviewed all cases of enterococcal bacteremia in NICU patients in a large metropolitan teaching hospital and found a three-fold increase in cases in 1983 to 1993 compared with the previous decade (McNeeley *et al.*, 1996). In 100 cases reviewed, the mean age of onset was approximately 45 days and the mean gestational age was 31 weeks. Of note, 64% of patients had other organisms isolated concurrently from blood culture. Another study also found a significant proportion of polymicrobial bacteremia associated with enterococcal sepsis in 83 pediatric patients, including 16 neonates (Christie *et al.* 1994). This may be due to the common association with central vascular catheters or NEC, found in 77 and 33% of cases, respectively. For reasons that are unclear at present, vancomycin resistance among *enterococci* has not become a significant problem in most NICUs (470), yet several studies have reported endemic or epidemic vancomycin-resistant enterococci (VRE) among hospitalized neonates. In the review by McNeely et al; six neonates had bacteraemia with VRE, and one died of the infection. Interestingly, none of the six had been given prior therapy with vancomycin, although they all had prolonged.

NICU stays prior to the VRE infection (mean age, 100 days). Other studies have found an association of prior antibiotic use and colonization or infection with VRE. In one study, 68% of pediatric patients infected with VRE had been treated with vancomycin within 90 days of detection of the organism (317). A recent case report describes a 4-month old ELBW infant with a central venous catheter who, after three 10-day courses of vancomycin for various infections including CoNS and *E. faecalis* bacteremia, developed endocarditis with vancomycin- resistant *E. faecium.*

Rapid spread of VRE among NICU patients was documented by Malik et al., who reported the spread of related strains of VRE to 40% of the NICU patient population. Two preterm infants developed bloodstream infection, and 33, including 11 of 13 babies who shared a room with the bacteremic babies, became colonized with VRE. Compared to noncolonized babies, those with colonization were more premature, had been in the hospital longer (requiring more intensive care interventions), and had more exposure to antibiotics including vancomycin. In both of these studies, outbreaks were successfully controlled after implementation of strict infection control measures including limiting the use of vancomycin. In another study, active surveillance and barrier precautions reduced VRE colonization in an NICU from 2.2 to 0.5%.

With longer hospitalization of extremely premature neonates, particularly those with surgical complications and prolonged central venous access, it is likely that VRE will become a more significant burden in the NICU in the future. Restricting the use of vancomycin should be a high priority for those caring for these patients.

***Listeria monocytogenes*: -** *Listeria monocytogenes*, a gram-positive bacillus, is a well-known and well-studied neonatal pathogen. Although neonates account for approximately one-third of cases of invasive listeriosis, the organism accounts for less than 5% of cases of EOS in preterm neonates in most studies. *L. monocytogenes* is commonly found in soil as well as other environmental sources, and farm animals may become infected through ingestion of spoiled silage. Most human cases of listeriosis are associated with ingestion of contaminated food such as undercooked or processed

meats, unwashed vegetables, and unpasteurized dairy products. The incidence of *L. monocytogenes* sepsis in neonates is approximately 13 per 100,000 live births in the United States as well as in Europe. The vast majority of cases represent perinatal transmission, although nosocomial transmission has been reported. *Listeria* infection during pregnancy may result in miscarriage, stillbirth, or chorioamnionitis, often with placental abscesses. Infection occurring after the fifth month of pregnancy commonly leads to premature labor and delivery, with up to 70% of cases delivering at less than 35 weeks' gestation. *Listeria* may infect the fetus through the ascending or hematogenous route, often leading to signs of severe sepsis at delivery. In contrast to nearly all other organisms causing neonatal sepsis, *Listeria* is an intracellular pathogen and primarily targets cells of the monocyte-macrophage lineage. Impaired cell-mediated immunity, characterized by deficient production of gamma interferon and IL-12, decreased number and function of natural killer cells, and immature chemotaxis, phagocytosis, and killing by mononuclear phagocytes predispose the VLBW infant to overwhelming infection with this intracellular pathogen.

3.8 Global scenario

A prospective study in Iran shows that there was found 11% positivity of blood culture among neonates suspected of sepsis. The boys/girls ratio was 1.67:1 and 69.9% of patients were premature. There were 164 (72.2%) cases of EOS and 63 (27.7%) cases of LOS. Coagulase negative staphylococcus (CONS) was the most common (54%) cause of both early and late onset neonatal sepsis and showed high degree of resistance to commonly used antibiotics; ampicillin (100%), ceftriaxon (65%), cefotaxim (67%) and gentamicin (51%), but comparatively low resistance to vancomycine (10%), imipenem (19%), and ciprofloxacine (23%) (Gheibi *et al*., 2008).

Awoniyi (2009) examined occurrence of bacterial pathogens associated with neonatal sepsis in hospital setting. It was found that among 100 samples collected and proceed, *Staphylococcus aureus* accounted for 28%, *Klebsiella* and *Pseudomonas* species 13% each, *Proteus* species 10%, other *Enterobacteriaciae* 9%, *Neisseria gonorrhoea* 8%,

beta-haemolytic *Streptococcus* 5 and 14% showed no bacterial growth. Antibiotic sensitivity patterns of the bacterial isolates showed that most were sensitive to oxfloxacin. However, a significant number of them showed resistance to commonly used antibiotics such as ampicillin and penicillin. Bacterial pathogens in neonates with sepsis vary from Gram-positive bacteria, mostly *S. aureus* to Gram-negative bacilli, mainly K*lebsiella* and *Pseudomonas* species.

A study from Italy (2000) confirmed that *S. epidemidis* is one of the leading causes of NICU acquired infections (villari et al., 2000).

Neonatal septicaemia caused by E. coli is still major health problem in industrialized country (Soto *et al.,* 2008).

A study shows the bacteriology of neonatal septicaemia in a tertiary care hospital of Northern India, out of 728 cases, 346 (47.5%) were positive on blood culture. The most frequent offender was *Klebsiella* spp (24.5%) followed by *Enterobacter* spp. (22.8%). There was over all predominance of gram negative organisms. CoNS (16.5%) were more frequent isolates than *S. aureus (14%)*. More than 89% of the staphylococci isolates were penicillin resistant. None were resistant to vancomycin. Ciprofloxacin and Amikacin resistant are infrequent (Roy *et al.,* 2002).

A Pakistani report shows that out of 100 cases, 64 belonged to early onset sepsis (EOS) and 36 belonged to Late onset Sepsis (LOS). Gram negative organisms were isolated from more than 80% of the cases. *E. coli* was the commonest isolate (n=34), followed by *Klebsiella* (n=30) and *Pseudomonas* (n=13), involving both early and late onset groups. No isolate of group B streptococci (GBS) was found. Out of 34 isolates of *E.Coli*, 14.70%(n=5), 17.6%(n=6), 41.17%(n=14), 61.76%(n=21), 79.4%(n=27) and 97.05% (n=33) were sensitive to ampicillin, gentamicin, cefotaxime, amikacin, ceftazidime and imipenem respectively. *Klebsiella* and *Pseudomonas* also showed a low sensitivity to ampicillin, gentamicin, and cefotaxime, while good sensitivity to

amikacin, ceftazidime and imipenem. The mortality was significantly high (P<0.05) in low birth weight infants (Wassem *et al,* 2005).

Center *et al.* (2003) reported that a total of 321 isolates of CoNS isolates seventy-five percent of the infants were colonized at admission, and virtually all were colonized thereafter. Common species were *Staphylococcus epidermidis* (69%), *S. warneri* (12%), *S. haemolyticus* (9.7%), and *S. hominis* (5.6%). A total of 3.9% of CoNS isolates had decreased vancomycin susceptibility (DVS) (MICs > 2.0 _g/ml); isolate recovery was associated with a stay in a neonatal intensive care unit for >28 days (P = 0.039), vancomycin exposure (P =0.021), and *S. warneri* colonization (P < 0.0001). Nine of 12 (75%) CoNS with DVS were *S. warneri*, had enhanceable high-level resistance in vitro, were indistinguishable or closely related by pulsed-field gel electrophoresis, and were different from 29 vancomycin-susceptible *S. warneri* isolates. Epidemiological analysis suggested unsuspected nosocomial spread. Species determination in certain settings may aid in the understanding of emerging nosocomial problems.

A retrospective study, which was performed to determine the incidence of bacterial sepsis with focus on Gram negative organisms in neonates admitted at Beheshti Hospital in Kashan, during a 3-yr period, from September 2002 to September 2005, shows from the 1680 neonates 36% had positive blood culture for *Pseudomans aeruginosa*, 20.7% for Coagulase negative *Staphylococci,* and *17%* for *Klebsiella* spp. Gram-negative organisms accounted for 72.1% of all positive cultures. The overall mortality rate was 19.8% (22 /111) of whom 63.6% (14 /22) were preterm. *Pseudomona aeruginosa* and *Klebsiella* spp. showed a high degree of resistance to commonly used antibiotics (ampicillin, gentamicin) as well as third generation cephalosporins. Continued local surveillance studies are urged to monitor emerging antimicrobial resistance and to guide interventions to minimize its occurrence (Movahedian *et al.,* 2006).

A total of 410 proved cases of neonatal septicaemia from seven Finnish hospitals seen between 1976 and 1980 were reviewed. The annual incidence of neonatal septicaemia

was 3 per 1000 births, and overall mortality was 23%. Onset was early in most patients. Symptoms of septicaemia occurred within the first 24 hours of life in 44% and within the first week of life in 90%. In the very early onset disease (within 24 hours) mortality was 30%, compared with 17% in all other cases. Group B streptococcus was the leading cause in very early onset disease (52%) but mortality from infection with this organism was similar to that in other very early onset cases. It is concluded that very early onset neonatal septicaemia, probably of intrauterine origin and caused by group B streptococcus in one half of the cases, constitutes the major form of neonatal septicaemia in Finland and should receive the highest priority in preventive measures (Vesikari *et al.,* 1985).

A study between 1979 and 1982 was reviewed 1000 consecutive admissions to the neonatal intensive care unit of Hammersmith hospital, London. Sixty five infants had positive blood cultures. Mortality was 70% among 17 infants who had septicaemia in the first 48 hours of life and for whom appropriate treatment may have been too late because of difficulties of early diagnosis. In the remaining 48 infants mortality was 12 %, septicaemia occurred later, and was associated with *Staphylococcus epidernmidis* (56 %) and with the presence of an intravascular catheter (50 %) (Pleczek *et al*., 1983).

3.9 National scenario

Jain et al. (2003) reported that among 106 neonates suspected of sepsis were studied, out of which 30 were culture positive. The most common organism was E. coli and the most common clinical presentation was the respiratory distress and letharginess.

A study from B. P. koirala institute of Health Science, Dharan shows that the factors which carried a significant risk for development of neonatal sepsis were premature rupture of membrane, meconium stained amniotic fluid, foul smelling liquor, low birth weight, prematurity. The blood culture was positive in 22% of cases. The commonest organisms isolated were *S. aureus* and *Klebsiella*. The overall mortality rate was 11%. The incidence of risk factor was almost equal in culture positive and culture negative cases (Shah *et al.,* 2006).

