Bacteriological profile of Neonatal septicemia with special emphasis on plasmid mediated Amp C β lactamase producing Gram negative isolates.

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DECLARATION

I, **Dr. B. SUBITHA** solemnly declare that this dissertation entitled **" Bacterological profile of Neonatal septicemia with special emphasis on plasmid mediated AmpC β lactamase producing Gram negative isolates"** was done by me at Coimbatore Medical College, Coimbatore during the period from June 2007 – July 2008 under the supervision and guidance of **DR. ANBU.N. ARAVAZHI, M.D.,** Professor and Head, Department of Microbiology Coimbatore Medical College, Coimbatore.

This dissertation is submitted to The Tamilnadu Dr.M.G.R. Medical University, Chennai towards the partial fulfillment of the requirement for the award of M.D. Degree (Branch- lV) in Microbiology to be held in March 2010.

I have not submitted this dissertation on any previous occasion to any University for the award of any degree.

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CERTIFICATE

This is to certify that the dissertation entitled **"BACTEROLOGICAL PROFILE OF NEONATAL SEPTICEMIA WITH SPECIAL EMPHASIS ON PLASMID MEDIATED AMPC B LACTAMASE PRODUCING GRAM NEGATIVE ISOLATES"** is a bonafide work done by **Dr. B. Subitha,** Post graduate student in the Department of Microbiology, under the supervision and the guidance of **DR.ANBU.N. ARAVAZHI, M.D.,** Professor and Head, Department of Microbiology, Coimbatore Medical College, Coimbatore, in fulfillment of the regulations of the Tamilnadu Dr.M.G.R. Medical University towards the award of M.D. Degree (Branch- lV) in Microbiology.

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LIST OF ABBREVIATIONS

1.	CLSI	Clinical Laboratory Standards Institute
2.	CONS	Coagulase Negative Staphylococci
3.	CRP	C Reactive Protein
4.	CSF	Cerebro Spinal Fluid
5.	DNA	Deoxyribo Nucleic Acid
6.	EOS	Early Onset Septicemia
7.	ESBL	Extended spectrum Beta Lactamase
8.	GBS	Group B Streptococci
9.	GNB	Gram Negative Bacilli
10.	GPC	Gram Positive Cocci
11.	IEF	Iso Electric Focusing
12.	IL6	Interleukin 6
1	IL8	Interleukin 8

14.	LOS	Late Onset Septicemia
15.	MHA	Muller Hinton Agar
16.	MIC	Minimum Inhibitory Concentration
17.	NICU	Neonatal Intensive Care Unit
18.	PABL	Plasmid Mediated Amp C β Lactamase
19.	PCT	Procalcitonin
20.	WBC	White Blood Cell Count
21.	WHO	World Health Organization

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INTRODUCTION

Neonatal septicemia **is a** major cause of morbidity and mortality in the newborn[1]. Neonatal septicemia is classified into early onset septicemia (EOS) and late onset septicemia (LOS) based on the age at onset[2, 3].

World Health Organization (WHO) estimates that there are about 5 million neonatal deaths occur globally per year. Ninety eight percent of them are occurring in developing countries. Neonatal Death rate varies in developing countries between 11-68/1000 live birth[2, 3].

The most common causes of death in neonatal period is infection (32%) followed by birth asphyxia (29%) and prematurity (24%). Neonatal infections currently cause 1.6 million deaths in developing countries[2.] In India septicemia occur in 10.97-27/1000 live births and is the leading cause of neonatal mortality, accounting for one quarter to nearly half of all neonatal deaths[4]. In Tamil Nadu as per the study Kuruvil et al the incidence of neonatal septicemia is 9.8 per 1000 live birth Vellore [5].

The microbial etiology of neonatal septicemia is variable and often changes temporally[6]. In developed countries Group B- Streptococci (GBS) and Coagulase Negative Staphylococci (CONS) are the most common etiologic agents for early onset and late onset septicemia respectively. In developing countries Escherichia coli, Enterobacter spp, Klebsiella spp and Acinetobacter precede GBS and CONS in causing EOS. Klebsiella spp, Pseudomonas spp, Salmonella spp, and Serratia precede CONS and Staphylococcus aureus in causation of LOS[3]. The source of infection in EOS is generally the maternal genital tract. Infants with EOS usually present with respiratory distress and pneumonia.The source of infection in LOS is either nosocomial or community acquired and the neonates usually present with pneumonia or meningitis [7].

Blood cultures are considered as the gold standard for diagnosis of neonatal septicemia. Nevertheless, their positivity varies widely (50 to 87%) and the results are not available rapidly for therapeutic management. For this reason, other, faster, laboratory tests like white blood cell count (WBC) and other biological markers as interleukin-8, C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and procalcitonin are used[8].

These biological markers, combined with clinical assessment, increase the probability of correct diagnosis and offer the physicians' greater confidence in promptly initiating antimicrobial therapy, in parallel with supportive care. On the other hand, they can also avoid the indiscriminate use of antibiotics thereby reducing the risk of developing multidrug resistant pathogens[8].

Resistance to commonly used drug is emerging as the most important problem globally. Antibiotic resistant bacteria are becoming an increasingly difficult problem in Neonatal Intensive Care Units (NICU) [2]. A major risk are the unwell or premature babies, those needing additional support such as ventilation, intravenous fluids, or blood products, and those babies who stay in hospital for more than 48 hours[3]. Multidrug resistant Gram negative bacilli belonging to the family Enterobacteriaceae have been increasingly responsible for infections among the neonates admitted to the NICU in many countries including India and Klebsiella pneumoniae constitutes a majority of these pathogens[9].

The most common mechanism of resistance in Gram negative bacteria is by the production of β-lactamases which inactivate β-

lactam antibiotics. Among the β-lactamases, Extended Spectrum β-lactamases (ESBLs) and AmpC β-lactamases are most commonly produced. AmpC β-lactamases are more important because they confer resistance to narrow, expanded, broad-spectrum Cephalosporins, β-lactam β-lactamase inhibitor combinations and Aztreonam, Earlier the AmpC β-lactamases were presumed to be chromosomally encoded. Recently the plasmid mediated AmpC β-lactamase has also arisen through the transfer of chromosomal genes for AmpC β-lactamase on to plasmids. This transfer has resulted in plasmid-mediated AmpC β-lactamases in isolates of E.coli, Klebsiella pneumoniae, Salmonella sp and Proteus mirabilis thus providing a new mechanism of resistance for those originally AmpC deficient bacterial strains[10,11,12,72].

Many clinical laboratories are not fully aware of the importance of plasmid mediated AmpC β-lactamases. Prevalence of this resistance mechanism appears to be increasing and has been responsible for nosocomial outbreaks, avoidable therapeutic failures and outbreaks of

multidrug resistant Gram negative pathogens that require expensive control efforts[13].

Neonatal septicemia is a life threatening emergency. **Diagnosis of neonatal septicemia is often difficult due to the minimal warning signs and symptoms both in preterm and term infants[2].** Delay in diagnosis and treatment with appropriate antibiotics may have devastating consequences. With early diagnosis and judicious use of antibiotics can bring down the neonatal morbidity and mortality substantially[14]. Surveillance is needed to identify the pathogens of neonatal septicemia as well as the antibiotic resistance patterns and the new mechanism of resistance[15]. Hence this prospective study is conducted to evaluate the bacteriological profile, antibiotic resistance pattern and plasmid mediated AmpC β-lactamase production in gram negative isolates of neonatal septicemia from the neonates admitted in NICU, Tertiary care Hospital, Coimbatore.

AIM:

The aim of the present study is to identify the common Bacterial isolates of neonatal septicemia and their antibiotic susceptibility patterns with special reference to plasmid mediated AmpC β-lactamase producing Gram negative isolates and also compare the CRP level with culture positivity.

OBJECTIVES:

1. To determine the bacteriological profile of neonatal septicemia.
2. To evaluate the antimicrobial susceptibility pattern of organisms isolated from neonatal septicemia.
3. To identify the prevalence of ESBL producers among the Gram negative isolates of neonatal septicemia.
4. To identify the prevalence of plasmid mediated AmpC β-lactamase producers among the Gram negative isolates of neonatal septicemia.
5. To compare the CRP level with culture positivity.

REVIEW OF LITERATURE

Neonatal septicemia is a clinical syndrome characterized by systemic signs of infection and accompanied by bacteremia in the first month of life[16, 17].Neonatal septicemia can be classified into two relatively distinct illnesses based on the postnatal age at onset. Early-onset neonatal septicemia (EOS) occurs in the first 7 days of life. Late-onset septicemia occurs (LOS) within 7-28 days of life[2, 3].

Epidemiology

The reported incidence of neonatal sepsis varies from 3.5 to 8.9 per 1000 live births in South America and the Caribbean, from 6.5 to 23 per 1000 live births in Africa, and from 7.1 to 38 per 1000 live births in Asia. By comparison, rates reported in the United States and Australasia range from 6–9 per 1000 live births for neonatal sepsis[3]. In the United States, septicemia accounts for 30% of the neonatal deaths.

In India Septicemia is the commonest clinical category with an incidence of 23 per 1000 live births while the incidence of meningitis is reported to be 3 per 1000 live births. Septicemia continues to be a major cause of neonatal mortality in India accounting for one-fourth to nearly half of neonatal deaths. Fatality due to neonatal septicemia ranges between 40 and 65%[16].

In developed countries, early onset neonatal septicemia is often more severe and case fatality rate is higher than late onset septicemia. As the

latter is usually caused by CONS, the associated morbidity and mortality are low. In developing countries, this may not be the case; in some series, LOS has a higher case fatality rate, particularly when Gram negative bacteria are involved[3]

.

Risk factors

Neonatal, perinatal and various maternal risk factors may predispose the neonate to development of septicemia. Most of these risk factors may be prevented, thereby reducing the incidence of neonatal septicemia[17]. Preterm and small for gestational age infants are more prone to develop septicemia than term and appropriate for gestational age infants[16].

Based on the studies from India, the following risk factors seem to be associated with an increased risk of EOS[7, 17].

1. Low birth weight (<2500 grams) or prematurity[63]
2. Febrile illness in the mother with evidence of bacterial infection within 2 weeks prior to delivery
3. Foul smelling and/or meconium stained liquor.
4. Rupture of membranes >24 hours.
5. Single unclean or > 3 sterile vaginal examinations during labor
6. Prolonged labor (sum of 1 and 2 stage of labor > 24 hrs)
7. Perinatal asphyxia (Apgar score <4 at 1 minute)

Presence of foul smelling liquor or three of the above mentioned risk factors warrant initiation of antibiotic treatment. Infants with two risk factors should be investigated and then treated accordingly.

