ANTIBACTERIAL RESISTANCE PROFILE OF *Streptococcus* spp. IN NEONATAL BLOOD SAMPLES FROM NEONATAL INTENSIVE CARE UNIT, EKITI STATE UNIVERSITY TEACHING HOSPITAL, EKITI STATE.

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CERTIFICATION

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DEDICATION

I dedicate this project to the ever loving God whose love is vividly radiant upon my life.

ACKNOWLEDGEMENT

I appreciate the ever faithful God for His sufficiency check in the face of lack and the instructor on the brink of vacillating. It would be an ungrateful act if I fail to acknowledge Mr and Mrs Fafiyebi for their imminent support from every angle in making me a useful tool for the reshaping of my immediate environment and Nigeria at large. It is no doubt a reality coming into play in due course.

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TABLE OF CONTENTS

LIST OF TABLES

ABSRACT

Neonatal sepsis is a systemic infection of neonates which causes high mortality rates in newborns. It is classified into two stages which is the early and late onset characterized by clinical features that varies and nonspecific making blood culture the gold standard of diagnosing neonatal sepsis. *Streptoccoccus* spp. is one of the major causative organisms of neonatal sepsis. The increasing resistance of causative organisms of neonatal sepsis has been a major challenge in treating neonates. The aim of this study is to isolate and characterize *Streptococcus* spp. from neonatal blood sample and ascertain their antibiotic resistance pattern. Following standard microbiological methods, 24 blood samples were obtained from Ekiti State Teaching Hospital, Ekiti State. The blood samples were transported in culture bottles in ice packs to the laboratory. Inoculum were standardized using 0.5McFarland tuprbidity standards. *Streptococcus* spp associated with neonatal sepsis blood samples were isolated and identified by phenotypic and biochemical tests. The antibiotic susceptibility pattern of isolates confirmed to be *Streptococcus agalactiae* (6) *and Streptoccous pyogenes* (8) strains were determined using the Kirby-Bauer disc diffusion method using Gram positive antibiotic disc. The *Streptoccocus agalactiae* strains showed 66.6% (4) resistance and 33.3% (2) susceptibility to Ceftazidime and varying resistance and susceptibility to other antibiotics. *Streptoccoccus pyogenes* showed 100% (8) and 0% (0) susceptibility to Ceftazidime while it has varying degree of resistance to other antibiotics. The results obtained from this study indicated that *Streptococcus agalactiae and Streptococcus pyogenes* are one of the causative organisms causing neonatal sepsis. In conclusion, the *Streptococcus* spp. strains from this study are of public health significance and their varying susceptibility and resistance shows an alarming development.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Globally, about forty percent of under-five deaths occur in the neonatal period resulting in 2.9 million newborn deaths each year (Kalathia *et al.*, 2013). The highest mortality rates for newborns are found in the poorest countries and a third of these deaths are attributed to infections acquired by the baby during labour and delivery or after birth (Kalathia *et al.*, 2013). Nigeria accounts for the highest number of neonatal deaths in Africa and third in the world (after India and China) with sepsis responsible for about 30% to 50% of deaths (Ahman *et al.*, 2007). In Nigeria, the prevalence of neonatal sepsis reported from previous hospital- based studies ranges between 7.04 and 22.9 per 1000 live births (Ogundare *et al.*, 2016). Also, mortality rates from neonatal sepsis have ranged from 26.7% in Abakaliki to 32.2% in Sagamu and 33.3% in Ile-Ife, over the last two decades (Ogunlesi and Ogunfowora, 2010).