Shaw et al. (2003) in one of his study said that one hundred nineteen cultures out of the 131 positives were obtained from blood (44.92%) and the remaining was isolated from urine (6.11 %) and CSF (4.58 %). The most common organism to be isolated was S*taphylococcus aureus* (42.75%) followed by *Klebsiella pneumoniae* (18.32%)and *Escherechia coli* (12.21%). *Staphylococcus* was isolated from 36.84%, 45.16% and 31.81% of the cultures obtained from neonates in the inborn, out-born and the nosocomial groups respectively while *Klebsiella pneumoniae* [18.32 %] was seen in 21.05 %, 17.39 % and 36.36 % in each of the three groups. *Pseudomonas aeruginosa* [6.11 %] was isolated from 13.64 % of the nosocomial cultures compared to 8.7 % of the out-borns while it was not seen in the in-borns. Other organisms isolated were much less in number, included - pathogenic streptococci, A*cinetobacter* and E*nterobacter* species. Coagulase negative staphylococci (CoNS) was seen in 4.39 % [n=4] and 9.09 % [n=4] of the same groups respectively. The gram positive organisms displayed a high degree of resistance to most penicillins and cephalosporins but glycopeptides and monobactams were effective in them. There was a high incidence of resistance noted with most third generation cephalosporins and aminoglycosides amongst most gram negative organism where-in cefepime and imepenem were effective in most cases.

A retrospective hospital based study was conducted over a 5-year period (Oct.2000 to Sept.2005) at Patan Hospital, Nepal with the main objective of finding out the common organisms in hospital born neonatal blood cultures and their antibiotic sensitivity pattern. All the blood cultures sent from neonatal unit during this period were analyzed. A total of 2463 blood cultures were sent; out of which 13.7% were positive. The commonest organism identified was coagulase negative *Staphylococcus* (48.4%) followed by *Enterobacter* (11.2%), *Acinetobacter* (9.7%) and *Klebsiella* (9.4%). Among the gram -ve organisms identified, gentamicin resistance was observed in 65.6% *Klebsiella*, 50% *Enterobacter*, 39.4% *Acinetobacter* and 25% *E. coli* isolates. Cefotaxime résistance was seen in 53% *Klebsiella*, 31.6% *Enterobacter*, 21.2% *Acinetobacter* and 16.6% *E. coli* isolates (Shrestha et al., 2006)

CHAPTER IV

METHODOLOGY

4.1. Materials

Various materials used in this study is enlisted in the appendix III

4.2. Methods

This study was conducted at Paropakar Maternity and Women's Hospital, Thapathali, Kathmandu. In this study 690 blood samples were collected from the suspected neonatal patients of septicemia and processed following the standard laboratory techniques in the Hospital.

4.2.1 Study design

A Prospective hospital based Cross sectional study design was used.

4.2.2. Population and sample size

Populations for the study were the neonates admitted in Neonatal intensive care unit (NICU) and Premature Baby care Unit (PBU) at Paropakar Maternity and Women's Hospital. Sample size was determined according to the existing prevalence as determined by the previous studies.

4.2.3 Sample size determination

The sample size for the study was according to the previous study. All the samples according to the criteria of the study were included in the study.

4.2.4 Inclusion criteria

The clinically suspected neonates with septicemia in the NICU and PBU were included in the study. During the research period, 690 blood samples were collected. The age of these patients ranged from neonates of 1 day to neonates of 28 days.

4.2.5 Exclusion criteria

The blood sample showing the contaminants growths were excluded from the study.

4.2.6 Site of study

Site for the collection of data was the Paropakar Maternity and Women's Hospital, Thapathali, Kathmandu and the sampling was done in the entire neonates admitted in NICU and PBU in the hospital.

4.2.7 Time frame

The collection of the data and laboratory work was conducted for six months from 15th June to 15th December 2009.

4.3 Collection of the data

The data regarding the neonates was collected directly by interview of mother by using semi-structured questionnaire, Clinical History of the neonates involved in the study. The history of all the neonates including age, weight, sex, gestation age, CRP level, symptoms was recorded in the data collection form. Thus collected data was analyzed and interpreted statistically by using Statistical Program for Social Science (SPSS) 16 software.

This study was carried out to isolate bacteria from neonatal septicemia and to study its antibiotic sensitivity pattern.

4.4 Collection of samples

The sample for this study was blood. The blood was collected by Child Specialist and well trained Nurses using standard aseptic technique described by Smith and Easmon

(1990) and Frobes *et. al*. (1998). First of all each patients was provide with one culture bottle containing Brain heart infusion (BHI) broth. One culture bottle contains about 10 ml of BHI broth. About 1 ml of blood was drawn from neonates and dispensed in the culture bottle.

Incubation of blood sample into the culture media was done immediately after collection.

4.5 Processing of samples

Immediately after blood culture bottle were received in the laboratory, they were provide with unique laboratory identification numbers and further proceeded.

4.5.1. Incubation of the samples

The culture bottles were incubated at 37ºC. Incubation was continued for 7 days unless the visible growth was obtained.

4.5.2. Macroscopic examination of Blood culture bottle

It was primary steps of sample processing. The culture bottle were examined visually daily for any visible growth such as turbidity, haemolysis of red cells, gas bubbles and clot formation or formation of discrete colonies. This step helps in the presumptive diagnosis of positive broth culture as shown in table.

Table 4.1: Macroscopic examination of Blood culture bottle (Forbes *et al.,* 1998)

S. No.	Observation	Presupmtive Diagnosis
1.	Uniform turbidity with gas bubbles formation	Enteric organism (gram negative bacilli)
2.	Partial hemolysis of blood with greenish tinge	*Streptococcus* (Gram positive cocci)
3.	Hemolysis with cotton ball colony	*Streptococcus* (Gram positive cocci)

4. Jelly like coagulum throughout the broth, descrete colonies on the surface of the red cell layer and in the broth — *Styphylococcus* (Gram positive cocci)

4.5.3. Microscopic examination

The microscopic examination for the macroscopically positive was performed by Gram-Staining methods. The microscopic examination of broth was used for presumptive diagnosis of bacteria as shown in table 4.2.

Table 4.2:

S. No.	Gram staining	Presumtive organism
1.	Gram-positive cocci in cluster	*Staphylococcus* spp.
2.	Gram positive cocci in chain	*Streptococcus* spp.
3.	Gram Negative bacilli	Members of *Enterobacteriaceae*

The gram staining procedure is mentioned in the appendix V.

4.5.4. Subculture from Broth culture

The broth cultures were sub-cultured on Blood agar, Chocolate agar, Mac Conkey agar and Nutrient agar plates. Reapeated sub-culture of the culture bottles were made at different times during their incubation from 24 hours to 7 days.

The composition and preparation of Blood agar and MacConkey agar are mentioned in the appendix IV.

4.5.5. Incubation of subculture plates

Nutrient agar and MacConkey agar plates were incubated aerobically at 37ºC, Blood agar and chocolate agar plates were incubated at CO_2 enriched humid atmosphere by using candle jar at 37ºC.

4.5.6. Examination of subculture plates

The subculture plates were examined after overnight incubation. Blood culture plates were examined for distinguishing the growth of hemolytic and non-hemolytic colony.

MacConkey agar plates were observed for the growth of lactose fermenter and non-fermenter organism.

4.5.7. Identification of isolated organism

The standard microbiological technique, which involves colony morphology, Staining reactions, biochemical properties, were followed for the identification of bacteria from positive subculture plates (Cheesbrough, 1984 and Barrow *et al.,* 1993).

4.5.8. Identification with Morphological characteristics

Since microorganism may produce colony that is not different from colonies of many other species; the total confidence is not given on colonial morphology for preliminary identification of isolates on primary media (Forbes et al, 1998). However colonial characteristics of some microorganisms were studied for preliminary identification as shown in table 4.3.

Table 4.3 Colonial characteristic of isolates

S. No.	Colonial characteristic on blood agar	Preliminary Identification
1.	Circular, opaque, Smooth, Yellow to cream or occasionally white colour colonies with 1-2 mm diameter.	*Staphylococcus* spp
2.	Translucent to milky, circular, small colonies (Approximately 1mm diameter) with a shiny surface.	*Streptococcus* spp.
3.	Large, Shiny, Grey colonies(may be hemolytic)	Members of *Enterobacteriaceae*

4.5.9 Identification with staining reaction

Gram-staining was performed for the presumptive identification of the isolates.

4.5.10. Identification with Biochemical Tests (Cheesbrouge, 1984; Forbes *et al.*, 1998; Colle *et al.*, 1996)

Appropriate biochemical tests were performed for the confident identification of the bacterial isolates. The pure isolated colonies from culture plates were picked up and inoculated onto different biochemical medium for different biochemical tests.

4.5.10.1 Indole test

A smooth bacterial colony was stabbed on SIM (Sulfur, indole, motility) media by a sterile stab wire and inoculated media was incubated at 37ºC for 24 hours. After 24 hours of incubation, 0.5 ml of Kovacs reagent was added. Appearance of red colour on the top of the media indicates indole positive.

4.5.10.2. Methyl red test

A pure colony of the test organism was inoculated into 2 ml of MR-VP medium and was incubated at 37ºC for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red colour, indicating acidity and negative with yellow colour.

4.5.10.3. Voges-Proskauer test

A pure colony of the test organism was inoculates into 2ml of MR-VP medium and was incubated at 37ºC for 24 hours. After incubation, about 5 ml of Barrit's reagent was added and shacked well and kept for 15 minutes, positive test shows development of pink red colour.

4.5.10.4. Citrate Utilization test

A loop full of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37 C for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue due to alkaline reaction.

4.5.10.5. Catalase test

A small amount of a culture from Nutrient agar plate was taken in a clean glass slide and about 2-3 drops of 3 % H_2O_2 was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e. g. Blood agar) or if iron wire loop is used.

4.5.10.6. Oxidase test

A piece of filter paper was soaked with few drops of oxidase reagent. Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple colour within 10 second.

4.5.10.7. Urease test

The test organism inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37ºC over night. Positive organism shows pink red due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in colour of the indicator to red pink.

4.5.10.8. O/F test (oxidation fermentation test)

The test organism was stabbed into the bottom of the two sets of tubes with Hugh and Leifson's media. The inoculated medium in one of the tubes was covered with a 10mm deep layer of sterile paraffin oil. The tube was incubated at 37ºC for 24 hours. After incubation the tubes were examined for carbohydrate utilization as shown by acid production.

Fermentation organism utilizes the carbohydrate in broth the open and sealed tubes s shown by a change in colour of the medium from green to yellow. Oxidative organism, however, are able to use the carbohydrate only in the open tube.