The source of infection in LOS is either nosocomial (hospital-acquired) or community-acquired and neonates usually present with septicemia, pneumonia or meningitis.

- Various factors that predispose to an increased risk of nosocomial acquired LOS include

1. Low birth weight[63]
2. Prematurity
3. Admission in intensive care unit
4. Mechanical ventilation
5. Invasive procedures
6. Administration of parenteral fluids, Use of stock solutions.

- Factors that might increase the risk of community-acquired LOS include

1. Poor hygiene,

2. Poor cord care

3. Bottle-feeding, Prelacteal feeds. In contrast, breastfeeding helps in prevention of infections.

Bacteriology

Microbiological spectrum of neonatal septicemia shows marked geographical varations[6].These organisms may even vary at different times within the same place.

Marico M Loureiro et al (2002) from Brazil reported that the most common organism was Klebsiella followed by Staphylococcus, Serratia marcescens and Pseudomonas aeruginosa in causing neonatal septicemia[18].

R N Musoke et al in the year 2000 from Kenya reported that the predominant organisms causing neonatal septicemia were Gram negative bacteria (73.6 % of isolates) with Klebsiella species topping the list at 31 %[19].

Ziba mosayebi et al in the year 2003 from Iran reported that the most common pathogen isolated from neonatal septicemia being Klebsiella followed by Staph.aureus, E.coli, pseudomonas, Serratia and Acinetobacter. In causing EOS Klebsiella was the common organism. In causing LOS Staphylococcus aureus was the common organism [20].

J L Yu et al in the year 2001 from China reported that the most common pathogen was Staphylococcus followed by Escherichia coli in causing neonatal septicemia [21].

Rahman et al in the year 2002 from Pakistan reported that the most common organism isolated from *neonatal septicemia being* Escherichia *coli* followed by *Staphylococcus, Pseudomonas and Klebsiella*[23].

Shaw CK et al in the year 2007 from Nepal reported that the most common pathogen isolated from Neonatal septicemia being Staphylococcus aureus followed by Klebsiella, and E.coli[22].

Indian scenario

In Indian studies, Gram negative organisms have been more frequently responsible for septicemia (65-85%) as compared to Gram positive organisms. Commonly found organisms are Klebsiella, E.coli, pseudomonas, Staph. Aureus, Coagulase negative Staphylococcus, Enterobacter, Citrobacter, Proteus mirabilis, Serratia and Group B Streptococcus[16].

According to .Mahabatra et al (2002) from Perhampur, Orrisa, Gram-negative bacteria (88.45%) and Gram positive bacteria (11.6%) were isolated from neonatal septicemia. Enterobacter.cloacae was maximally isolated bacteria followed by K.pneumoniae and E.coli[1].

Diwakar et al in the year 2002 from Kerala reported that the most common pathogen isolated from Neonatal septicemia being CONS followed by Klebsiella, Pseudomonas aeruginosa, Enterobacter and Acinetobacter[24].

In a study by Vinodkumar et al (2007) from Karnataka, among the isolates of neonatal septicemia, Klebsiella (26.9%) was the most common infective organism followed by Staphylococcus aureus (20%), CONS (13.4%), E.coli (10.5%) and Acinetobacter species (6.1%) [17].

According to Kurien Anil Kuruvilla et al from Tamil Nadu, E. coli and E. fecalis were the predominant organisms causing EOS, while Klebsiella and E. fecalis were the predominant organisms in LOS[5].

Patho physiology

The fetus is protected from bacterial exposure by the membranes and placenta. It has also been shown that the amniotic fluid has inhibitory properties against bacterial growth. However, fetal bacteremia may occur in preterm labor, and term neonates may have bacteremia or present symptoms at birth, suggesting that bacterial colonization may take place before birth. Some bacteria (e.g. Listeria monocytogenes) cause transplacental infections via the mother's bloodstream. More commonly, however, bacterial exposure takes place in the amniotic cavity, or during the passage through the birth canal. Pyati et al reported that preterm neonates may be exposed to GBS in utero, whereas term neonates often may be exposed during the passage through the birth canal. Fetal colonization is likely to take place by aspiration of contaminated amniotic fluid, or by bacteria penetrating through injured skin or natural body openings. In most cases this colonization proceeds without causing disease. The mechanism by which bacterial colonization converts to invasive disease is not fully understood, but it is likely to reflect bacterial virulence, maternal immunological factors, and the competence of the neonatal immune system[25, 26].

Once bacteria gain access to the bloodstream, mechanisms are activated by the host for their elimination. Usually the bacteria are efficiently cleared by the monocyte-macrophage system after opsonization by antibody and complement. Sometimes, however, a systemic inflammatory response syndrome is established and can progress independently of the original infection.

Earlier it was believed that the bacteria or their cell wall components (endotoxins of Gram-negative organisms and lipoteichoic acid-peptidoglycan

complex of Gram-positive bacteria) were largely responsible for the direct toxic effects on tissues. Recent research indicates that the physiologic effects generated by bacterial infections are largely mediated by interaction of pro-inflammatory cytokines activated in response to the presence of microbial components within the vascular compartment[16].

Biological markers

As Marshall et al. pointed out, while many circulating or cell-associated molecules such as white blood cell count (WBC) ,interleukin 8, C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and procalcitonin have been proposed as useful markers of the presence, severity, or response to therapy of septicemia. But none has been demonstrated to be 100% sensitive and specific, to have a clear utility in directing therapeutic decision-making[27].

Procalcitonin

Procalcitonin (PCT) is a protein of 116 amino acids and a molecular weight of 13 kDa. It is a prohormone of calcitonin that is produced by the parafollicular cells of the thyroid gland, and is intracellularly cleaved by proteolytic enzymes to form the active hormone[50]. This marker is also produced by macrophage, and monocyte cells of various organs in severe bacterial infection and sepsis. In 1993, a study identified elevated level of PCT in patients with bacterial infection[52]. Since then, PCT has become the most widely studied and reported putative biomarker for sepsis in children. While circulating levels of PCT in healthy subjects are below the limit of detection, production of PCT during

inflammation correlates with both the presence of bacterial endotoxin and inflammatory cytokines[27].

PCT levels of 0.5-2 ng/ml, 2.1-10 ng/ml and >10 ng/ml were considered as weakly positive, positive, and strongly positive, respectively[64].

Interleukins 6 and 8

Interleukin-6 (IL-6) levels correlate with the severity of the inflammatory response, although they are not specific for bacterial infection. The concentration of IL-6 increases in children with sepsis, and a persistent IL-6 concentration >500 pg/mL may be useful in identifying pediatric patients with intra-abdominal sepsis who are likely to have a prolonged length of stay and increased morbidity. In liver cells, it stimulates the synthesis of C-reactive protein, serum amyloid A, α1-acid glycoprotein, α1-antitrypsin, and fibrinogen, and is also responsible for myocardial depression. IL-8 is a chemokine responsible for migration of neutrophils and macrophages to the site of inflammation and is not normally present in high quantities in healthy children. It has been shown to serve as a diagnostic marker for bacterial sepsis in febrile, neutropenic children[27].

C-reactive protein

C-reactive protein (CRP) is a real-time and low-cost biomarker. CRP is a major acute phase plasma protein, is synthesized by hepatocytes in response to infections or tissue injuries[28].

CRP was originally discovered by Tillett and Francis in 1930 as a substance in the serum of patients with acute inflamation has reacted with the *C* polysaccharide of Pneumococcus

Pepys and Hirschfield (2003) and Marnell et al (2005) reported CRP as an indicator of acute infection and inflammation. Hengst(2003), Black et al (2004) and Sierra et al(2004) reported that Systemic infection by Gram-positive and Gram negative bacteria raises CRP level up to 1000-fold suggesting its involvement in the immune response against most pathogens[29]. It falls quickly after efficient elimination of microbial stimulus, due to its short half-life of 19 hours[28].

CRP directly binds to Streptococcus pneumonia (Volanakis and Kaplan, 1971), Neisseriae lactamica (Serino and Virji, 2000) and Haemophilus influenzae (Weiser et al, 1998) via repetitive phosphoryl choline moieties on the lipoteichoic acid or the lipopolysaccharide (LPS) of these pathogens has been shown. Binding of CRP recruits phagocytic cells, and activates the classical complement pathway (Kaplan and Volanakis, 1974; Siegel et al, 1974), causing bacterial clearance. However CRP during systemic infection in vivo is not completely understood[29].

Sensitivity and specificity of CRP varies in different studies in screening neonatal septicemia.

In a study by garland Suzanne et al [30], sensitivity was 67% and negative predictive value was 86% in screening neonatal septicemia. In a study by khassawneh et al[31], sensitivity was 95% and negative predictive value was 98% in screening neonatal septicemia. In a study by Beger et al, CRP had 75% of sensitivity and 86% of specificity. In a study by Nutnar,et al, sensitivity was 100% and specificity was 94%, PPV and NPV were 91.6% and 100% respectively. In

Tehran study, sensitivity was 79% and negative predictive value was 97%, but poor positive predictive value of (36%), the specificity of it was 85%[31].

Antibiotic resistance

In neonatal intensive care units, infection by multidrug resistant Gram negative microorganisms is a threat for survival of premature and term newborns, since the therapeutic choices are limited for this group of patients and susceptibility of isolates in neonatal septicemia to the most commonly used antibiotics is decreasing around the world. Since emergence of resistance is a characteristic of the evolution of bacteria, it has been claimed as unavoidable[33,51,55,85].

. The various mechanisms of drug resistance in Gram-negative bacilli include enzyme production efflux mechanisms and porin deficiency[11]. Among them enzyme β lactamase production remains the most important contributing factor to β lactam resistance. The four major groups of β lactam antibioticss Penicillin, Cephalosporins, monobatams and carbapenems have a β lactam ring which can be hydrolyzed by β-lactamases resulting in microbiologically ineffective compounds[35].

Over the last two decades many new β lactam antibiotics have been developed that were specifically designed to be resistant to hydrolytic actions of β-lactamase. However, with this new class of drug that has been used to treat patients, new types of β-lactamases emerged by overproduction and mutation of lactamases[10]. AmpC β-lactamase is one of these new types of β-lactamases. In Gram negative bacteria these enzymes remain in the periplasmic space, where they attack the antibiotic before it can reach its receptor site[35].

Classification of β lactamase enzymes.

The β lactamase enzymes are a diverse group of more than 340 discrete types that have been categorized into different functional groups, depending on their antimicrobial substrate profile, enzyme inhibition profile, enzyme net charge (PI), hydrolysis rate (Vmax), binding affinity (Km), isoelectric focusing, protein molecular weight and aminoacid composition[36]. Ambler's molecular classification is based on the nucleotide and amino acid sequences in these enzymes. To date, four classes are recognized (A to D), correlating with the functional classification. Classes A, C and D act by a serine-based mechanism whereas class B (metallo beta lactamases) needs divalent cation zinc for their action. However the most recent and comprehensive classification is the one given in 1995 by Bush, Jacoby and Medeiros. This classification system takes into account a number of properties simultaneously[36,43].

Bush Jacoby-Medeiros Functional Classification of Beta lactamases with correlation to Ambler molecular classification scheme[36, 43, 74, 78].

	Molecular Class	Preferred Substrate	Inhibited by CA*	Examples
1	C	Cephalosporins	-	AmpC, MIR-1
2a	A	Penicillins	+	Gram positive penicillinases
2b	A	Penicillins, Cephalosporins,	+	TEM-1 and 2, SHV-1
2be	A	Penicillins,extended spectrum Cephalosporins,monobactams	+	TEM-3 to 26, SHV-2 to 6
2br	A	Penicillins	+/-	TEM-30 to 36
2c	A	Penicillins, Carboxypenicillins	+	PSE-1, 3 and 4

2d	D	Penicillins, Cloxacillin	+/-	OXA-1 to 11
2e	A	Cephalosporins	+	*P. vulgaris*
2f	A	Penicillins, Cephalosporins, carbapenems	+	NMC-A
3	B	Beta-lactams	-	CcrA
4	?	Penicillins	-	*B. cepacia*

Amongst the mechanisms of resistance to third generation cephalosporins, production of ESBLs and AmpC β-lactamases are the most common. AmpC β-lactamases are clinically important because they confer resistance to narrow expanded, and broadspectrum cephalosporins, β lactam β lactamase inhibitor combinations and Aztreonam[10, 11, 12].

Plasmid mediated AmpC β-lactamases

AmpC β-lactamases are Group I cephalosporinases that confer resistance to a wide variety of β lactam antibiotics including alpha methoxy β lactams such as Cefoxitin, narrow and broad spectrum cephalosporins, Aztreonam, and are poorly inhibited by β-lactamase inhibitors such as clavulanic acid.

Genes for AmpC β-lactamases are commonly found on the chromosomes of the several members of the family Enterobacteriaceae, including Enterobacter, Shigella, Providencia, Citrobacter freundii, Morganella morganii, Serratia and Escherichia coli. Plasmid mediated AmpC β-lactamases has arisen through the transfer of chromosomal genes for the inducible AmpC β-lactamases on to plasmids. This transfer has resulted in plasmid mediated AmpC

β-lactamases in isolates of Esch. coli, Klebsiella pneumoniae, Salmonella species, Citrobacter freundii, Enterobacter aerogenes and Proteus mirabilis[12].

History

In 1976, Bobrowski et al[46] described a plasmid-mediated β lactamase indistinguishable from the AmpC enzyme of E. coli in a strain of P. mirabilis. Unfortunately, the original plasmid was lost, there was some doubt about the transfer experiments, and molecular studies were not done.

In 1982, Levesque et al[47] reported a plasmid-mediated cephalosporinase in Achromobacter spp. Regrettably, the original strain was lost, the β lactamase gene was not sequenced, and in retrospect the biochemical properties of the enzyme resembled those of a group 2b broad-spectrum enzyme.

In 1983, Knothe et al[48] reported the transfer of Cefoxitin resistance from Serratia. marcescens to Proteus or Salmonella spp., but resistance segregated on transfer to E. coli and no biochemical or molecular studies were done.

In 1989, Bauernfeind et al [49] described a K. pneumoniae isolate from South Korea that could transfer resistance to Cefoxitin and Cefotetan as well as to Penicillins, oxyimino-cephalosporins, and Monobactams to E. coli. The enzyme, termed CMY-1 for its cephamycinase activity, had an isoelectric point (pI) of 8.0 and was more sensitive to inhibition by Sulbactam than by Clavulanate or Tazobactam, suggesting that it might be a class C enzyme.

However, the first proof that a class C β lactamase had been captured on a plasmid was provided by Papanicolaou et al., who described transmissible resistance to α methoxy- and oxyimino-β lactams mediated by an enzyme (MIR-1) with the biochemical properties of a class 1 β lactamase and showed that part of the MIR-1 gene was 90% identical to the amp C gene of Enterobacter. cloacae. Subsequently, plasmid-mediated class C β- lactamases have been discovered worldwide[37].

Nomenclature

AmpC β-lactamases have been named with inconsistency typical of β lactamase nomenclature according to the resistance produced to Cephamycins (CMY), Cefoxitin (FOX), and Moxalactam (MOX) or Latamoxef (LAT), to the type of ßlactamase, such as Amp C type (ACT) or Ambler class C (ACC), and to the site of discovery, such as the Miriam Hospital in Providence, R.I. (MIR-1) or Dhahran hospital in Saudi Arabia (DHA). BIL-1 was even named after the patient (Bilal) who provided the original sample (D. J. Payne, personal communication) [37].

Epidemiological features

Plasmid mediated class C β-lactamases have been discovered most frequently in isolates of K. pneumoniae and also in other naturally AmpC species such as *K*. oxytoca, Salmonella, and *P*. mirabilis. Some enzymes have been found in E. coli, although this species can also increase production of its normally weakly expressed chromosomal AmpC enzyme by gene duplication or

mutation in the amp C promoter or attenuator with consequent enhanced gene expression.

In Greece, plasmid-mediated LAT-2 (CMY-2) has been found in clinical isolates of Enterobacter aerogenes simultaneously with its appearance in clinical strains of K. pneumonia and E. coli.

In France plasmid-mediated ACC-1 has been found in both E. coli and Proteus. mirabilis isolates obtained from the same urine sample.

In the United States, Ceftriaxone- resistant Salmonella have begun to appear that owe their resistance to plasmid-mediated CMY-2 β lactamase.

A striking feature is the global distribution of strains producing plasmid-determined cephalosporinases. They have been found in Africa, Asia, Europe, Saudi Arabia, and America

Most strains producing plasmid-determined AmpC enzymes have been isolated from patients after several days in the hospital. Affected patients have often had prolonged stays in intensive care units. Some patients had one or more surgical procedures, an underlying disease such as leukemia or cancer or were immune compromised after liver or kidney transplantation. Sources of organisms included cultures of urine (about 50% of isolates), blood, wounds, sputum, or stool. Some isolates were recovered in mixed cultures with other potential pathogens. A majority of the patients had been treated with β lactam antibiotics including Cefoxitin, Moxalactam, Cefmetazole, Cefotetan, or Imipenem. Many strains with plasmid-determined AmpC enzymes also produce TEM-1, TEM-2, or even an ESBL, such as SHV-5[37].

Susceptibility patterns.

Strains with plasmid-mediated AmpC enzymes were consistently resistant to Aminopenicillins (Ampicillin or Amoxicillin), Carboxypenicillins (Carbenicillin or Ticarcillin), and Ureidopenicillins (Piperacillin) and, among the Penicillins, these strains were susceptible only to Amdinocillin or Temocillin. The enzymes provided resistance to Cephalosporins in the oxyimino group (Ceftazidime, Cefotaxime, Ceftriaxone, Ceftizoxime, Cefuroxime) and the 7α methoxy group (Cefoxitin,Cefotetan, Cefmetazole, Coxalactam). MICs were usually higher for Ceftazidime than for Cefotaxime and for Cefoxitin than for Cefotetan. The enzymes were also active against the Monobactam Aztreonam. Susceptibility to Cefepime or Cefpirome was little affected and was unchanged for Carbapenems (Imipenem, Meropenem) [81,82, 84].

Alterations in antibiotic access to the enzyme can markedly change the susceptibility profile. With loss of outer membrane porin channels, Klebsiella. pneumoniae strains carrying plasmids determining AmpC enzymes can have Imipenem resistance. Although commercially available inhibitors especially Sulbactam, caused a modest reduction in MICs of Cefoxitin. Plasmid-mediated AmpC enzymes were not susceptible to inhibitor combinations, with the possible exception of Piperacillin- Tazobactam. Resistance was, however, blocked by BRL 42715 and Ro 47-8284

Enzymatic properties.

Plasmid-mediated AmpC type β lactamases have isoelectric points between 6.4 and 9.4.The apparent molecular size of the mature plasmid-mediated AmpC β-lactamases vary from 38 to 42 kDa with 378 to 386 amino acid residues. Kinetic properties were characteristic of chromosomal AmpC enzymes with relative *V*max values generally 10-fold or greater for Cephalothin and Cephaloridine than for Ampicillin and Penicillin, greater activity with Penicillin than with Ampicillin, and low hydrolysis rates for oxyimino- or α methoxy compounds. On the other hand, *Km* values for Cefoxitin, Cefotetan, Cefotaxime, Moxalactam, or Aztreonam were generally less than those for Penicillin or Ampicillin and much lower than the *Km* values for Cephaloridine, Cephalothin, or Cefepime as is typical of group 1 Cephalosporinases. The amino acid sequence of the enzymes revealed an active site serine in the motif Ser-X-X-Lys (where X is any amino acid) at residues 64 to 67 of the mature protein. A Lys-Ser/ Thr-Gly motif has been found at residues 315 to 317 and plays an essential role in forming the tertiary structure of the active site.

The plasmid-mediated enzymes can be divided into five or six clusters: the *C*. freundii group with LAT types and certain CMY types, the Enterobacter group with MIR-1 and ACT-1, the M. morganii group with DHA-1 and DHA-2, the H alvei group represented by ACC-1, and the Aeromonas group with MOX-, FOX-, and other CMY-type enzymes[37, 42.]

Genetic features

Genes for the AmpC enzymes have been located on plasmids of sizes varying from 7 to 180 kb. A few of the plasmids have not been self-transmissible but are transferable by transformation or mobilization. Plasmids encoding AmpC enzymes often carry multiple other resistances, including resistance to Aminoglycosides, Chloramphenicol, Sulfonamide, Tetracycline, Trimethoprim, or mercuric ion. A plasmid encoding a FOX-type enzyme even carried a gene for Fluoroquinolone resistance. Clinical isolates often produce other β lactamases in addition to an AmpC enzyme. The *bla* genes may be on different plasmids, but often they coexist on the same plasmid[37, 42].