4.5.10.8. Triple Sugar iron Agar (TSI agar)

The test organism was streaked and stabbed on the surface of TSI and incubated at 37ºC for 24 hours. The results are interpreted as follows.

a. Yellow (Acid) / Yellow (Acid) , Gas. H_2S - glucose, lactose/ sucrose fermenter, H_2S producer
b. Red (Alkali) / Yellow (Acid) , no gas , no H_2S - Only Glucose, not lactose/ Sucrose fermenter, not aerogenic, No H_2S production
c. Red (Alkali) / No change – Glucose, Lactose and sucrose non fermenter.
d. Yellow (Acid) / No change – glucose – oxidizer
e. No change / no change – Non-fermenter

4.5.10.8. Coagulase test

Slide Coagulase test

For slide coagulase test, a drop of physiological saline was placed on three places of a slide, and then a colony of the test organism was emulsified in two of the drops to make thick suspensions. Later a drop of plasma was added to one of the suspensions and mixed gently. Then a clumping was observed within 10 second for positive coagulase test. No plasma was added in second suspension. This test was used for the differentiation of any granular appearance of the organism from true coagulase clumping. The third drop of saline was used for a known strain of coagulase positive *Staphylococci*.

Tube coagulase test

For the organism showing positive test on slide coagulase test, the tube coagulase test was performed. In the tube caogulase test, 0.5 ml of diluted plasma (1:10 in physiological saline) was pipetted into 18-24 hours broth culture tube inoculated with test organism. After mixing gently, the tube was incubated at 37C for 2-6 hours. The clotting was observed by gently tilting the tube for positive coagulase test.

4.5.10.9. Optochin (5 µg) Sensitivity Test

A loop full of test organism was spread on a surface of Blood Agar with cotton swab. After spreading, 5 **µg** of optochin (ethylhydrocupriene hydrochloride) was placed on the surface of the agar. The plate was incubated in a candle jar. The Pnumococcus shows sensitivity to optochin and viridans streptococci show resistance to optochin disc.

4.5.10.10. Bacitracin Test

Bacitracin is an antibiotics which acts only on certain bacteria killing them. The test helps to identify β-haemolytic streptococci group A from the BA plates. A loop full of test organism was spread on a surface of Blood Agar with cotton swab. After spreading, 0.04unit Bacitracin disc was placed on the surface of the agar. The plate was incubated in a candle jar. The β-haemolytic streptococci group A shows sensitivity to bacitracin and other group streptococci show resistance to bacitracin disc.

The composition and preparation of biochemical media and reagent used in the biochemical test are mentioned in the annex.

4.6. Purity test

The inoculum used for the biochemical tests was pure culture. The purity was used to ensure that the inoculum used for the biochemical tests was pure culture. So, before performing biochemical test, the same inoculum was subcultured in respective medium in order to confirm the purity of the inoculum.

4.7. Sensitivity tests for isolated organisms

Antibiotics selection criteria

Antibiotics are chosen on the basis of frequent use in treatment of bacterial neonatal septicaemia. For gram positive and gram negative organism antibiotics are chosen on the basis of spectrum. However, some antibiotics were used for the research purposes on the basis of available literature.

After identification of isolated organism, the sensitivity test in vitro was performed for the clinically significant organism. This test was performed by following Kirby-Bauer disc diffuse technique. In this technique the antimicrobial agent diffuse from the disc into the medium. Following overnight incubation, the culture was examined for areas of

no growth around the disc. Bacterial strains sensitive to the antimicrobial are inhibited at a distance from the disc where as resistant strain grows up to the edge of the disc.

Isolated colony of organism was transferred to a test tube containing 4-5 ml nutrient broth and incubated at 37ºC for 24 hours. After 24 hours, it was swabbed on Mueller-Hinton Agar plates with the help of sterile cotton swab and left for 15 minutes at room temperature for drying. For Streptococcal isolates Blood agar were used for antimicrobial susceptibility tests. With the help of flamed forceps, selected antibiotic discs were then placed on the inoculated plate, no closer than 15 mm from the edge of the plate and 24 mm apart from each other and also from centre to centre. Then the plate was allowed for 30 minutes at room temperature for diffusion and incubated at 37ºC for 24 hours. After incubation, the zone of inhibition of test organism was measured and observed into sensitive, intermediate, and resistant categories by referring in an interpretative chart table supplied by the disc manufacturers.

Multi drug resistant isolates were defined as those which showed resistance to two or more than two antibiotics disc used for the sensitivity testing.

The preparation and composition of Mueller-Hinton Agar medium is mentioned in the appendix IV.

The detail about antibiotic discs used and its interpretative chart are mentioned in the Appendix III

4.8. Quality control of the test

Quality control is considered as one of the important factor for the correct result interpretation. During this study, quality control was applied in various areas.

a. During sample collection, Aseptic technique was followed using sterile syringe and needle, disinfecting the skin over the vein of the patient and collecting blood in sterile bottles in order to avoid contamination.

b. During sample processing, the entire tests were carried out appropriately in aseptic condition.

c. While using readymade dehydrated media, the manufacturer's instruction for preparation, sterilization and storage were followed to prevent the alteration of the nutritional, selective. Inhibitory and biochemical properties of the media.

d. The performance of newly prepared media was tested using control species of bacteria (i.e. known organism giving positive and negative reactions).

e. For strain and reagents, whenever a new batch of them were prepared, a control smear was stained to ensure correct staining reaction.

f. Control strains of E. coli (ATCC 25922), Staphylococcu aureus (ATCC 25923) and Pseudomanas aeruginosa (ATCC 27853) were used for the standardization of the Kirby-Bauer test to adjust the diameter of the inhibition zone.

4.9 Data analysis

All the results were entered in the worksheet of Statistical Package of Social Sciences (SPSS 14.0). Chi-square test was used to determine the association of independent variables. Chi square is very useful test which can be applied to find significant in the same type of data. It is most commonly used when data are in frequencies such as in the number of responses in two or more categories.

CHAPTER V

5. RESULTS

A total of 690 neonates admitted in Neonatal Intensive Care Unit (NICU) and Premature Baby Care Unit (PBU) of Paropakar Maternity and Women's Hospital, Thapathali from 15st June 2009 to 15th December 2009 were included in this study. The prevalence of neonatal septicaemia was found 28.1%.

5.1 BACTERIOLOGICAL PATTERN OF THE RESULT

5.1.1 Growth Pattern of Bacteria

Out of 690 blood samples, 194 samples showed culture positive result and 496 blood samples did not show growth of organisms in culture. The total prevalence of neonatal septicemia is 28.1%.

Table 5.1 Growth Pattern of Bacteria

S. No.	Growth	No. of Samples	Percentage of Samples
1	Culture positive	194	28.1
2	No Growth	496	71.9
Total		**690**	**100.0**

5.1.2 Distribution of Pathogens in Neonatal Septicemia

Of the 194 organisms isolated nearly two third (67.0%, 130/194) were gram negative bacilli; and nearly one third were gram positive organism (33.0%, 64/194). Among the gram negative organism *E. coli* was the most prominent isolates (51.0%, 99/194) followed by *Klebsiella pneumoniae* (12.4%, 24/194), *Enterobacter cloaecae* (3.1%, 6/194) and *Pseudomonas aeruginosa* (0.5%, 1/194). CoNS (26.3%, 51/194) and

Staphylococcus aureus (4.6%, 9/194) were the major isolates of gram positive bacteria followed by *Streptococcus* spp. (2.1%, 4/194).

Table 5.2 Distribution of Pathogens in Neonatal Septicemia

Organisms	**Number**	**Percent**
Gram Negative		
E. coli	99	51.0
Klebsiella pneumoniae	24	12.4
Enterobacter cloaecae	6	3.1
Pseudomonas aeruginosa	1	0.50
Gram Positive		
Coagulase negative Staphylococcus (CoNS)	51	26.3
Staphylococcus aureus	9	4.6
Streptococcus spp.	4	2.1
Total	**194**	**100**

5.2 CLINICAL PATTERN OF THE RESULT

5.2.1 Sex wise distribution of Neonates Requesting for Blood Culture

Out of 690 neonates requested for blood culture, 400 (57.9%) were male and 290 (42.1%) were female. The table 5.3 shows that male neonates had requested more blood culture than female neonates. 27.6% of total male showed the culture positive result while 28.6% of total female showed culture positive. There is no significant difference between growth of organisms and sex of the neonates (p = 0.544).

Table 5.3 Association of Sex and growth of the organisms

Variables	**Growth of Organism**		**Total (N=690)**		**p-value[b]**
	n	**%**	**n**	**%[a]**	
Sex					
Male	110	27.6	400	57.9	0.544[b]
Female	84	28.9	290	42.1	

a: Column percentage to indicate the distribution of overall characteristics
b: Compare the significance difference in Growth patterns of organisms

5.2.2 Association of C-reactive protein level and growth of the organism

Of 690 blood samples requested for blood culture, 150 (21.7%) were CRP positive remaining 540 (78.3%) were CRP negative. 31.3% of CRP positive neonates showed culture positive results while 27.2% CRP negative neonates showed culture negative. But there is no significance difference between CRP level and growth of the organisms in culture (p = 0.254).

Table 5.4 Association of C-reactive protein level and growth of the organism

Variables	Growth of Organism		Total (N=690)		p-value[b]
	n	%	n	%[a]	
CRP test					
Positive	47	31.3	150	21.7	0.254
Negative	147	27.2	540	78.3	

a: Column percentage to indicate the distribution of overall characteristics
b: Compare the significance difference in Growth patterns of organisms

5.2.3 Distribution of neonates among culture positive according to their weight and gestation age.

Out of 690 neonates, 61 (11.3%) neonates were born very low birth weight, 213 (30.8%) were born low birth weight, and remaining 416 (60.2%) were good birth weight baby. Growth of the organism was seen maximum in VLBW neonates (36.1%). But there was no significance difference between weight group and growth of organism (p = 0.274).

In terms of gestation age, out of 690 neonates, 32.1% (221/690) were preterm baby and growth of organism was highest in preterm baby 38.4% (77/194). There is significance difference between growth of organism and gestation age of neonates (p = 0.024).

Table 5.5 Distribution of neonates among culture positive according to their weight and gestation age

Variables	Growth of Organism		Total (N=690)		P-value[b]
	n	%	n	%[a]	
Weight of Baby					
VLBW(<1500g)	22	36.1	61	11.3	
LBW(1500-2500g)	62	29.1	213	30.8	0.274
GBW(>2500g)	110	26.4	416	60.2	
Gestation age					
Preterm	77	34.8	221	32.1	0.024
Term	108	25.2	428	62.0	
Post term	9	22.0	41	5.9	

a: Column percentage to indicate the distribution of overall characteristics
b: Compare the significance difference in Growth patterns of organisms

5.2.4 Difference of weight of baby and gestation age between the growths of organism in blood culture

Table 5.6 shows that there was significance difference of weight of baby between the growth of organism (P<0.05). So the babies having the growth of organism had lower weight than babies having without growth of organism. There was also significance difference of gestation age between the growth of organism (P<0.05). So the babies having the growth of organism had lower gestation age than babies having without growth of organism.