Detection

There is no clear consensus regarding guidelines for performing tests for the phenotypic screening or confirmatory tests for the isolates that contain AmpC β-lactamases[66]. Sensitivity to Cephamycin such as Cefoxitin 30 µg disk (Hi-Media,) is tested by disk diffusion method and interpreted as per the Clinical Laboratory Standard institute (CLSI).Isolates with resistance or with decreased susceptibility (intermediate by CLSI criteria) to Cefoxitin are selected for further study. Furthermore, not all strains with plasmid-mediated AmpC enzymes meet the CLSI criteria for resistance to cephamycins and oxyimino-cephalosporins. Isolates resistance to Cefoxitin can be studied further for cephamycin hydrolysis with the three-dimensional test, AmpC disk test and for β lactamase inhibitor effects. Unfortunately, inhibitors (BRL 42715, Ro 47-8284, Ro 48-1220, Ro 48-1256) that are active against AmpC enzymes are not readily

available, but Cloxacillin or Cefoxitin have been used to block AmpC activity selectively after isoelectric focusing. Lack of inhibition of activity against oxyimino- β lactams or cephamycins by Clavulanate is indirect evidence for the presence of an AmpC enzyme, but some AmpC enzymes are unusually susceptible to inhibition by Tazobactam

A reference laboratory is needed for β-lactamase isoelectric focusing or gene localization[37, 40].

Isoelectric focusing.

Crude AmpC β-lactamase extracts are subjected to analytical IEF on an ampholine polyacrylamide gel (pH 3.5 to 9.5; Pharmacia, Uppsala, Sweden). Preparations from standard strains known to harbor TEM-12 (pI 5.25), TEM-10 (pI 5.6), TEM-29 (pI 6.1), SHV-1 (pI 7.6), and ACT-1 (pI 9) plasmid-mediated β-lactamases are used as controls. Then β-lactamases are visualized with a 0.2mg/ml nitrocefin solution (Oxoid Ltd., Basingstoke, England). A 1 mM solution of potassium clavulanate and a 0.3 mM solution of Cloxacilin were used to visualize general inhibitor characteristics[9, 38, 67,68 and 69].

PCR and sequencing.

Multiplex PCR is performed to amplify six PABL group genes. A series of specific primers are used for the detection of DHA-1, CMY-2, MOX, ACC, EBC, FOX, TEM, SHV, and CTX-M. The PCR products are purified with a QIAquick

PCR purification kit (Qiagen, Hilden, Germany) and cloned into DH5. Plasmid DNA is prepared by using Qiagen columns and is sequenced on an ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, CA)[38,67,68,69.]

DNA fingerprinting.

Random amplified polymorphic DNA (RAPD) analysis is applied to type the PABL-producing isolates with the primer ERIC. Amplified PCR products are separated using 1.5% agarose gels and visualized by UV transillumination. DNA fingerprints are compared by visual inspection RAPD patterns are regarded as different if there are different bands on visual inspection.

Conjugation,plasmid analysis

Escherichia coli **K12** J53 Azi^{r} **is** used as the recipient strain. Cultures of donor and recipient cells are grown to saturation, and 0.1 ml of each is added to the brain heart infusion broth (5 ml) and allowed to stand at 37°C for 2 h. The mixture is then incubated with shaking for 3 h, and 0.1-ml samples were streaked onto **T**rypticase Soy Agar incorporated with 250 µg/ml of sodium azide and 10 µg/ml of Cefotaxime. To confirm the presence of plasmids and to estimate their sizes, plasmids from clinical isolates and transconjugants are extracted, electrophoresed on a 0.7% agarose gel, and subjected to Southern hybridization[38, 79,91].

Southern hybridization.

Southern blot hybridizations are performed by standard methods with a *bla*CMY-2-specific and *bla*DHA-1-specific digoxigenin (DIG)-labeled probe. Briefly, purified plasmid DNA is transferred onto a positively charged Millipore nylon membrane by capillary action. DIG-labeled *bla*CMY-2-specific and *bla*DHA-1-specific detection probes are generated according to the directions of the manufacturer. After prehybridization, hybridization of the membrane with the denatured, DIG-labeled probe
(25 ng/ml of hybridization solution) is done overnight at 65°C. The hybridized probes are immunodetected with anti-DIG-alkaline phosphatase[38].

Detecting a plasmid-mediated AmpC enzyme in a strain with a native inducible ß lactamase or a coexisting ESBL is even more challenging. Given the difficulty in detecting plasmid-mediated AmpC β-lactamases, their prevalence is currently likely to be underestimated[37].

Yi Li et al[38] (2003) from China reported that plasmid mediated AmpC β-lactamase were detected in 4.29% (29 isolates of DHA-1 and 1 isolate of CMY-2) of K. pneumoniae, 1.91% (11 isolates of DHA-1 and 12 isolates of CMY-2) of E. coli, and 3.03% (1 isolate of DHA-1) of Klebsiella. oxytoca isolates.

Coudron et al[83] Studied 1,286 consecutive, non repeat isolates from Omaha Medical Center collected between 1995 and 1997 and estimated that 1.6% of E. coli isolates, 1.1% of K. pneumoniae isolates, and 0.4% of P.

mirabilis isolates were Cefoxitin-resistant AmpC β-lactamase producers, mostly via transmissible plasmids.

The occurrence of AmpC β-lactamases in Delhi was reported by Manchanda & Singh, who found 20.7 per cent of the clinical isolates harboring Amp C β-lactamases[39].

A. Subha et al[44] (2003) from Chennai reported that, twenty eight isolates (24.1%) of Klebsiella spp. and 12 (37.5%) of Esch. Coli were AmpC β-lactamase producers.

A.K. Ratna et al[45] (2003) from Karnataka reported sixteen (3.3%) isolates were positive for AmpC β-lactamases. Based on the species 9 (3.3%) Escherichia coli, 4 (2.2%) Klebsiella pneumoniae, 2(5%) Citrobacter freundii and 1 (5.5%) isolate of Enterobacter aerogenes harbored Amp C enzymes.

In a study by V. Hemalatha et al[40] (2007) from Chennai, AmpC β-lactamase was detected in 47.3 per cent isolates, four-fifths of which occurred in combination with ESBLs. Pure AmpC β-lactamases were detected only in 9.2 per cent of the isolates[40].

MATERIALS AND METHODS

This prospective study was conducted on 273 Neonates with signs and symptoms of septicemia admitted in Neonatal Intensive Care Unit at Coimbatore medical college & Hospital, Coimbatore over the period of 14 months from May'2008 to June'2009.

Approval was obtained from the ethical committee prior to conduct of this study. Informed consent was obtained from the parents of neonates.

Patient selection

Inclusion criteria

Age<28 days
>30 weeks of gestation & Full term babies
Presence of three or more clinical symptoms and signs of septicemia like lethargy, poor feeding, irritability, fever, vomiting, abdominal distension, jaundice, respiratory distress, hypothermia, cyanosis and convulsions.

Exclusion criteria

Extreme prematurity <30 wks of gestation
Gross congenital anomalies
Undergone surgery.

The name, age, sex, address, date of admission, inpatient number and detailed clinical history of the patients were noted. A thorough head to foot general examination, systemic examination and Respiratory Distress Syndrome (RDS) scoring were carried out by the pediatricians.

Specimen collection

Samples collected

a.i. **Blood**

a.ii. **CSF (in case of suspected meningitis)**

Blood sample collection [75, 76].

Venepuncture site was cleansed thoroughly with alcohol, followed by povidone- iodine, and again by alcohol. Povidone-iodine was applied in concentric circles moving outward from the centre. The skin was allowed to dry for at least 1 minute. Two ml of blood was withdrawn aseptically. One ml of collected blood sample was inoculated into a blood culture bottle containing 10 ml Brain Heart Infusion broth for cultural isolation and remaining one ml of blood was preserved for serological test.

Cerebrospinal fluid specimen collection

Term infants were placed in a seated position on the edge of the table, with trunk flexed forward, stabilised from the front by the assistant. The infant's shoulders and hips were held in order to maintain vertical alignment of the hips and shoulders during the procedure. Lumbar Punctures were performed at or below the L4 level. CSF was allowed to drip into at least two tubes. A minimum of 10 drops/tube was collected in separate sterile containers for microbiological and biochemical analysis.

Safety precautions

All samples were treated as potentially infectious and leak proof containers were used for collection and transportation of samples. The containers were disinfected by treating with freshly prepared 1 to 2% sodium hypo chlorite solution or sterilized by autoclaving.

Specimen processing

Separation of serum from blood

The 1 ml blood preserved for serological test was kept undisturbed at room temperature for 30 to 45 minutes to allow clot formation and transferred to 4-8^{0}c for clot retraction for a minimum of 1to 2 hours till serum was separated.

The clot was removed aseptically using sterile Pasteur pipette and the supernatant was centrifuged at low speed (1500 rpm for 5 to 10 minutes) to remove the residual blood cells. Clear portion/serum was used for CRP Latex agglutination test.

CSF

The CSF specimen for microbiological work was centrifuged at 1500-2000 rpm for 10-15 minutes. After centrifugation the deposit was used for Gram film and the remainder of the deposit used for cultural isolation.

Gram stain [75, 76.]

The gram staining was done as per the standard procedure mentioned in Appendix I and the stained smear was examined microscopically under oil immersion field.

Cultural isolation [75, 76.]

The brain heart infusion broth with inoculated blood was incubated aerobically at 37°C. A subculture was done after 18 hours. The bottles were kept and observed for turbidity, gas production, haemolysis and any color change for

seven consecutive days in case of absence of growth. Media used for sub culturing included Chocolate agar, Blood agar and Mac Conkey agar (HiMedia).

The remaining sediment of CSF was inoculated on Blood agar,Choclate agar and Mac Conkey agar. The inoculated plates were incubated at 37^0c for 18 to 24 hours.

The isolates were identified by colony morphology, Gram staining, motility and the following biochemical reactions as per the standard procedure[77] mentioned in Appendix II.

1. **Catalase test**
2. **Oxidase test**
3. **Coagulase test**
4. **Indole production test**
5. **Methyl red test**
6. **Voges –proskauer test**
7. **Urease test**
8. **Citrate utilization test**
9. **Triple sugar iron agar test**
10. **Carbohydrate utilization test**

I. Antibiotic sensitivity testing [75, 76.]

Anti microbial susceptibility testing was performed by Kirby Bauer disk diffusion method. Commercially available Muller Hinton Agar culture medium and antibiotic disks (Himedia) were used.

Inoculum preparation

Using a sterile wire loop, 3-4 well isolated colonies of similar appearance from primary culture plate were inoculated into 2-3 ml of normal saline and subsequently emulsified. The turbidity was adjusted so as to correspond to the 0.5 Mc Farland standards.

Preparation of barium sulfate 0.5 Mc Farland standard.

A 0.5 ml of aliquot of 0.048mol/l(1.175%w/v $Bacl_2 2H_2O$)is added to 99.5 ml of 0.18 mol/l H_2SO_4 (1%V/V) with constant stirring to maintain a suspension.

Inoculation of test plates

After adjusting the turbidity of the inoculum suspension, a sterile cotton swab dipped in to the suspension. Excess fluid was removed by pressing and rotating the swab against the side of the tube above the level of the suspension. The surface of the Muller Hinton agar plate was streaked with the swab evenly over in three directions. Using sterile forceps the appropriate antimicrobial disks were placeded over the inoculated plate and not closer than about 25mm from disc to disc. Then the plates were incubated at 37^0c for 18 -24 hrs.

Interpretation of zone sizes

After overnight incubation, examined the plates to ensure the confluent or near confluent growth. Using a ruler held on the underside of the plate measured the diameter of each zone of inhibition in mm. The end point of inhibition was where growth starts. The sizes of zone of inhibition were

interpretated by referring to the CLSI standards, and the organisms were reported as susceptible, intermediate, or resistant to the agents that have been tested.

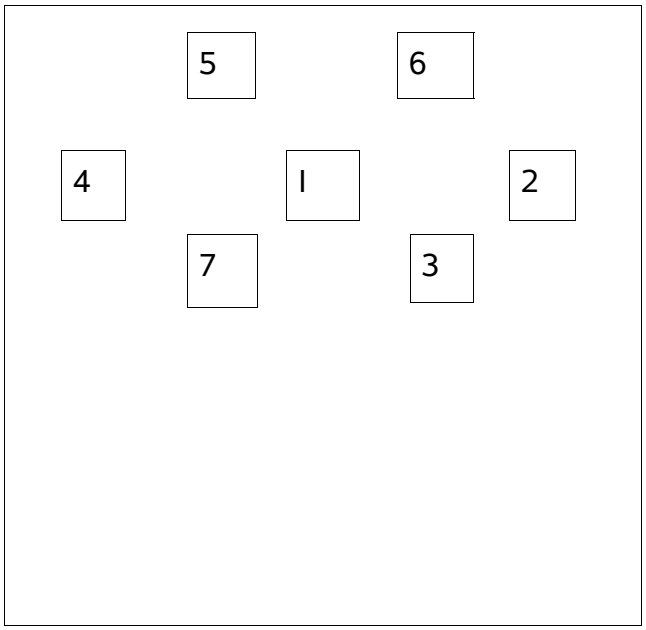
II. Screening for ESBL and AmpC β-lactamase producing isolates.

Gram negative isolates resistant to third generation Cephalosporins (Cefotaxime, Ceftazidime,& Ceftriaxone) were screened for ESBL production and AmpC β-lactamase production by Novel Predictor Disk Placement Method.

Novel predictor disk placement method [87.]

C.Rodrigues **et al (2004) followed this procedure to detect** ESBL and AmpC β-lactamase production.

The disk placement was designed in a novel fashion to assess ESBL and AmpC β-lactamases.The Ceftazidime and Ceftazidime +Clavulanic acid disks were kept 15-20 mm apart from each other. Imepenam ,an inducer was placed in the centre and on either side of it at a 15 mm distance, were placed Ceftazidime and Cefotoxime (indicators of induction)in addition another inducer Cefoxitin was placed at 15mm from Cefotaxime .This was placed opposite to that of Ceftazidime +Clavulanic acid to avoid any effect of inducible β lactamases on the zone of inhibition of the latter.



1Imepenam, 2Cefotaxime,

3Cefoxitin, 4Ceftazidime,

5Ceftazidime+Calvulanic acid, 6Azteronam, 7Ceftritriaxone.

ESBL

- **Zone diameters for Cefotaxime<27 mm,Ceftazidime<22mm and Ceftriaxone <25mm**
- **Susceptible to Cefoxitin**
- **Increase in zone size with addition of inhibitor by ≥ 5mm**

AmpC

- **Resistanse to Cefoxitin**
- **No increase in zone size with addition of an inhibitor**
- **(Inducible Amp C-Blunting of zone towards inducers (imepenam. Cefoxitin)**

III. Detection of ESBL production in gram negative isolates

ESBL production in gram negative isolates was further confirmed by Double disk potentiation test as per CLSI Standards.

Phenotypic confirmatory test. (Double disk potentiation test)[9, 10]

Ceftazidime (30 µg) disk and a Ceftazidime plus Clavulanic acid (Ca 30 µg + Caz 10 µg) disk were placed at a distance of 20 mm apart on a lawn of culture of the suspected ESBL producing clinical isolates on MHA. **The plates were incubated at 37°C overnight.**

Interpretation

The test organism was considered to produce ESBL if the zone size around the Ceftazidime plus Clavulanic acid increased >5 mm in comparison to the third generation Ceftazidime disk alone. This increase occurred because the β lactamases produced by the isolates were inactivated by Clavulanic acid.

IV. Detection of plasmid mediated AmpC β-lactamases in Gram negative isolates

Gram negative isolates positive for AmpC β-lactamase production in screening test were subjected to plasmid mediated AmpC β-lactamase detection by the Modified Three Dimensional Extract Method and AmpC Disk Test.

1. Modified Three Dimensional Extract Test [10,39,44,45,66].

AMPC **enzyme production was tested by a modified three-dimensional test as described by Manchanda & Singh.**

Preparation of AmpC enzyme extract.

10 to 15 mg fresh overnight growth from MHA was taken in a centrifuge tube. Peptone water was added and centrifuged 3000 rpm for 15 min .Crude enzyme extract was prepared by repeated freeze thawing for five to seven times

Procedure

Lawn cultures of E.coli ATCC 25922 were prepared on MHA plates and Cefoxitin (30 µg) disks were placed on the plate. Slits were cut using a sterile surgical blade 3 mm away from the Cefoxitin disk.10µg enzyme extract was added to a well made at the outer edge of the slit .The plates were incubated at 37°C overnight.

Interpretation

After incubation, plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of Cefoxitin (positive result), or the absence of a distortion, indicating no significant inactivation of Cefoxitin (negative result).

2. AMPC Disk test [11, 62,66.]

Plasmid mediated AmpC β-lactamases production was further tested by the AmpC disk test as described by Black et al[62].

Amp C disk preparation

AmpC disks were prepared by applying 20ml of a 1:1 mixture of saline and 100Xtris-EDTA to sterile filter paper disks. Several colonies of each test organism were applied to a disk.

procedure

The surface of a Mueller-Hinton agar plate was inoculated with a lawn of 0.5 McFarland matched suspension of Cefoxitin susceptible E. coli ATCC 25922. A 30 mcg Cefoxitin disk was placed on the inoculated surface of the Mueller-Hinton agar. The AmpC disk was then placed almost touching the antibiotic disc with the inoculated disk face in contact with the agar surface. The plate was then inverted and incubated overnight at 35°C.

Interpretation

After incubation, plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of Cefoxitin (positive result), or the absence of a distortion, indicating no significant inactivation of Cefoxitin (negative result).

V. Confirmation of plasmid mediation in AmpC β-lactamases production .

Conjugation assay [44, 79, 81, 91].

Transfer of cefoxitin resistance and AmpC β-lactamase production:

Medium used for conjugation assay

Trypticase soy agar

Trypticase peptone	**17g**
Phytone peptone	**3g**
Nacl	**5g**
K2HPO4	**2.5g**
Glucose	**2.5g**
Distilled water	**1L**

P.H **7.4**

Supplemented with Sodium Azide 250 µg/ml and **Cefotaxime** 10 µg/ml

Mating procedure

Transconjugation experiments were done following the procedure by Abigail et al with few modifications. Mating was performed with Escherichia coli **K12** J53 Azi ʳ(kindly provided by Professor Dr Raju B Appala, HOD, Department of Microbiology, PSG Medical College &Hospital, Coimbatore.) **as the recipient strain. Overnight brain heart infusion (BHI) broth cultures (0.5 Mac Farland turbidity matched) of the donor and the recipient strains were mixed in the ratio of 1:10. This mixed culture was incubated overnight at 37°C.**

Selection of transconjugants.

Transconjugants were selected on Trypticase Soy Agar plates containing 250 µg/ml of sodium azide and 10 µg/ml of Cefotaxime. **200 µl of the mixed culture after overnight incubation was spread on the selection plates with the help of the L-rod. The plates were incubated overnight at 37°C. The plates were then observed for the presence of the transconjugants, which were then biochemically identified. The transconjugants were then tested for their antibiotic sensitivity pattern by the disc diffusion technique.**

VI. Minimum inhibitory concentration determination

MIC of Ceftazidime and Cefepime was determined for ESBL and AmpC β-lactamase producing Gram negative isolates by an Epsilometer test (E test). A saline

suspension of each isolate was adjusted to a McFarland standard of 0.5 and inoculated over the surface of the Muller Hinton agar plate by using sterile swab to produce an even inoculum. The Himedia Hicomb MIC strip was applied on the agar surface using sterile forcep. Then the plates were incubated for 18 hrs at 35°C and the MIC was the point where the elliptical zone of growth inhibition intersected the MIC scale on the E test strip. The concentration range of antimicrobial on the E test strip corresponds to two fold dilutions in a conventional MIC method.

VII. C reactive protein estimation

C reactive protein level was determined qualitatively and semi quantitatively by using CRP latex kit (Mediclone biotech pvt ltd)

Principle

When a serum positive for C reactive protein is mixed with a latex reagent (specially selected polystyrene latex particles are coated with monospecific goat anti human CRP antibodies), a positive reaction is indicated by a distinctly visible agglutination of the latex particles in the test cell of the slide used. In specimen negative for C reactive protein the latex remains in a smooth suspension form, in the test cell.

Procedure

One drop of test serum, positive control serum and negative control serum were placed on the separate cells of the slide. Then CRP latex reagent was added to all cells. Mixed and spread the fluid over the entire area of the

particular cell with separate mixing sticks. The slide was tilted back and forth for 2 minutes. CRP content of more than 6 mg/litre in undiluted serum specimen showed distinct agglutination. Sera with positive results in the screening were tested in the titration test (semi quantitative analysis).

Semi quantitative analysis

0.9% saline solution was prepared. One drop of saline was placed on the separate cells of the slide. One drop of positive serum was added to the saline in the first cell. Mixed and took one drop from the first cell and added it into second cell. The same was done in subsequent cells. Finally one drop was discarded from the last cell. Then CRP latex reagent was added to all cells. Mixed and spread the fluid over the entire area of the particular cell with separate mixing sticks. The slide was tilted back and forth for 2 minutes. Last dilution which showed distinct agglutination was noted.

Results were interpreted as per the following table present in the leaflet attached with a kit.