Table 5.6

Variables	**Growth**(N = 194)	**No growth** (N = 496)	**p- value**
Weight of baby	2588.43 ± 753.803	2674.95 ± 695.38	0.022
Gestation age of baby	260.37 ± 27.884	268.34 ± 24.670	<0.01

5.2.5 Distribution of neonates with mode of delivery

Table 5.7 shows the distribution of neonates according to their mode of delivery. Normal delivery was found to be occurring in highest number 46.1% followed by caesarian delivery (33.8%). In terms of culture positive cases, it was found that baby having vacuum delivery had maximum culture positive (36.4%, 12/33) followed by premature delivery (34.9%, 29/83). It was found that there was no significant difference between growth of organism and mode of delivery (p = 0.514).

Table 5.7 Association between growth of organism and mode of delivery

Variables	**Growth of Organism**		**Total (N=690)**		**P-value[b]**
	N	**%**	**n**	**%[a]**	
Mode of delivery					
Normal	85	26.7	318	46.1	
Caesarian	64	27.5	233	33.8	0.514
Premature	29	34.9	83	12.0	
Vacuum	12	36.4	33	4.8	
Breech	3	15.8	19	2.8	
Episiotomy	0	0.00	1	0.10	
Home	1	33.33	3	0.40	

a: Column percentage to indicate the distribution of overall characteristics
b: Compare the significance difference in Growth patterns of organisms

5.2.6 Common clinical manifestation of blood culture positive cases

Table 5.8 shows blood culture positive cases and common clinical manifestation on neonates suspected of sepsis. Maximum blood sample was taken from neonates with birth asphyxia which was 19.8% of total. Out of 194 blood culture positive cases, baby with chorioalantosis showed highest culture positive result (50.0 %, 2/4) followed by fever (41.4%, 46/111). It was found there was significant difference between clinical

manifestation and growth of organism ($p = 0.004$). The detail of which was shown in following table 5.8.

Table 5.8 Common Clinical Manifestations of the 194 Blood Culture Positive Cases of Neonatal Septicemia

Variables	Growth of Organism		Total (N=690)		P-value[b]
	n	%	n	%[a]	
Sign and Symptoms					
Fever	46	41.4	111	16.0	
Poor cry	7	36.8	19	2.7	
Grunting	21	22.3	94	13.6	
Low birth weight	6	40.0	15	2.1	
Poor feeding	5	23.8	21	3.0	
IUGR	0	0.00	15	2.1	0.004
Birth Asphyxia	28	20.4	137	19.8	
Respiratory distress	6	27.3	22	3.1	
Jaundice	2	33.3	6	0.8	
Mother CRP positive	2	14.3	14	2.0	
Baby CRP positive	8	30.8	26	3.7	
PV leacking	4	22.2	18	2.6	
TMSL	2	12.5	16	2.3	
Chorioalantosis	2	50.0	4	0.5	
Other	19	24.4	78	1.3	

a: Column percentage to indicate the distribution of overall characteristics
b: Compare the significance difference in Growth patterns of organisms

5.3 Types of organisms found in 194 cases of positive blood culture according to mode of delivery.

In relation to organism *E. coli* was present in 54.1% in normal, 55.2% in premature and 51.6% in caesarian delivery while CoNS was present maximum in vacuum (50.0%) and breech (66.7%) delivery. Detail about the types of organisms in 194 cases of positive blood culture according to mode of delivery is shown in table 5.9.

Table 5.9 Types of organisms found in 194 cases of positive blood culture according to mode of delivery

Organism	**Normal**		**Caesarian**		**Premature**		**Vacuum**		**Breech**		**Epi**		**Home**	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
E. coli	46	54.1	33	51.6	16	55.2	3	25.0	1	33.3	0	0	0	0
CoNS	20	23.5	14	21.9	9	31.0	6	50.0	2	66.7	0	0	0	0
K. pneumoniae	9	10.6	11	17.2	3	10.3	0	0	0	0	0	0	1	100
S. aureus	5	5.9	2	3.1	0	0	2	16.7	0	0	0	0	0	0
E. cloacae	4	4.7	1	1.6	0	0	1	8.3	0	0	0	0	0	0
Streptococcus	1	1.2	2	3.1	1	3.4	0	0	0	0	0	0	0	0
P. aeruginosa	0	0	1	1.6	0	0	0	0	0	0	0	0	0	0
Total	85	100	64	100	29	100	12	100	3	100	0	0	1	100

5.4 Types of organisms isolated in 194 cases of positive blood culture in relation to birth weight, gestation age, CRP level and sex of the baby.

Table 5.10 shows distribution of organisms in different birth weight categories together with gestation age, CRP, and sex. For detail about number and types of organisms, refer below table.

Table 5.10 - Organisms Isolated in 194 Cases of Blood Culture Positive Septicemia in Relation to Birth Weight, Gestational Age, CRP level and sex of the baby

Organisms	**Birth Weight(g)**			**Gestation age**			**C- Reactive protein**		**Sex of baby**		**Total**	**P**
	<1500	1500-2500	>2500	Pre-term	term	Post-term	Positive	negative	male	Female		
E. coli	13	32	54	39	54	6	20	79	48	51	99	5
CoNS	5	21	25	23	27	1	8	43	34	17	51	2
K. pneumoniae	2	6	16	7	16	1	13	11	16	8	24	1
S. aureus	1	0	8	2	7	0	3	6	7	2	9	4
E. cloacae	1	1	4	3	2	1	1	5	3	3	6	3
Streptococcus spp.	0	2	2	2	2	0	2	2	1	3	4	2
P. aeruginosa	0	0	1	0	1	0	0	1	1	0	1	0
Total	22	62	110	77	108	9	47	147	110	84	194	1
Percent of total	11.3	32.0	56.7	32.2	56.2	4.6	24.2	75.8	56.5	43.5	100	

5.4 ANTIBIOTIC SUSCEPTIBILITY PATTERN OF THE ISOLATES

Generally all of the isolates were having a low sensitivity to ampicillin, gentamycin, and tobramycin, while most of the organisms were sensitive to ofloxacin, chloramphenicol, amikacin and cloxacillin. The *E. coli* showed sensitivity of 73.7%, 60.6%, 35.5%, 22.2%, 21.2%, 39.4%, 90.9%, and 94.9% to amikacin, ciprofloxacin, gentamycin, ampicillin, tobramycin, cefotoxime, ofloxacin and choramphenicol respectively. The sensitivity of the remaining organism has been shown in table 5.11.

Table 5.11: Antibiotics susceptibility pattern of bacterial isolates in **Percent** from blood of neonates to selected antibody

Organism	**No.**	**Amik**	**Cipro**	**Genta**	**Ampi**	**Tobra**	**Cefot**	**Oflox**	**Chlor**	**Cloxa**
E. coli	99	73.7	60.6	35.5	22.2	21.2	39.4	90.9	94.9	-
K. pnumoniae	24	66.7	29.1	25.0	8.3	4.2	12.5	83.3	87.5	-
E. cloaecae	6	66.7	50.0	1.7	0.0	1.7	33.3	33.3	77.8	-
P. aeroginosa	1	100.0	0.0	0.0	0.0	0.0	100	0.0	100	-
CoNS	51	90.2	68.7	43.1	39.2	33.3	51.0	-	-	70.6
S. aureus	9	44.4	55.6	44.4	44.6	22.2	55.6	-	-	77.8
Streptococcus	4	100.0	75.0	50.0	50.0	50.0	75.0	-	-	100

5.4.1Antibiotic sensitivity pattern of some common isolates are shown in below tables

The *E. coli* isolates from blood wass most sensitive to chloramphenicol (94.9%) followed by ofloxacin (90.9%) and amikacin (73.7%) While least sensitive to tobramycin (21.2%) and ampicillin (22.2%).

Table 5.12: Antibiotic susceptibility pattern of *E. coli*

Organism	Antibiotics	Sensitive		Resistant	
		n	%	N	%
E .coli (N = 99)	Amikacin	73	73.7	26	26.3
	Ciprofloxacin	60	60.7	39	39.3
	Gentamicin	35	35.3	64	64.7
	Ampicillin	22	22.2	77	77.8
	Tobramycin	21	21.2	78	78.8
	Cefotoxime	38	39.3	61	60.7
	Ofloxacin	90	90.9	9	9.1
	Chloramphenicol	94	94.9	5	5.1

The *Klesiella pneumoniae* isolates from blood was most sensitive to chloramphenicol, (87.5%) followed by ofloxacin (83.3%) and Amikacin (66.7%), while least sensitive to tobramycin (4.2%) and ampicillin (8.3%).

Table 5.13: Antibiotic susceptibility pattern of *K. pneunoniae*

Organism	Antibiotics	Sensitive		Resistant	
		N	%	n	%
K. pneumoniae **(N = 24)**	Amikacin	16	66.6	8	33.4
	Ciprofloxacin	7	29.2	17	70.8
	Gentamicin	6	25.0	18	75.0
	Ampicillin	2	8.3	22	91.7
	Tobramycin	1	4.2	23	98.8

	Cefotoxime	3	12.5	21	87.5
	Ofloxacin	20	83.3	4	16.7
	Chloramphenicol	21	87.5	3	12.5

The CoNS isolates from blood was most sensitive to amikacin (90.9%) and cloxacillin (70.6%) followed by ciprofloxacin (68.7%) and while least sensitive to tobramycin (33.3%) and ampicillin (39.2%).

Table 5.14: Antibiotic susceptibility pattern of CoNS

Organism	**Antibiotics**	**Sensitive**		**Resistant**	
		N	**%**	**n**	**%**
CoNS (N=51)	Amikacin	46	90.2	5	9.8
	Ciprofloxacin	35	68.7	16	31.3
	Gentamicin	22	43.1	29	56.9
	Ampicillin	20	39.2	31	60.8
	Tobramycin	17	33.3	34	66.7
	Cefotoxime	26	60.0	25	49.0
	Cloxacillin	36	70.6	15	29.4

The *Staphylococcus aureus* isolates from blood was most sensitive to cloxacillin (77.8%) followed by ciprofloxacin (55.6%) and cefotoxime (55.6%), while least sensitive to tobramycin (22.2%).

Table 5.15: Antibiotic susceptibility pattern of *S. aureus*

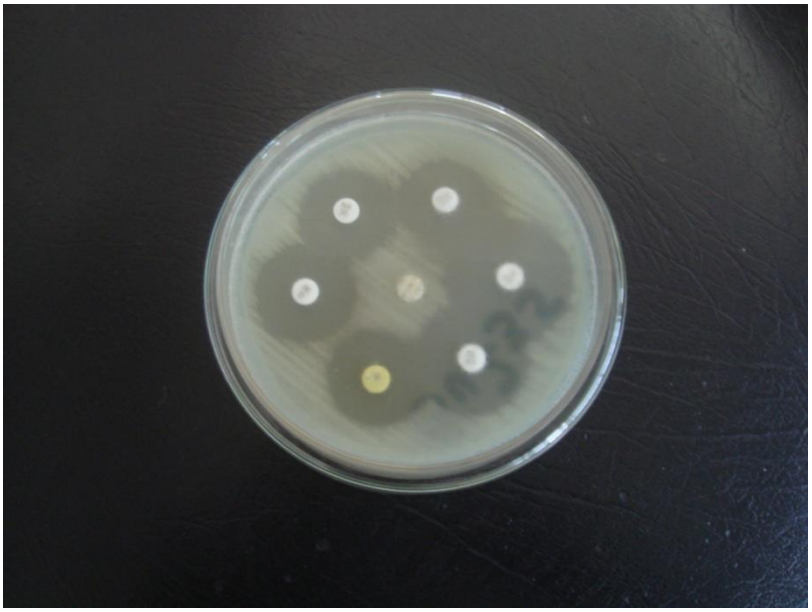
Organism	**Antibiotics**	**Sensitive**		**Resistant**	
		N	**%**	**n**	**%**
***S. aureus* (N = 9)**	Amikacin	4	44.4	5	55.6
	Ciprofloxacin	5	55.6	4	44.4
	Gentamicin	4	44.4	5	55.6
	Ampicillin	4	44.4	5	55.6

	Tobramycin	2	22.2	7	77.8
	Cefotoxime	5	55.6	4	44.4
	Cloxacillin	7	77.8	2	22.2

Photograph 1. Pure culture of *E. coli* isolated on Mac Conkey agar plate

Photograph 2. Biochemical test for *E. coli*

Photograph 3. Pure culture of *S. aureus* on Nutrient agar



Photograph 4. Antibiotic susceptibility test for *E. coli*

CHAPTER VI

6. DISCUSSION

Infants are generally more susceptible to infections than adults (Anah *et al.*, 2008). This is due to a number of factors including an inadequately developed immune system making sepsis a risk of the newborn especially under poor hygienic conditions. Despites improvement in diagnosis and management of neonatal sepsis in recent years, it is still a major cause of neonatal morbidity and mortality especially in developing countries. The incidence of neonatal septicaemia depends on geographic area and may vary from country to country as well as within the same country from time to time. The varying microbiological pattern of neonatal septicaemia warrants the need for an ongoing study of the causative organism and their antibiotic sensitivity pattern.

The present study was conducted to find the prevalence of septicaemia in the neonates admitted in NICU and PBU of PAROPAKAR MATERNNITY AND WOMEN'S HOSPITAL, THAPATHALI and to determine their antibiotic sensitivity pattern and also to assess the different factors associated with the neonatal septicaemia. All together 690 blood samples were collected from the neonates and were subjected to standard microbiological procedure.

Among 690 blood samples, 496 (71.9%) blood samples didn't showe any growth of bacteria and 194 (28.10%) samples were culture positive. Similar previous studies from home and abroad have reported the growth positivity varies from 10% to 50%. The incidence of neonatal septicaemia depends not only upon counties but also varies at different time. Similar study from Patan hospital has showed 13.7% culture positive (Shrestha S *et al.*, 2005). This might be due to the inclusion of only hospital born neonates and inclusion of neonates with risk factor. One international report shows that

neonatal sepsis varies from 7.1% to 38% per 1000 live births in Asia, from 6.5% to 23 per 1000 live births in Africa, and from 3.5% to 8.9% per 1000 live births in South America and Caribbean (Vergnano *et al.*, 2002) which is more or less similar with our study. Comparing with a study carried out at another institute within the country, B. P. Koirala Institute of Health Science at Dharan, in 1999 they had positive blood cultures in 59.7% cases of neonatal sepsis (Karki *et al.*, 1999). This rate is higher than our study; this may be due to most of the neonates in our study were hospital born. Other studies from home also supported this result such as Jain *et al*. (2003) reported that among 106 neonates suspected of sepsis were studied, out of which 30 (28.3%) were culture positive (Jain *et al*., 2003)

In our study, Gram negative organisms have found to be responsible for about two third (67.0%) of total septicaemic case. This high predominance of gram negative organism is consistence with most of the data from different parts of world. Among gram negative organisms, *E coli* were the most common (51.0%) followed by *Klebsiella pneumoniae* (12.4%). Nearly one third of the total isolates were gram positive. Among gram positive organisms, Coagulase negative Staphylococcus were the most common organism counting 26.3 % of total bacterial isolates, *Staphylococcus aureus* were the second most common gram positive organism.

Comparing the neonatal blood culture results in different studies, the common isolates may vary from place to place and institution to institution. In a study carried out in GTB hospital, India the most common isolate was *Klebsiella* (33.8%) while other two Indian studies, in Kasturba Gandhi Hospital and Coimbatore had *Staphylococcus aureus* as the most common isolates accounting for 61.4% and 50.6% respectively (Karthikeyan *et al.,* 2001). In study done at Peshawar, Pakistan (Rahaman *et al*., 2004) and University of Utah, United States (Glasgow TS) the most common isolates were *E. coli* accounting for 36.6% and 59% respectively. Another study at Karachi, Pakistan (Mahamood *et al.,* 2002), *Klebsiella* were the most common isolates contributing to 34.4% of the total positive blood culture.

Altogether, there were 7 different bacterial species were isolated, in which 4 species were gram negative and 3 species were gram positive. Among the total isolates, *E. coli* (51.0%) was the most prevalent followed by CoNS (26.3%), *K. pneumoniae* (12.4%), S. *aureus* (4.6%), *E. clocaea* (3.1%), *Streptococcus* spp. (2.1%) and *Ps. aeruginosa* (0.50%).

The high evidence of gram negative organism is consistent with most of the data from country and abroad (Waseem *et al*., 2005 Mathur *et al.*, 1994 Kaufman and Fairchild, 2004 Ray et al., 2002 Ahmed *et al.* 2002). One of the study of from Pakistan shows, gram negative organisms were isolated from more than 80 % of the cases. *E. coli* was the commonest isolates (34%) followed by *Klebsiella* (30%), (waseem *et al.*, 2005). This result is more or less similar with this study. The finding of high percentage of *E.coli* is consistent with results by Ahmed *et al.* (2002) and Millar *et al.* (2002). CoNS has been isolated in 26.3% (51) cases of total. Initially thought to be a contaminant this organism has now been recongnised as a considerable cause of neonatal sepsis. In many studies CoNS is one of the leading cause of NICU- acquired infection. (Villari *et al.*, 2000 Shrestha *et al.,* 2006 Gheibi *et al.,* 2008). CoNS is common commensals organisms but premature neonates are particularly susceptible to invasive infection. (Kaufman and Fairchild, 2002). Pro-inflammatory response to CoNS are dependent on gestation age in preterm infants, thus the predominance of neonatal sepsis in pre term newborns in this study may be one reason to this finding. (Gheibi *et al*., 2008).

Pseudomonas aeruginosa is not common isolates from neonatal blood sample. In this study one *P. aeruginosa* was isolated which may come from ventilators or bed used in NICU rather some studies (Movahedian et al., 2006 and Koutouby et al., 1995) showed that it is a mojor pathogens of neonatal septicaemia with very high mortality rate.

Out of 690 blood sample investigated for microbial growth, 400 (57.9%) were from the male neonates and 290 (42.1%) were from the female neonates. The growth of the organism is higher in male (56.5%) than in female (43.5%). But this study did not

found significant association with sex of neonates with growth of organism. Some studies found that male infants have an approximately higher incidence of sepsis than female infants but this study did not find any evident about this.

C-reactive protein (CRP) is a prototype acute-phase protein whose serum level increases 1000 fold during an acute-phase response. The CRP test is a sensitive indicator for inflammatory processes such as septicaemia. In our study out of 690 blood samples, only 150 (21.7%) blood sample shows CRP positive. Among 194 culture positive cases, 47 (24.2%) positive cases had CRP positive. The association of CPR with growth of organism is not significant statistically ($p > 0.05$). This means CRP test alone was not sufficiently reliable to use as predictor of neonatal septicaemia. In the similar study conducted by H. Borna and S. Borna shows similar result (Borna and Borna, 2005).

In our study weight group from 2001 gram to 3000 gram had maximum request for blood culture (45% of total) with mean (± SD) weight of 2588.43 ± 753.803 gm showed growth while mean weight of 26.74.95 ± 695.38 gm showed no growth. There was significant difference of weight of baby between the growth of organism ($p<0.05$). So the baby having the growth of organism has lower weight than baby having without growth of organism. Further when weight of baby is categorized according to low body weight, the study did not find any significant difference between weight categories and growth of organisms ($p>0.05$). This may be due to other factors which were not considered during analysis such as prematurity and weight of baby. Another parameter is gestation age of neonates. In our study neonates with gestation age between 251-300 days had maximum request for blood culture (73.20% of total) with mean (± SD) days of 260.37 ± 27.844 showed growth of organism in culture while mean days of 268.34 ± 24.670 showed no growth in culture. There are also significant difference of gestation age between the growth of organism ($P<0.05$). So the babies having the growth of organism have lower gestation age than babies having without growth of organism. Further when gestation age of babies categorized into preterm (<37 weeks), term (37-42

weeks), and post-term (>42 weeks), preterm babies showed 34.8% culture positive result while term babies showed 25.20% culture positive result which was also signified statistically (P<0.005). According to Nelson textbook of pediatrics (Behrman et al., 1992) the most important factor predisposing to infection in neonates is prematurity or low-birth weight. Preterm infants have a 3 to 10 fold higher incidence of infection than full term normal birth weight infants which was also verified by different studies carried out worldwide. One of the studies carried out in University of Virginia Health System, Virginia shows approximately 20% of low-birth weight preterm infants experience a serious systemic infection during their initial hospital stay. On studding various literature it is well known that the incidence of neonatal sepsis is inversely proportional to birth weight and gestational age. This evident was also shown by our study.

Regarding mode of delivery, 318 babies were born by normal delivery which is highest in our study (46.1% of total). 43.8% of babies born normally showed the growth of the organism while 33.8% of babies born by cesarean mode showed the growth of organism. There is not any association between the mode of delivery and growth of the organisms which is also verified statistically (p>0.05). Similar results were obtained in other types of research. One of the studies carried out in BP Koirala Institute of Health Sciences, Dharan, Nepal in Department of Paediatrics and Adolescent Medicine (Shah GS et al., 2006) shows mode of delivery has not any effect on sepsis of neonates which is also verified by our studies.

On studding the common clinical manifestation of the blood culture positive cases of neonatal septicaemia we found babies with birth asphyxia, fever, low-birth weight, premature rupture of membrane (PROM) had higher rate of culture positive with 20.40%, 41.40%, 40.00%, and 43.62% of total culture positive result respectively. This study also found significant difference between clinical manifestation and blood culture positive cases of neonatal septicaemia (P <0.05). The results were also verified by similar studied carried out in home and abroad. One home (Shah GS et al., 2006) study

shows prolonged leaking and premature rupture of membranes is considered as a major risk factor for sepsis because of the danger of ascending infection. The same study also shows the significant difference between low birth weights and growth of organism. Premature and low birth weight babies are relatively immune deficient, which predispose them to infections. Similar observations were also reported by other workers (Wong et al., 2000 and Oddie et al., 2002)). Other risk factors reported by our study were grunting, poor cry, poor feeding, CRP positive mother, chorioalntotosis and respiratory distress. One study from Kerala, India (Jayan et al., 2003) in Department of community medicine, government medical college shows odds ratio for asphyxia is 27.136; odd ratio for PROM is 14.793; odd ratio for low-birth weight is 6.047; odd ratio for Prematurity is 4.520. These results showed similarity with this study.