Dilution	CRP (mg/litre)
(1:2)	12
(1:4)	24
(1:8)	48
(1:16)	96
(1:32)	192

RESULTS

During the study period, 273 neonates with signs and symptoms of septicemia admitted at Neonatal Intensive Care Unit, Coimbatore Medical College & Hospital were investigated with Blood culture & sensitivity and CRP assay. The Resistant Bacterial isolates were analyzed for ESBL and plasmid mediated AmpC β-lactamase enzyme production by conventional Microbiological methods.

The Male:Female Neonates ratio of this study was 1.1:1. Out of 273 neonates, 173 (63 %) were less than 7 days old (EOS) and 100(37 %) were in between 7-28 days after birth. (LOS) (Ref: Table 1).

The Blood culture isolation rate was only 33% (n=91). Out of this, culture positivity was more in EOS cases 60 %(n=55) than the LOS cases 40 %(n=36) (Ref: Table 2).

Gram negative bacterial isolates constituted 64 %(n=59) of the total bacterial isolates whereas Gram positive bacterial isolates constituted 36 %(n =32) (Ref: Table 4).

Klebsiella pneumoniae (40%) was the most common organism among the GNB isolates followed by Escherichia coli (10%), Enterobacter spp (7%), pseudomonas aeuriginosa (5%), and Acinetobacter spp (2%).In case of GPC isolates, Staphylococcus aureus constituted (19%), followed by CONS (13%) and streptococci sp (2%).

The most common organism isolated from both early and late onset septicemia was Klebsiella pneumoniae followed by Staph. aureus. (Ref: Table 5).

The serum sample from all the suspected neonatal septicemic cases were tested for CRP using Latex agglutination test. It was found to be positive in 131 cases, of which only 64 cases showed culture positivity. The sensitivity of CRP was 49% and specificity was 80%. Negative predictive value and positive predictive value were 63% and 70% respectively (Ref Table 3).

The antibiotic susceptibility testing for Gram positive organisms revealed 100% sensitivity to Vancomycin, followed by 82% to Amikacin & Cefepime. (Ref: Table 6).

Gram negative organisms were 100% sensitive to Imepenam followed by 96% to Cefoperazone - sulbactum, 83% to Cefepime and 61% to Amikacin (Ref: Table 6).

Out of 59 Gram negative isolates tested, 31 (53 %) were found to be resistant to third generation Cephalosporins (Cefotaxime, Ceftazidime,& Ceftriaxone). Third generation Cephalosporins resistance was more common in Klebsiella pneumoniae (24) followed by E. coli (5), Pseudomonas aeruginosa (1) and Enterobacter sp (1) (Ref: Table 7).

Gram negative isolates (n=31) resistant to third Generation Cephalosporins were screened for ESBL and Amp C β lactamase production by Novel Predictor Disk Placement method. In screening test, 12 Klebsiella isolates, 3 Escherichia coli isolates and 1 Pseudomonas aeuriginosa were positive for ESBL production and 12 Klebsiella isolates, 2 Escherichia coli isolates and 1 Enterobacter sp were positive for AmpC β-lactamase production. (Ref: Table 7).

ESBLs production among 16 GNB isolates were further confirmed by Disk Potentiation method using Ceftazidime (30 µg) disk and a Ceftazidime

plus Clavulanic acid (Ca 30 µg + Caz 10 µg) as per CLSI guidelines and the results were found to be similar to ESBL screening test.

The 15 GNB isolates showed Amp C β-lactamase production in screening test was further tested for confirmation by the Modified Three Dimensional Extract Method and AmpC Disk method.

It was observed 9 (25 %) Klebsiella isolates and 2 (22 %) Escherichia coli isolates were positive for AmpC β-lactamase production, but Enterobacter sp failed to produce Amp C β-lactamase in confirmatory test.

The AmpC β- lactamase producing 9 Klebsiella isolates and 2 Escherichia coli isolates were subjected to Conjugation assay to demonstrate whether the Amp C β- lactamase is plasmid mediated one by using Escherichia coli **K12** J53 Azi^r (Recipient strain) as per the procedure described by Abigail et al with few modifications. The results showed transfer of Cefoxitin resistance (marker of Amp C) to the recipient strain from all the 9 AmpC β-lactamase producing Klebsiella pneumoniae strains. In case of Escherichia coli, only one strain showed the transfer of Cefoxitin resistance in the Conjugation assay. (Ref: Table 8).

ESBL and AmpC β-lactamase producing Gram negative isolates were further subjected to MIC determination by Ceftazidime and Cefepime Epsilometer test strip. MIC of Ceftazidime was ≥32 µg /ml for both ESBL and AmpC β-lactamase producing Gram negative isolates.MIC of Cefepime was ≥4µg /ml for ESBL producing Gram negative isolates and MIC of Cefefime was ≤1µg for AmpC β-lactamase producing Gram negative isolates.

Table -1:
Age and Sex wise distribution of septicemic neonates

Characteristics	Male	Female	Total
< 7 days old EOS	95(55%)	78(45%)	173(63%)
7-28 days old LOS	63(63%)	37(37%)	100(37%)
Total	158(58%)	115(42%)	273 (100%)

Table -2:
Comparison of Blood culture positivity & time of onset of septicemia

Culture	EOS	LOS
Culture Positive 91(33%)	55 (60%)	36 (40%)
Culture Negative 182(67%)	118(65%)	64(35%)
Total	173(63%)	100(37%)

Table -3:
Correlation of culture positivity with C Reactive protein level

Culture	CRP positive	CRP negative	Total
Culture positive	64	27	91
Culture negative	67	115	182
Total	131	142	273

Table-4:
Comparison of Gram positive organisms with Gram negative organisms in neonatal septicemia.

Bacterial isolates	No/Percentage
Gram positive	59 (64%)
Gram negative	32(36%)

Table-5: Pathogens isolated in neonatal septicemia.

Name of the organisms	EOS NO (%)	LOS NO (%)	Total NO (%)
Klebsiella pneumoniae	21 (38%)	15(42%)	36 (40%)
Staphylococcus aureus	11 (20%)	6 (16%)	17 (19%)
CONS	7 (13%)	6 (16%)	13 (15%)
Escherichia coli	7 (13%)	2(6%)	9 (10%)
Enterobacter spp	4 (7%)	3 (8%)	7 (7%)
Pseudomonas aeruginosa	3 (5%)	2(6%)	5 (5%)
Streptococci spp	2(4%)	-	2 (2%)
Acinetobacter spp	-	2(6%)	2 (2%)

Table -6:

Antibiotic sensitivity pattern of organisms isolated from neonatal septicemia.

Drugs	Klebsiella	E.coli	Entero bacter	Pseudo monas	Acineto bacter	Staphylo coccus
Gentamycin	14%	33%	28%	20%	50%	-
Amikacin	44%	78%	43%	80%	100%	88%
Cephalexin	33%	44%	72%	80%	100%	76%
Cefotaxime	33%	44%	86%	80%	100%	76%
Ceftazidime	44 %	56%	86%	60%	100%	-
Ceftriaxone	31%	44%	72%	80%	100%	59%
Cefoxitin	67%	78%	86%	80%	100%	59%
Cefepime	67%	66%	100%	80%	100%	88%
Aztreonam	44%	44%	86%	80%	100%	-
Ciprofloxacin	64%	66%	86%	80%	100%	82%
Ofloxacin	72%	56%	86%	80%	100%	82%
Imepenam	1oo%	100%	100%	100%	100%	-
Piperaziline	33%	56%	-	80%	-	76%
Cs+sulbactum	91%	100%	-	-	-	-
Ampicillin	-	-	-	-	-	23%
Cloxacillin	-	-	-	-	-	23%
Amox-Cal	67%	78%	86%	80%	100%	59%
Erythromycin	-	-	-	-	-	59%
Azithromycin	-	-	-	-	-	88%
Oxacillin	-	-	-	-	-	59%
Vancomycin	-	-	-	-	-	100%

Table -7:
Screening for ESBL and AmpC β-lactamase production

Bacterial isolates	No of 3rd Gen Cep resistance isolates screened	No of ESBL positive isolates	No of Amp C β lactamase positive isolates
K. pneumonia (36)	24(67%)	12(33%)	12(33%)
E. coli 9	5(55%)	3(33%)	2(22%)
Enterobacter 7	1(14%)		1(14%)
P. aeruginosa 5	1(14%)	1(20%)	
Total 59	31(53%)	16(27%)	15(25%)

Table -8:
Detection of AmpC β-lactamase production

Bacterial isolates	Screening test positive	Modified Three D ‘imensional test	Amp C disk test	Conjugation assay
Klebsiella pneumoniae (36)	12(33%)	9 (25%)	9(25%)	9(25%)
Escherichia coli (9)	2 (22%)	2 (22%)	2(22%)	1 (11%)
Enterobacter (7)	1 (14%)	-	-	-

DISCUSSION

Septicemia is still a major cause of mortality and morbidity in the neonates. Nowadays Gram negative microorganisms are increasingly reported as the major cause of neonatal septicemia particularly in Asian countries. The emergence of multidrug resistant Gram-negative bacteria is mainly due to inadvertent use of broad-spectrum antibiotics.

Two seventy three suspected septicemic neonates were investigated for septicemia. Among which 91 were culture positive. The positivity rate of Blood cultures was 33%.This culture positivity rate (33%) is comparable to that reported by E Malakan Rad and N Momtazmanesh et al (2004)[33] (30%), Mahabatra et al (2002)[1] (40%) and by Bhattacharjee et al (2008)[58] (48%).In contrast Shahanam ghelbi et al (2008)[34] reported 11% and Ziba Mosayebi et al (2003)[20] reported 13% of culture positivity rate: this discrepancy might have arisen due to the administration of antibiotics before blood collection either to the mother or to the baby or the possibility of infection with viruses, funguses or anaerobes .

In the present study, early onset septicemia was observed in 61% and late onset septicemia in 39% of the neonates. Early onset septicemia was more common than late onset septicemia which is compatible with the reports from Choudry habibur rasul et al (2007)[2] (70.7%-EOS, 30% -LOS), A.H.Movahedion et al (2006)[32] (77.5 % -EOS, 22.5 %-LOS) and Vinod kumar et al (2008)[17](55%-EOS, 47%-LOS).

Contrasting results were published by kuruvilla et al (1997)[5] who reported that late onset septicemia was more common than early onset septicemia. Shaw et al (2007)[22] and Ziba Mosayebi et al (2003)[20] also reported that late onset septicemia was more common than early onset septicemia. Kuruvilla et al's & Shaw's et al study group comprised of babies delivered at their Institution and treated with prophylactic antibiotics. All babies referred to our neonatal center with suspected septicemia were included in this study which explains the high incidence of early onset septicemia.