Antimicrobial resistance is a global problem. It is now generally accepted as major public health issue and has significant implication on health and patient care. Resistance to antimicrobial drugs is associated with high morbidity and mortality, high health-care cost and prolonged hospitalization. The problem antimicrobial resistance is more troublesome to developing countries. WHO and the European Commission (EC) have recognized the importance of studying the emergence and determinants of resistance and the need for strategies for its control.

In this study, amikacin (75.2%) of the cephalosporin antibiotics was found most efficient, followed by ciprofloxacin (59.3%) and cefotoxime (39.2%). Gentamycin (36.0%), ampicillin (25.7%) and tobramycin (22.1%) were found to be least sensitive. In the similar study performed by Awoniyi et al (2009) at Cape Peninsula University of Technology, South Africa almost all bacterial isolates were sensitive to ofloxacin while most were found to be resistant to commonly used antibiotics such as ampicillin. Recent data from developed countries also indicates increasing resistance to ampicillin. One of the studies (Waseem et al., 2005) also shows very low sensitivity to ampicillin, gentamycin and cefotoxime.

Antimicrobial sensitivity patterns differ in studies and at different times. This is due to emergence of resistant strains as a result of indiscriminate use of antibiotic (Baser and Gharebaghi., 2001; Dawodu et al., 2002). The high resistance rates in our study may be associated with frequent use of antibiotics for both prophylaxis and treatments of neonates in hospital.

In gram negative isolates chloramphenicol (93.1%) and ofloxacin (88.5%) were found more sensitive followed by amikacin (71.5%) and ciprofloxacin (54.6%). Ampicillin (18.5%) and tobramycin (16.9%) were the least sensitive.

In gram positive isolates amikacin (82.8%) and cloxacillin (70.3%) were found more sensitive followed by ciprofloxacin (68.7%) and cefotoxime (54.7%). Here also ampicillin (40.6%) and tobramycin (32.2%) were the least sensitive. In general susceptibility pattern of gram negative bacteria shows they are more resistant than gram positive organisms.

In the blood isolate *E. coli*, tobramycin (21.2%) was found the least susceptible followed by ampicillin (22.2%). In other antibiotic chloramphenicol (94.9%) was found most efficient followed by ofloxacin (90.9%) and amikacin (73.7%). ciprofloxacin (60.6%) showed moderate sensitivity. The results found in this study is strongly supported by different other researches.

K. pneumoniae was found most susceptible to chloramphenicol (87.5%) and least susceptible to tobramycin (4.2%) followed by ampicillin (8.3%) and cefotoxime (12.5%). Ofloxacin (83.3%) and amikacin (66.6%) were also showed higher susceptibility against *K. pneumonia.* In the similar study done by E malakan Rad (2004) about 23% of *Klebsiella* isolates were found to be resistance to amikacin and 90% of *klebsiella* were found to be resistant to ampicillin. These results are quite similar to our studies (Rad *et al*., 2004).

In CoNS, amikacin was most sensitive antibiotics with susceptibility of 90.2% followed by cloxacillin (70.6%) and ciprofloxacin (68.7%). Again tobramycin is least sensitive with susceptibility of 33.3% followed by ampicillin (39.2%). Cefotoxime is found to be moderately susceptible (60.0%). In the similar study done by Gheibi et al. (2008) in Urmia, Iran, CoNS showed high degree of resistance to commonly used antibiotics ampicillin (100%), cefotoxime (67%) and comparatively low resistance to ciprofloxacin (23%) (Gheibi et al., 2008). These results are more or less similar to our study.

In this study *Staphylococuus aureus* showed comparatively more resistance towards commonly used antibiotics among gram positive organisms. In *S. aureus* there was unusual high resistance towards amikacin (55.6%). cloxacillin showed maximum susceptibility (77.8%) towards *S. aureus*. Again tobramycin showed least susceptible to *S. aureus* with 22.23% sensitive. Cefotoxime and ciprofloxacin showed moderately sensitive with 55.6% susceptibility each. This result is similar with study carried out by Waseem *et al*. (2005) in Lahore Medical College, Lahore, Pakistan.

Antibiotic resistance is now a global problem. Reports of multi drug resistant bacteria causing neonatal sepsis in developing countries are increasing, particularly in intensive care. Spread of resistant organisms in hospitals is a recognized problem, although babies admitted from the community may also carry resistant pathogens. The wide availability of over the counter antibiotics and the inappropriate use of broad spectrum antibiotics in the community may explain this. More studies are needed to compare patterns of resistance in babies born in and out of hospital. It is difficult to compare antibiotic resistance between countries because the epidemiology of neonatal sepsis is extremely variable. Few studies compare antibiotic susceptibility over time in the same unit, but where data are available they show increasing resistance to commonly used antibiotics (Vergnano *et al.*, 2002).

Due to the small sample size and hospital based design of this study, findings cannot be generalized to other places. Limitation of laboratory facilities at hospital, strict anaerobes cannot be grown on culture media. There is need of community based case control studies with larger sample size to identify risk factors and preventive measures for neonatal septicaemia.

6.1 Conclusion

From this study, the prevalence of Neonatal Septicaemia Maternity hospital was found 28.1%. The prevalence of septicaemia was comparatively found higher in male than in female neonates. Form the blood sample of 690, a total of 194 pathogens belonging to seven different species were isolated. Gram negative pathogens were found predominant. Among Gram negative, *E. coli* was the major isolates. In gram positive CoNS and *S. aureus* were found maximum.

Low-birth weight babies and preterm babies showed higher frequency of sepsis which was also significant statistically. But this Study did not find any significant difference between CRP level and growth of organism.

In Gram negative, ampicillin and tobramycin were least susceptible whereas chloramphenicol and ofloxacin were found most potent antimicrobial agents. In Gram positive, amikacin was found most susceptible followed by cloxacillin.

CHAPTER VII

7. SUMMARY AND RECOMNENDATIONS

7. 1 SUMMARY

1. The study was conducted in the microbiological laboratory of Paropakar Maternity and Women's Hospital Nepal from Asadh 1st to Mansir 30th 2066, in the Neonates suspected of septicaemia. A total of 690 samples were collected, in which 194 were found to culture positive.
2. The prevalence of neonatal septicaemia was found to be 28.10%.
3. Among the isolates 7 different bacteria were isolated. In which nearly 67% (130/194) belongs to Gram negative and 33.0% (64/194) were from Gram positive. *E. coli* (51.0%) was found most predominant in Gram negative isolates followed by followed by *K. pneumonia* (12.4 %%), *E. cloacae* (3.1%), *Ps. aeroginosa* (0.5%). In Gram positive, CoNS (26.3%), *S. aureus* (4.6%) and were the major isolates followed by *Streptococcus* spp. (2.1%).
4. Among different factors weight and gestation age of babies were associated with growth of organism in culture ($P<0.05$).
5. Sex of the neonates, CRP level of neonates and mode of their delivery had not any association with growth of organism ($p>0.05$)
6. The most efficient antibiotics for gram negative was found chloramphenicol (93.1%) followed by ofloxacin (88.0%), amikacin (71.5%), ciprofloxacin (54.6%). The isolated gram negative organism showed least sensitive towards ampicillin (18.5%) and tobramycin (16.9%). *K. pneumoinae* showed comparatively higher resistance than *E. coli*.
7. The most efficient antibiotics for gram positive were found amikacin (82.8%), cloxacillin (70.3%) and ciprofloxacin (68.7%). The isolated gram positive organism showed least sensitive towards ampicillin (40.6%) and tobramycin (32.2%). *S. aureus* show comparatively higher resistance than CoNS

7. 2 RECOMMENDATION

1. The highly risk groups such as premature babies, low-birth weight babies and mother or babies showing signs and symptoms should be regularly screened.
2. High risk pregnancy should be carefully screened.
3. Nearly more than two third of the blood isolates were found to be resistant toward commonly used antibiotics, so regular surveillance and monitoring of such drug resistant bacteria should be made by the authoritative bodies.
4. There is need for community based studies to identify risk factors and preventive measures for neonatal septicaemia.

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APPENDIX I

QUESTIONNAIRE

1. **Patient's ID:**
2. **Date:**
3. **Patient's Name: B/O …….**
4. **Age:** **…….**Days
5. **Sex:** Male ☐ Female ☐
6. **Gestational age:** Preterm ☐ term ☐
7. **Weight: …….. gm**
8. **Residence:**

   Address**: ……. (**Rural ☐ Urban ☐ **)**

9. Feeding:

Breast ☐ Spoon ☐ Mixed ☐

10. C-Reactive protein test: Positive ☐ Negative ☐

11. Symptoms and signs:

Symptoms	**Yes**	**No**	**Signs**	**Yes**	**No**
Refusal to feed			Tachypnoea		
Lethargy			Grunting		
Poor cry			Jaundice		
Fever			Cyanosis		
Diarrhea			Vomiting		
Excessive cry			Seizures		
Others			Poor capillary refill		
			Hypothermia		
Abdominal distension					
Conjunctivitis					
Others					

APPENDIX II

CLINICAL AND MICROBIOLOGICAL PROFILE OF PATIENT

Microbiological Profile

DAY 1

Time of sample collection: …….

Specimen: …….

Method of sample collection: ……..

Macroscopic Observation (if necessary): ……

Enrichment of blood sample on: a. BHI broth

Incubation: a. Aerobic b.Anaerobic c. Microaerophillic

Incubation temperature and period: …….

DAY 2

Macroscopic Observation of BHI broth: ……

Gram-staining test: ……..

Subculture of specimen on: a. MacConkey Agar b. Nutrient Agar

c. Blood agar d. Chocolate agar

Incubation: a. Aerobic b.Anaerobic c. Microaerophillic

Incubation temperature and period: …….

DAY 3 on ward

Reading of Culture Plates

Colony Characteristics on MacConkey Agar/Blood Agar/Nutrient agar/Chocolate agar

Media used	Shape	Size	Color	Texture	Lactose fermentation	Growth

Gram-staining test: ……..

Catalase test: …… Oxidase test: …….

Coagulase test: …….. Others: …….

Provisional Identification of Organism: …….

Biochemical Tests:

Results:

a. TSI: ……. b. SIM: …….. c. Citrate: ……..

d. Urea Hydrolysis: …….. e. MR: …… f. VP: ……..

Organism Identified as: ……

Antibiotic Sensitivity Test (Kirby- Bauer Method)

Antibiotics used	Zone of inhibition (mm)	Interpretation

Performed by Checked by

APPENDIX III

LIST OF EQUIPMENTS AND MATERIALS USED DURING STUDY

Media and chemicals

Media

Nutrient Agar	Hi media, India
MacConkey Agar	Hi media, India
Mueller Hinton agar	Hi media, India
Triple Iron Sugar Agar	Hi media, India
SIM (Sulphide Indole Motility) media	Merck, Germany
MR-VP Broth	Hi media, India
Citrate Agar	Hi media, India
Urea agar base	Merck, Germany
Urea crystal	Merck, Germany
O/F Agar	Hi media, India
Mannitol Salt Agar	Hi media, India

Chemical

H_2O_2	Barritt's Reagent A
Oxidase	Barritt's Reagent
Kovac's Reagent	Alpha –napthalamine
Methyl Red	Crystal Violet

Dehydrated alcohol	Phenol red
Hydrocloric acid	Safranin
Iodine	

Equipments

Autoclave	Life, India
Electronic weighing pan	OHAUS, USA
Glasswares	Pyrex and Borax
Hot water bath	Universal, India
Incubator	Universal, India
Microscope	Olympus
Hot Air Oven	Life, India
pH meter	Universal, India
Refrigerator	
Shaker	
Centrifuge	

Test organisms

Escherichia coli ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumonia* ATCC 700603, *Enterococcus faecalis* ATCC 29212

ANTIBIOTIC DISCS

All the antibiotics discs used for the susceptibility tests were from Hi-Media Laboratories Pvt. Limited, Bombay, India. The antibiotics used were as follows

Ampicillin (10 mcg)
Gentamicin (10 mcg)
Ciprofloxacin (5 mcg)
Ofloxacin (5 mcg)
Tobramycin (10 mcg)
Amikacin (30 mcg)
Vancomycin (30 mcg)
Ampicillin (10 mcg)
Cloxacillin (5 mcg)

E. MISCELLANEOUS

Conical flasks, Cotton, Distilled water, Droppers, Forceps, Glass slides and cover slips, Immersion oil, Inoculating loop, Inoculating wire, Lysol, Measuring cylinder, Petri dishes, Pipettes, Plastic containers, Spatula, Test tubes, Wooden applicator sticks

APPENDIX IV

A. COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

Brain heart infusion broth

Ingredients	**gm/liter**
Calf Brain infusion	200.0
Beef Brain infusion	250.0
Protease peptone	10.0
Dextrose	2.0
Sodium chloride	5.0
Disodium Phosphate	2.5
Final pH (at 25^{O}C)	7.4±0.2

Direction: 37 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^{O}C (15 lbs pressure) for 15 minutes. The medium was poured into sterile culture bottle.

MacConkey Agar

Ingredients	**gm/liter**
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral Red	0.075
Agar	12.0
Final pH (at 250C)	7.4±0.2

Direction: 52 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 1210C (15 lbs pressure) for 15 minutes. The medium was poured into sterile petriplates.

Nutrient Agar

Ingredients	**gm/liter**
Peptone	5
Sodium Chloride	5
Beef extract	1.5
Yeast extract	1.5
Agar	15
Final pH	7.2

Direction: 37 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

Mueller Hinton Agar

Ingredients	**gm/liter**
Beef, Infusion form	300.0
Casein Hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 250C)	7.4±0.2

Direction: 38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve completely. It was sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes and poured into sterile petriplates.

Mueller Hinton Broth

Ingredients	**gm/liter**
Beef, Infusion form	300.0
Casein Hydrolysate	17.5
Starch	1.5
Final pH (at 250C)	7.4±0.2

Direction: 21 grams of the medium was dissolved in 1000 ml of distilled water, boiled and dispensed into small containers. It was then sterilized by autoclaving at 121˚C for 15 minutes.

B. COMPOSITION AND PREPARATION OF DIFFERENT BIOCHEMICAL TESTS MEDIA

MR-VP Medium

Ingredients	**gm/litre**
Peptone	5.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 250C)	6.9±0.2

Direction: 15 gm powder was dissolved in 1000 ml of distilled water & mixed well. 3 ml of medium was distributed in each test tube and autoclaved at 1210C for 15 minutes.

Simmon's Citrate Agar

Ingredients	**gm/litre**
Ammonium dihydrogen phosphate	1
Dipotassium hydrogen phosphate	1
Sodium chloride	5
Sodium citrate	2

Magnesium sulphate	0.2
Bromothymol blue	0.08
Agar	15
Final pH	6.9

Direction: 23 grams of the medium was dissolved in 1000ml of distilled water. 3ml medium was distributed in test tubes and sterilized by autoclaving at 1210C for 15 minutes. After autoclaving, tubes containing medium were tilted to form slant.

SIM(Sulphide Indole Motility) Agar

Ingredients	**gm/litre**
Peptone	30
Beef extract	3
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.025
Agar	3
Final pH	7.3

Direction: 30 grams of the medium was suspended in 1000 ml of distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized by autoclaving at 121ºC for 15 minutes.

Triple Sugar Iron Agar (TSI)

Ingredients	**gm/litre**
Lab-lemco powder	3.0
Yeast Extract	3.0
Peptone	20.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0

Ferric citrate	0.3
Sodium chloride	5.0
Sodium thiosulphate	0.3
Phenol red	0.025
Agar	12.0
Final pH (at 250C)	7.4±0.2

Direction: 65 grams of the medium was dissolved in 1000ml of distilled water and sterilized by autoclaving at 15 lbs (1210C) pressure for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch of length.

Urea Broth Base

Ingredients	**gm/litre**
Monopotassium phosphate	9.1
Dipotassium phosphate	9.5
Yeast extract	0.1
Phenol red	0.01
Sterile 40% urea solution	5ml

Direction: As directed by manufacturing company, 1.87 grams of the medium was suspended in 95 ml of distilled water and sterilized by autoclaving at 1210C for 15 minutes. After cooling to about 550C, 5 ml of sterile urea solution was added aseptically, mixed well and distributed 5 ml amount in sterile test tubes.

C. COMPOSITION AND PREPARATION OF DIFFERENT STAINING AND TEST REAGENTS

For Gram's Stain

(a) Crystal Violet solution

Ingredients	**gm/litre**
Crystal Violet	20.0 g

Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml
Distilled Water (D/W) to make 1 litre	

Direction: In a clean piece of paper, 20 gm of crystal violet was weighed and transferred to a clean brown bottle. Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Final volume was made 1 litre by adding D/W.

(b) Lugol's Iodine

Ingredients	**gm/litre**
Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

Direction: To 250 ml of D/W, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Final volume was made 1 litre by adding D/W.

(c) Acetone-Alcohol Decoloriser

Ingredients	**gm/litre**
Acetone	500 ml
Ethanol (Absolute)	475 ml
Distilled Water	25 ml

Direction: To 25 ml D/W, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

(d) Safranin (Counter Stain)

Ingredients	**gm/litre**
Safranin	10.0 g

Distilled Water	1000 ml

Direction: In a clean piece of paper, 10 gm of safranin was weighed and transferred to a clean bottle. Then 1 litre D/W was added to the bottle and mixed well until safranin dissolved completely.

Biochemical Test Reagents

(a) Catalase Reagent (For Catalase test)

Ingredients	**gm/litre**
Hydrogen peroxide	3 ml
Distilled Water	97 ml

Direction: To 97 ml of D/W, 3 ml of hydrogen peroxide was added and mixed well.

(b) Oxidase Reagent (impregnated in a Whatman's No. 1 filter paper)

(For Oxidase Test)

Ingredients	**gm/litre**
Tetramethyl *p*-phenylene diamine dihydrochloride (TPD)	1 gm
Distilled Water	100 ml

Direction: This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. To that solution strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

(c) Kovac's Indole Reagent (For Indole Test)

Ingredients	**gm/litre**
Isoamyl alcohol	30 ml
p-dimethyl aminobenzaldehyde	2.0 g
Conc. Hydrochloric acid	10 ml

Direction: In 30 ml of isoamylalcohol, 2 g of *p*-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of conc. HCl was added and mixed well.

(d) Methyl Red Solution (For Methyl Red Test)

Ingredients	**gm/litre**
Methyl red	0.05 g
Ethyl alcohol (absolute)	28 ml
Distilled Water	22 ml

Direction: To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml D/W was added to that bottle and mixed well.

(e) Barritt's Reagent (For Voges-Proskauer Test)

Solution A

Ingredients	**gm/litre**
α-naphthol	5.0 g
Ethyl alcohol (absolute)	100 ml

Direction: To 25 ml ethanol, 5 g of α-naphthol was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

Solution B

Ingredients	**gm/litre**
Potassium hydroxide	40.0 g
Distilled Water	1000 ml

Direction: To 25 ml D/W, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

McFarland tube (No. 0.5)

0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.H_2O$) was added to 99.5 ml of 0.18 M H_2SO_4 (1% w/v) with constant stirring. The McFarland standard was thoroughly mixed to ensure that it is evenly suspended. Using matched cuvettes with a 1 cm light path and water as a blank standard, the absorbance was measured in a spectrophotometer at a wavelength of 625 nm. The acceptable range for the turbidity standard is 0.08-0.13. The standard was distributed into screw-cap tubes of the same size and volume as those used to prepare the test inoculum. The tubes were sealed tightly to prevent loss by evaporation and stored protected from light at room temperature. The turbidity standard was then vigorously agitated on a vortex mixer before use. Standards may be stored for up to 6 months, after which time they should be discarded.

APPENDIX V

GRAM-STAINING PROCEDURE

First devised by Hans Christian Gram during the late 19th century, the Gram-stain can be used effectively to divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal dye to was out easily with the decolorizer alcohol or acetone (Gram-negative). The following steps are involved in Gram-stain:

1. A thin film of the material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 1 minute.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
8. The slide was flooded with counter-stain (safranin) for 1 minute and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

APPENDIX VI

METHODOLOGY OF BIOCHEMICAL TESTS USED FOR IDENTIFICATION OF BACTERIA

A. Catalase test

This test is performed to demonstrate the presence of catalase, an enzyme that catalyzes the release of oxygen from hydrogen peroxide. During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* spp.

Procedure: A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g. Blood Agar) or if an iron wire loop is used.

B. Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test. The test is used for screening species of *Neisseria, Alkaligenes, Aeromonas, Vibrio, Campylobacter* and *Pseudomonas* which give positive reactions and for excluding the Enterobacteriaceae, all species of which give negative reactions.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (Whatman's No. 1 filter paper impregnated with 1% tetramethyl-p-phenylene diamine

dihydrochloride). Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

C. Indole Production test

This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatole (methyl indole) and indole acetic acid. The enzyme tryptophanase catalyses the deamination reaction attacking the tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole.