In the present study sensitivity of CRP was 49%. Specificity was 80%. Negative predictive value and positive predictive value were 63% and 70% respectively. Similar results were obtained in a study by Garland Suzane et al (2003)[30].In contrast Khassawneh et al (2007)[31], reported that CRP had a sensitivity of (95%) and negative predictive value of (98%) in screening neonatal septicemia[31].Intriguingly Nuntnar,et al reported that CRP had a sensitivity of 100% ,specificity of 94% ,PPV and NPV of 91.6% and 100% respectively. This difference could be due to the fact that these studies measured CRP quantitatively with different cut off points and different times from the onset of the signs of infection. However, it can be emphasized that a single CRP level measured at the onset of infection lacks sufficient sensitivity to be useful in identifying neonate with septicemia [56]. In addition CRP cannot be recommended as a sole indicator of neonatal sepsis, but it may be used as part of a sepsis workup and in combination with other laboratory tests.

The pathogens most often implicated in neonatal septicemia in developing countries differ from those seen in developed countries. In developed countries GBS and CONS are the more common etiological agents of neonatal septicemia. But in developing countries Gram negative organisms are more common and are mainly represented by Klebsiella pneumoniae followed by Escherichia coli and pseudomonas aeuriginosa. It is not known whether these differences reflect the true differences in pathogens across the world, reflecting the epidemiological transition in some countries or whether it reflects an epidemiological bias.

In the present study, Gram negative organisms constituted 64% of the total bacterial isolates causing neonatal septicemia whereas Gram positive organisms constituted 36%. This distribution pattern correlates well with the results published by Nalini Agnihotri et al (2004)[90] from Chandigarh who reported that Gram-negative organisms accounted for 59% of all positive cultures. Another study conducted by Mahapatra et al (2002)[1] in Orissa also reported that Gram-negative bacilli were isolated in maximum percentage (88.45%) of cases whereas gram-positive bacteria in 11.6% of culture. In contrast Shahsanam et al (2008)[34] and Reza et al (2007)[54] from Iran reported that Gram positive organisms constituted the major group of isolates (62.1%), 68% respectively.

In the present study, among the Gram negative organisms Klebsiella pneumoniae (40%) was the most common organism followed by Escherichia coli (10%), Enterobacter spp (7%), pseudomonas aeuriginosa (5%), and Acinetobacter spp (2%).Among the Gram positive organisms Staphylococcus

aureus constituted (19%), followed by CONS (13%) and Streptococci sp (2%). This distribution pattern correlates well with the results published by Vinodkumar CS et al (2008)[17] who reported that Klebsiella (26.9%) was the most common infective organism followed by Staphylococcus aureus (20%), CONS (13.4%), E.coli(10.5%) and Acinetobacter spp (6.1%). Same distribution pattern was also observed by kuruvilla et al (1997)[5]. In contrast Shaw et al (2007)[22] reported that Staphylococcus aureus was the most common organism isolated from neonatal septicemia. Malakan rod et al (2004)[33] also reported that pseudomonas aeruginosa as the most common causative organism of neonatal septicemia.

Neonatal surveillance in developed countries generally identifies GBS and E coli as the dominant early onset septicemia pathogens and CONS the dominant late onset septicemia pathogen followed by GBS and Staphylococcus aureus. In developing countries E.coli, GBS, Enterobacter, Enterococcus and Listeria are mostly associated with early onset septicemia. Klebsiella, Acinetobacter, Staphylococcus aureus and CONS are associated both with early onset septicemia & late onset septicemia. Pseudomonas aeruginosa, Salmonella and Serratia are more often associated with late onset septicemia. There appears to be a wide variety of bacteria causing early onset septicemia and late onset septicemia in developing countries. This variation may be true, but important confounders may include different definitions of early onset septicemia and late onset septicemia, different inclusion criteria for studies, inability to culture certain organism, small numbers and short period of surveillance.

In the present study, Klebsiella pneumoniae was the most common organism in early and late onset septicemia. Mahapatra et al (2002)[1] reported

that there was no significant difference in bacteriology between early onset septicemia and late onset septicemia, which was also reported by Nalini Agnihorti et al (2004)[90].

Majority of the Gram negative and Gram positive organisms isolated in the present study were resistant to one or more antibiotics. This is in concurrence with other studies by Tallur et al (2000)[4] and kuruvilla et al (1998)[5]. Present study revealed a very high degree of resistance of Gram negative bacilli not only to commonly used antibiotics, but also predominantly to broad spectrum Cephalosporins. These findings were compatible with other studies by Joshi et al (2000)[60] and A.H Movahedian et al (2006)[32]. This is probably due to emergence of new variant of the existing strain as a result of mutations or may be plasmid borne.

In the present study, Gram positive organisms were 100% sensitive to Vancomycin and Gram negative organisms were 100% sensitive to Imepenam. Similar findings were observed by Shaw C.K et al (2007)[22].

. In the present study, resistance rate of Klebsiella pneumoniae, E.coli, pseudomonas and Enterobacter to third generation Cephalosporins were 67%, 56%, 20% and 14%. respectively. Acinetobacter spp were 100% sensitive to third generation Cephalosporins..These findings were similar to the study by Ziba mosayebi et al (2003)[20] reported that resistance rate of Klebsiella pneumoniae, E.coli, Enterobacter to third generation Cephalosporins were 60%,30% and,20%.Pseudomonas and Acinetobacter were 100% sensitive to third generation Cephalosporins.

Amongst the mechanisms of resistance to third generation Cephalosporins, production of ESBLs and AmpC β-lactamases are the most common. AmpC β-lactamases are clinically important because they confer resistance to narrow expanded and broad spectrum Cephalosporins, β lactam β lactamase inhibitor combinations and Aztreonam[10, 11, 12].

In the present study, Gram negative isolates resistant to third generation Cephalosporins were screened for ESBL production and AmpC β-lactamase production by Novel Predictor Disk Placement method.

ESBL production was further confirmed by Disk potentiation test method as per CLSI guidelines. In the present study, ESBL production was observed in 33% of Klebsiella isolates, 33% of Escherichia coli isolates and 20% of Pseudomonas isolates. These findings were lower than the results published by Bhattacharjee et al (2008)[54] (ESBL production by Klebsiella. pneumoniae 62.7%, E. coli 46.5%), B.V.Krishna et al (2007)[9] (ESBL production by Klebsiella pneumoniae 71.93 %) and Amita jain et al (2003)[6] (ESBL production by Klebsiella sp 86.6%, E. coli 63.6%) but higher than the result published by Vinod Kumar et al (2004) (13.4 %) [89].

Currently, CLSI documents do not indicate the screening and confirmatory tests that are optimal for detection of AmpC β-lactamases. However, several studies have been done on various test methods namely, the Three Dimensional test, Modified Double Disk test, AmpC disk test, Inhibitor based method[71] employing inhibitors like boronic acids[88], Broth micro dilution method and Cefoxitin Agar method[82]. In spite of many phenotypic tests, isoelectric focusing and genotypic characterization by various molecular methods are

considered gold standard as the results with the phenotypic tests can be ambiguous and unreliable[40].

However these techniques are expensive and the requiring reagents are costly and not easily available. Hence simple, reliable and inexpensive phenotypic methods like Modified three dimensional test method and Amp C disk test method were used in this study. Singhal et al (2005)[66]and Parul Sinha et al (2008) [86] also reported that Amp C disk test was easier, reliable and rapid method of detection of isolates that harboring AmpC β-lactamases[65].

AmpC β-lactamases production in screen positive isolates was further confirmed by the modified three dimensional extract method and Amp C disc method. Isolates harboring AmpC β-lactamases were tested for plasmid mediation by conjugation assay.

In present study, 9 (25 %) Klebsiella isolates and 2 (22 %) Escherichia coli isolates were positive for AmpC β-lactamase production. In K. pneumonia AmpC β-lactamase production is only plasmid mediated but in Esch. Coli hyper production of chromosomal mediated AmpC and plasmid mediated. Transfer of Cefoxitin resistance to recipient strain was observed in all AmpC producing Klebsiella isolates and 1 of 2 AmpC producing Escherichia coli isolate.

Cefoxitin resistance in non AmpC producing Klebsiella pneumoniae is often due to porin deficient mutants. The interruption of a porin gene by insertion sequences is a common type of mutation that causes the loss of porin expression and increased Cefoxitin resistance in Klebsiella. pneumonia[73]. In Esch. Coli hyper production of chromosomal AmpC with OMP F porin loss can produce similar resistance phenotype[45].

In the present study, Plasmid mediated AmpC β-lactamase production was observed in 25% of Klebsiella isolates and 11% of Escherichia coli isolates. Similar findings were observed by A. Subha et al (2003)[44] from Chennai reported that, twenty eight isolates (24.1%) of Klebsiella spp. and 12 (37.5%) of Esch. Coli were plasmid mediated AmpC β- lactamase producers. Manchanda & Singh et al from Delhi also reported 20.7 per cent of the clinical isolates were harboring AmpC β-lactamases[39.] In contrast A.K. Ratna et al (2003)[45] from Karnataka reported sixteen (3.3%) isolates were positive for plasmid mediated AmpC β-lactamases. Based on the species 9 (3.3%) Escherichia coli, 4 (2.2%) K.pneumoniae, 2 (5%) Citrobacter freundii and 1 (5.5%) isolate of Enterobacter aerogenes harbored Amp C enzymes.

In the present study MIC of Ceftazidime was≥32 µg /ml for ESBL and AmpC β-lactamase producing Gram negative isolates.MIC of Cefepime was ≥4µg /ml for ESBL producing Gram negative isolates and MIC of Cefefime was ≤1µg for AmpC β-lactamase producing Gram negative isolates. Similar results were obtained with the study by Hyunjoo Pai et al (2004)[81] and Jing-Jou Yan et al (2002)[70].

SUMMARY

- Two seventy three suspected septicemic neonates admitted in NICU, Coimbatore medical college, Coimbatore were studied over a period of 14 months from May 2008 to June 2009.
- One fifty eight male babies and one fifteen female babies were in the study.
- Blood Culture positivity rate was 33%
- Early onset septicemia (61%) was more common than late onset septicemia (39%).
- Comparing with culture positivity, sensitivity of CRP was 67% and specificity was 60% in diagnosis of neonatal septicemia.
- Gram negative organisms constituted 64% of the total bacterial isolates. Klebsiella pneumoniae (40%) was the commonest organism isolated. This was followed by Staphylococcus aureus (19%), CONS (13%), Escherichia coli (10%), Enterobacter spp (7%), Pseudomonas aeuriginosa (5%), Aceinetobacter (2%) and streptococci (2%).
- Klebsiella pneumonia was the most common organism in early and late onset septicemia.
- All Gram positive cocci were 100% sensitivity to Vancomycin
- All Gram negative bacilli were 100% sensitivity to Imepenam followed by Cefoperazone - sulbactum 96%, Cefepime 83% and Amikacin 61%.

- Gram negative isolates (53%) resistant to third generation Cephalosporins were screened for ESBL production and AmpC β-lactamase production by Novel Predictor Disk Placement Method.
- The confirmation of ESBL production was done by Double Disc Potentiation Test.
- 33% of Klebsiella isolates, 33% of Escherichia coli isolates and 20% of Pseudomonas isolates were ESBL producing strains.
- The detection of plasmid mediated AmpC β-lactamase production was done by Modified Three Dimensional Test and Amp C Disk Test.
- Plasmid mediation in AmpC β-lactamase production was confirmed by Conjugation assay.
- 25% of Klebsiella pneumoniae isolates and 11% of Escherichia coli isolates were plasmid mediated AmpC β-lactamase producing strains.

CONCLUSION

The study of bacteriological profile of neonatal septicemia revealed early onset septicemia as more common than late onset septicemia. Klebsiella pneumoniae was the most common bacterial isolate followed by Staphylococcus aureus, CONS, Escherichia coli, Enterobacter spp, Pseudomonas aeuriginosa, Acinetobacter spp and Streptococci spp in causing neonatal septicemia. Single CRP level measured at the onset of infection lacked sufficient sensitivity to beuseful in identifying neonatal septicemia. Antimicrobial susceptibility revealed Imepenam as the most effective antibiotic followed by Cefoperazone + Sulbactum, Cefepime and Amikacin against Gram negative isolates. Among the Gram negative isolates of the neonatal septicemia 27% were ESBL producers and 22% were plasmid mediated AmpC β-lactamase producers. Failure to distinguish AmpC β-lactamase producers from ESBL producers will lead to inappropriate antimicrobial treatment and may result in increased mortality. The ability to detect and distinguish between AmpC and ESBL producing organisms has therapeutic importance as well as epidemiological significance.

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APPENDIX-I:

Gram staining

Prepare a smear by placing 1 to 2 drops of sediment on a clean grease free glass slide

Air dry and heat fix the slide

Cover the slide with methyl violet solution and allow to act for about 1 minute

Wash with clean water

Then cover the smear with freshly prepared Grams iodine solution and wait for 1 minute

Then wash the smear with clean water

Decolorize with 100% acetone for 2- 3 seconds

Wash the slide immediately

Apply counter stain dilute carbol fuschin for 30 seconds

Wash with water and blot dry

The stained smear was examined microscopically under oil immersion field

APPENDIX- II:

Catalase test

Principle

The test is performed to determine the presence of catalase enzyme in an organism.

Method

Pure growth of the organism was transferred from the agar to a clean slide with a glass rod. A drop of 3% hydrogen peroxide was added to the growth.

Interpretation

Evolution of bubbles from the colony was considered as a positive test.

Oxidase test

Principle

The test is performed to determine the presence of enzyme oxidase in an organism.

Reagent

Tetra methyl para phenylene diamine dihydrochloride 1% aqueous solution.

Method

A small portion of colony was removed with wooden stick and rubbed over the filter paper soaked with oxidase reagent

Interpretation

Positive oxidase test was indicated by developmentof a purple color.

Slide Coagulase test

Principle

The test is performed to determine the ability of an organism to clot plasma by the action of the enzyme coagulase, and is used to differentiate between Staphylococcus aureus and CONS. This test detects bound coagulase.

Method

A single colony was emulsified in a drop of saline on a clean glass slide. Similar suspensions were made for positive and negative control stains to confirm the proper reactivity of plasma.Traces of plasma was added to milky suspensions of test and control strains.

Interpretation

The test was read as positive if visible clumping occurs within 10 seconds and read as negative if clumping was absent.

Indole test

Principle

The test is performed to determine the ability of an organism to split indole from the tryptophan molecule Indole reacts with aldehydes to produce a red color

Medium -Peptone water

Method

Pure single colony was inoculated into peptone water and incubated at 37°c for 18-24 hrs 0.2 ml of Kovacs reagent paradimethyl aminobenzaldehyde was added to culture broth.

Interpretation

A positive reaction was indicated by the formation of pink ring at the junction.

Citrate utilization test

Principle

The test is performed to determine the ability of the microorganisms utilize citrate as sole carbon source and grow. The usage of citrate can be tested for alkali production by using a suitable PH indicator.

Medium

Simmons citrate medium: Magnesium sulfate 0.2gram,Ammonium dihydrogen phosphate 1 gram,Dipottasium phosphate 1 gram, Sodium citrate 2 gram, Sodium chloride 5 gram, Agar 20 gram, Bromothymol blue 0.08 gram, Distilled water 1000 ml.

Method

A drop of 4-6 hrs growth of bacterium in broth was inoculated on the Simmons citrate agar slant and incubated at 37^0c for 18-24 hrs.

Interpretation

The test was read as positive reaction when the indicator turned blue and negative reaction when no change in color occurred.

Methyl red test

Principle

To test the ability of the organism to produce and maintain stable acid end products from glucose fermentation.

Method

Test organism was inoculated into glucose phosphate broth. Then the broth was incubated at 37^0c for 48 hrs.

Then O.2 ml of methyl red reagent was added to the broth.

Interpretation

Reddening of the supernatant was considered as positive.

Urease test

Principle

The test is performed to determine the ability of microorganisms to produce the enzyme urease.The occurrence of this enzyme can be tested for by growing the organism in the presence of urea and subsequently testing for alkali (NH3) production by using a suitable pH indicator.

Medium

Christensen's urease medium: Peptone 1.0 gram, Glucose(o.1%) 1.0 gram, Sodium chloride 5.0 grams, Mono potassium phosphate 2.0 grams, Phenol red 0.012 gram,agar 15-20 grams, distilled water1000 ml

Method

A drop of 4-6 hours old bacterial broth culture was inoculated over the entire slope surface. Then the medium was incubated at 37 ^{0} c for 18 to 24 hours.

Interpretation

The test was read as positive reaction when the indicator turned purple – pink, and negative reaction when no change in color occurred.

Voges –Proskauer test:

Principle

The test is performed to determine the ability of the micro organisms to ferment glucose with the production of acetyl methyl carbinol (acetoin).

Medium- Glucose phosphate broth

Method

Test organism was inoculated into glucose phosphate broth. Then the broth was incubated at 37^0c for 48 hrs. Subsequently 0.2ml of VP reagent A (5% alpha naphthol) and 0.2 ml of VP reagent B (40% KOH) were added and the contents were shaken vigorously for 30 seconds.

Interpretation

Reddening of the supernatant within 5-10 minutes due to acetoin production was read as positive reaction. A negative reaction was indicated by the development of yellow color.

Carbohydrate utilization test

Principle

The test is used to determine the ability of an organism to ferment a specific carbohydrate which is incorporated in a basal medium, and to produce acid or acid with visible gas. Sugars tested included glucose, lactose, sucrose, maltose, mannitol, trehalose, xylose, arabinose, sorbitol and starch.

Medium

Sugar 1.0 gram, Nutrient broth base 100 ml, Bromothymol blue indicator 1.2 ml

Method

Test organism was inoculated into each sugar medium and subsequently incubated at 37^0c for 24 hrs

Interpretation

A positive test was shown by yellow coloration of the medium due to acid production:

no color change, due to inability of the microorganisms to ferment sugars, was read as negative reaction.

Triple sugar iron agar test

Principle

The test is performed to determine the ability of an organism to ferment a specific carbohydrate incorporated in a basal medium, with or without the production of gas along with the determination of possible hydrogen sulfide production

Method

Test organism was inoculated on TSI agar by stab the butt and streak the slant with straight wire and incubated subsequently for 24 hours.

Interpretation

Glucose fermentation was shown by a yellow butt and red slant .Glucose, sucrose and lactose fermentation were shown by a yellow butt and slant. Gas production was indicated by Bubbles in the medium. Hydrogen sulfide production was identified by presence of black precipitate.

APPENDIX-III:

Antibiotics	Disc content µg	Sensitive mm ≥	Intermediate mm	Resistant mm≤
Gentamycin	10	15	13-14	12
Amikacin	30	17	15-16	14
Amox-cal	20/10	18	14-17	13
Cephalexin	30	18		14
Cefotaxime	30	23	15-22	14
Ceftazidime	30	18	15-17	14
Ceftriaxone	30	21	14-20	13
Cefoxitin	30	18	15-17	14
Aztreonam	30	22	16-21	15
Cefepime	30	18	15-17	14
Ciprofloxacin	5	21	16-20	15
Ofloxacin	5	16	13-15	12
Imepenam	10	16	14-15	13

Gram negative Isolates were tested for following antimicrobial antibiotic discs and the zone size interpretative chart as comparable with CLSI standards

Gram positive isolates were tested for following antibiotic discs and the zone size interpretative chart as comparable with CLSI standards

antibiotics	Disc content mcg	Sensitive mm ≥	Intermediate mm	Resistant mm ≤
ampicillin	10	29		28
Cloxacillin	5	18		17
Amox-cal	20/10	20		19
Cephalexin	30	18		14
Cefotaxime	30	23	15-22	14
Cefoxitin	30	18	15-17	14
Ceftriaxone	30	21	14-20	13
Cefepime	30	18	15-17	14
Erythromycin	15	23	14-22	13
Azithromycin	15	18	14-17	13
Amikacin	30	17	15-16	14
Ciprofloxacin	5	21	16-20	15
Ofloxacin	5	18	15-17	14
Oxacillin	1	13	11-12	10
Vancomycin	30			15-17

PROFORMA

Name
Age <7 days
>7days<28 days
Sex F/M
Parent/guardian
Address
Birth weight
Term
Nature of Delivery
Present history
H/O Poor **cry, Poor feeding, Irritability, Fever, Vomiting**
H/O convulsions
H/O Antibiotic use
Examination of newborn

Cry	**HR**
Activity	**RR**
Color	**SPO2**
Tone	
Posture	
Temperature	

RDS SCORING
RR, Cyanosis, Retraction, Grunting & Airway

Systemic examination

CVS	**SIS2 heard**
	No murmur
RS	**Normal vesicular birth sound heard**
	No added sound
Abdomen	**Soft, mild distension**
	Spleen not palpable
CNS	**Tone**
	Reflexes

Investigations
Haemogram
CRP
Blood culture &Sensitivity