Procedure: A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37°C for 24 hours. After 24 hours incubation, 2-3 drops of Kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

D. Methyl Red test

This test is performed to test the ability of an organism to produce and maintain stable acid end product from the fermentation of glucose to give a red color with the indicator methyl red and to overcome the buffering capacity of the system. Medium used in the study was Clark and Lubs medium (MR/VP broth, pH 6.9). Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions over a pH range of 4.4- 6.0.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red color, indicating acidity and negative with yellow color.

E. Voges-Proskauer (VP) test

The principle of this test is to determine the ability of some organisms to produce a acetyl methyl carbinol, a neutral end product (acetoin) or its reduction product 2, 3-butanidiol during fermentation of carbohydrates. An organism of the Enterobacteriaceae group is usually either methyl red positive and Voges- proskauer-negative or methyl red negative and Voges-Proskauer positive. The Voges proskauer test for acetoin is used primarily to separate *E. coli* from *Klebsiella* and *Enterobacter* species

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of Barritt's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red color.

F. Citrate Utilization test

This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. The medium used for citrate fermentation (Simmon’s Citrate medium) also contains inorganic ammonium salts. Organisms capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

Procedure: A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

G. Motility test

This test is done to determine if an organism was motile or non-motile. Bacteria are motile by means of flagella. Flagella occur primarily among the bacilli; however a few cocci forms are motile .Motile bacteria may contain a single flagella. The motility media used for motility test are semisolid, making motility interpretations macroscopic.

Procedure: Motility of organism was tested by hanging drop and cultural method. In cultural method, the test organism was stabbed in the SIM medium and incubated at 37ºC for 48 hours. Motile organisms migrate from the stabline and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stabline, and the surrounding media remains colorless and clear.

H. Triple Sugar Iron (TSI) Agar Test

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation of these carbohydrates and it gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours .Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

a. Yellow (Acid)/ Yellow (Acid), Gas, H_2S → Lactose/ Sucrose fermenter, H_2S producer.
b. Red (Alkaline) / Yellow (Acid), No Gas, No H_2S → Only Glucose, not lactose/
c. Sucrose fermenter, not aerogenic, No H_2S production.

d. Red (Alkaline) / No Change → Glucose, Lactose and Sucrose non-fermenter.
e. Yellow (Acid)/ No Change → Glucose- Oxidiser.
f. No Change / No Change →Non-fermenter.

I. Urea Hydrolysis test:

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator (phenol red) incorporated in the medium.

Procedure: The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37°C overnight. Positive organism shows pink red color due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in color of the indicator to pink.

J. Coagulase test

This test is used specifically to differentiate species within the genus *Staphylococcus*: *S. aureus* (usually positive) from *S. saprophyticus, S epidermidis* (negative). A positive coagulase test is usually the final diagnostic criterion for the identification of *S. aureus.* Free coagulase and bound coagulase are the two types of coagulase possessed by this organism; most strains possess both free and bound coagulase.

Slide Coagulase Test

Bound coagulase (Clumping Factor) is detected by slide test. The bound coagulase is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in alteration of fibrinogen so that it precipitates on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma.

Procedure: For slide coagulase test, a drop of physiological saline was placed on three places of a slide, and then a colony of the test organism was emulsified in two of the drops to make thick suspensions. Later a drop of plasma was added to one of the suspensions and mixed gently. Then a clumping was observed within 10 seconds for the positive coagulase test. No plasma was added in second suspension. This was used for the differentiation of any granular appearance of the organism from true coagulase clumping. The third drop of saline was used for a known strain of coagulase positive staphylococci.

Tube Coagulase Test

This test is carried out to detect production of free coagulase. Plasma contains coagulase reacting factor (CRF) which activates free coagulase. The activated coagulase acts upon prothrombin thus converting it to thrombin. Thrombin converts fibrinogen into fibrin which is detected as a firm gel (clot) in the tube test. Tube test is performed when negative or doubtful results are obtained in slide coagulase test.

Procedure: In the tube coagulase test, plasma was diluted 1:10 in physiological saline. Four small tubes were taken, one for test organism, one for positive control, one for negative control, and one to observe self clotting of plasma. Then 0.5 ml of the diluted plasma was pipetted into each tube and 0.5 ml of test organism, 0.5 ml of positive control (*S. aureus* culture), and 0.5 ml negative control (*S. epidermidis* culture) was added to three tubes, to the fourth tube, 0.5 ml sterile broth was added. After mixing gently, all tubes were incubated at 37^0C on a water bath for 6 hours and observed for gel formation in every 30 minutes. The clotting is observed by gently tilting the tube for positive coagulase test.

APPENDIX VII

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF BACTERIA ISOLATED FROM URINE SAMPLE

Bacteria	Morphological Characteristics	Cultural Characteristics
Escherichia coli	Gram negative rod of 1-3µm×0.4-0.7µm size, aerobic and anaerobic, nonsporing, motile, noncapsulated	On BA: Large 1-4 mm in diameter, grayish white, moist, smooth, convex and opaque. The colonies may appear mucoid and some strains are haemolytic. On MA: Bright pink colonies due to lactose fermentation, smooth, glossy and translucent.
Klebsiella spp.	Gram negative, short and thick rods of 1-2µm × 0.8µm size, nonsporing, nonmotile and capsulated.	Large dome shaped moist and usually viscid or mucoid colonies when cultured on BA and MA. Most Klebsiella species are lactose fermenting.
Proteus spp.	Gram negative rods of 1-3 µm × 0.4-0.6µm size, non capsulated, nonsporing motile rods.	On BA: when cultured aerobically, most strains are swarming type and have a characteristic fishy odour. On MA: *Proteus* species produce individual non-lactose fermenting colonies after overnight incubation at 35ºC to 37ºC. Swarming is prevented on MA because this media contains bile salts.

Enterobacter spp.	Gram negative rods, non sporing, noncapsulated.	About 2 to 3 mm in diameter, moist, yellowish coloured, LF, motile organism.
Citrobacter freundii	Gram negative, non sporing, noncapsulated, motile rods.	Smooth, convex 2-4 mm colonies, sometimes rougher mucoid forms occur On BA: Non-haemolytic On MA: Non-lactose fermenting colonies.
Acinetobacter spp.	Gram negative, short, stout, non- motile rods that become almost coccoid, frequently capsulated, strict aerobes.	They grow well on ordinary media and form white of cream, glistening smooth and often rather viscid colonies about 1mm in diameter.
Alcaligenes spp.	Gram negative rod actively motile by peritrichate flagella, non-capsulated, strict aerobes.	Colonies on NA are grayish white. On BA: Non-haemolytic
Staphylococcus aureus	Gram positive, spherical cocci, 0.8-1 µm in diameter, non sporing, facultative anaerobe, non-motile, except for rare strains, non capsulated. They	On BA: Large, 2-4 mm diameter. Circular, smooth with glistening surface, entire edge, soft butyrous consistency and opaque. The pigmentation is golden yellow to cream coloured.Some strains are beta-haemolytic when grown

	are arranged in characteristics grape like clusters or in small groups, pairs, singles and short chain(less than five cocci in line).	aerobically. On MA: Small (pin head size), 0.1-0.5mm, pink or pink orange due to lactose fermentation. Some strains are non-lactose fermenting.
Coagulase Negative *Staphylococcus* species	Morphology of CoNS is similar to *Staphylococcus aureus*.	Colonies on media are similar to that of *Staphylococcus aureus* although often smaller and are grey or white in color, though some may be pigmented usually cream to yellow.

APPENDIX VIII

DISTINGUISHING REACTIONS OF THE COMMONER AND PATHOGENIC ENTEROBACTERIACEAE

Table: Distinguishing reactions of the commoner and pathogenic Enterobacteriaceae

Species	Test/ substrate											
	lac	mot	gas	ind	VP	cit	PDA	ure	lys	H_2S	inos	ONPG
E. coli	+	+	+	+	-	-	-	-	+	-	-	+
Shigella groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-
Sh. sonnei	-	-	-	-	-	-	-	-	-	-	-	+
Salmonella (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-
S. typhi	-	+	-	-	-	-	-	-	+	+	-	-
S. paratyphi A	-	+	+	-	-	-	-	-	-	-	-	-
C. freundii	±	+	+	-	-	+	-	±	-	±	-	+
C. koseri	±	+	+	+	-	+	-	±	-	-	-	+
K. pneumoniae	+	-	++	-	+	+	-	+	+	-	+	+
K. oxytoca	+	-	++	+	+	+	-	+	+	-	+	+
E. aerogenes	+	+	++	-	+	+	-	-	+	-	+	+
E. cloacae	+	+	+	-	+	+	-	±	-	-	-	+
Hafnia alvei	-	+	+	-	+	-	-	-	+	-	-	+
Serratia marcescensb	-	+	±	-	+	+	-	-	+	-	±	+
P.mirabilis	-	+	+	-	±	±	+	++	-	+	-	-
P. vulgaris	-	+	+	+	-	-	+	++	-	+	-	-
M. morganii	-	+	+	+	-	-	+	++	-	±	-	-
Providencia rettgeri	-	+	-	+	-	+	+	++	-	-	+	-
P. stuartii	-	+	-	+	-	+	+	±	-	-	+	-
P. alcalifaciens	-	+	+	+	-	+	+	-	-	-	-	-
Yersinia enterocoliticac	-	-	-	±	-	-	-	±	-	-	±	+
Y. pestis	-	-	-	-	-	-	-	-	-	-	-	±
Y. pseudotuberculosis	-	-	-	-	-	-	-	+	-	-	-	±

[a] lac, inos, fermentation of lactose, inositol; mot, motility; gas, gas from glucose; ind, indole production; VP, Voges-Proskauer; cit, Citrate utilization (Simmons'); PDA, phenylalanine deaminase; ure, urease; lys, lysine decarboxylase; H_2S, H_2S produced in TSI agar; ONPG, metabolism of *o*-nitrophenyl-β-D-galactopyranoside.

[b] Some strains of Serratia marcescens may produce a red pigment

[c] *Yersinia* are motile at 22°C.{Key: +, ≥85% of strains positive; -, ≥ 85% of strains negative; 16-84% of strains are positive after 24-48 hour at 36°C}

(Source: Collee *et al.* 1996)

APPENDIX IX

ZONE SIZE INTERPRETATIVE CHART

Antimicrobial Agents used	Symbol	Disc Content	Resistant (mm or less)	Intermediate (mm)	Susceptible (mm or more)
Ampicillin When testing gram-negative enteric organisms When testing *Staphylococci*	A	10 mcg	13 28	14-16	17 29
Ciprofloxacin	Cf	5 mcg	15	16-20	21
Cloxacillin	Ob	5 mcg	12	12-13	14
Gentamicin	G	10 mcg	12	13-14	15
Ofloxacin	Of	5 mcg	12	13-15	16
Amikacin	Ak	30 mcg	14	15-16	17
Chloramphenicol	C	30 mcg	12	13-17	18
Tobramycin	Tb	10 mcg	12	13-14	15
Cefotoxime	Ce	30 mcg	14	15-22	23

(Source: Product Information Guide, Hi-Media Laboratories Pvt. Limited, Bombay, India)