The term neonatal sepsis refers to systemic infection of neonates including septicaemia, pneumonia, meningitis, arthritis, osteomyelitis, and urinary tract infection (Shalini and Malik, 2010). It is a clinical syndrome characterised by systemic signs of circulatory compromise (e.g., poor peripheral perfusion, pallor, hypotonia, poor responsiveness) (Edmund and Zaidi, 2010). Neonatal sepsis could be from early onset, within the first 72 h of life, and presumed to be acquired through prenatal and intrapartum maternal transmission, or late onset from the fourth day to fourth week of life (Shalini and Malik, 2010). Sepsis-related case fatality rates are largely preventable with proper antimicrobial use and aggressive supportive care. However, neonatal

sepsis has no pathogenomonic features and the clinical presentation varies as well (Shalini and Malik, 2010). Recorded clinical features include fever, hypothermia, lethargy, bulging fontanelle, irritability, vomiting, jaundice, convulsion, respiratory distress, abdominal distension seizures, apnoea and failure to thrive for the first 28 days of life. The clinical signs and symptoms associated with neonatal infections are non-specific because of an over lapped disease that comes with infections occurred by other diseases, and hence prior detection and treatment becomes crucial for the better neonatal outcomes (West and Tabansi, 2014). Poor or delayed laboratory services also make laboratory diagnosis difficult in poor resource settings. As a result, neonatal healthcare providers in resource limited settings make tentative diagnosis and empirical treatment of neonatal sepsis especially using the new neonatal WHO International Management guidelines (Edmund and Zaidi, 2010). However, the diversity of organism causing neonatal sepsis varies significantly across different regions and changes over time, even in the same place. This variation may affect the success of empirical management (Edmund and Zaidi, 2010). Combination of clinical signs, non-specific laboratory tests and microbiological confirmation by the detection of bacteria in blood culture is relevant for clinically diagnosing Neonatal sepsis (Marchant *et al.,* 2013). The gold standard for diagnosis of septicaemia is blood culture. Sick newborn show nonspecific signs and symptoms which makes diagnosis and treatment of neonates difficult in many developing countries (Lawn *et al.*, 2005). In developed countries, the most common causes of neonatal sepsis are Group B *Streptococci* (GBS), *Escherichia coli* and *Listeria monocytogenes* while Gram negative bacteria and coagulase-negative staphylococci are the most common in developing countries (Palazzi *et al.*, 2006). It is noteworthy that the growing incidence of drug resistant bacterial isolates has also made treatment more difficult and costly (Roy *et al.*, 2002). Many scientists and healthcare providers, as well as policymakers, believe

that the resistance levels of microbes to antibiotics has now put patients in danger. Particularly, Gram-negative rods such as *Escherichia coli* have become resistant to almost all current antibiotics. Recent reports have shown that antibiotic resistance is a global threat (Kumarasamy *et al.*, 2010). Inappropriate and unnecessary use of antibiotics has only increased the rate of antibiotic resistance in our communities and around the world. Nearly half of all antibiotic use in human medicine is not appropriately conducted Thus, education about the causes of antibiotic resistance and appropriate use of antibiotic drug use, as well as drug history, is needed. (Sanchez *et al.*, 2010).

It is imperative, therefore, that the epidemiology of neonatal sepsis should be regularly updated to provide information required for regular review of the choice of drugs most suitable for the treatment of neonatal sepsis in different places and at different times (Kumarasamy *et al.*, 2010).

1.2 STATEMENT OF RESEARCH PROBLEM

Neonatal sepsis has been a major problem globally; an occurrence leading to death among neonates resulting from increased drug resistance to commonly used antibiotics by microorganisms implicated in neonatal sepsis. This increased resistance is highly aided by indiscriminate and irrational use of antibiotics, over the counter sale of antibiotics and ineffective infection control in maternity centers.

1.3 AIM AND OBJECTIVES

The aim of this study is to isolate and characterize implicated microorganisms of bacterial origin from neonatal sepsis samples and ascertain their antibiotics resistance pattern.

The specific objectives of this study are:

1. To isolate causative organisms of *Streptococcus* spp. origin from samples of neonates diagnose of neonatal sepsis via the using of selected media.
2. To characterize isolates of *Streptococcus* spp. origin using various biochemical tests
3. To determine the antimicrobial resistance pattern of isolates obtained from the blood sample

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Description of Neonatal Sepsis

Neonatal sepsis is systemic infection of including septicemia, pneumonia, meningitis, arthritis, and urinary tract infection. Sepsis is more common in extramural admissions (Sankar *et al.*, 2008). Neonatal sepsis is more common in developing countries in comparison of developed countries; it is a disseminated disease with the positive blood culture during the first month of life after birth (Vergnano *et al.*, 2005). Neonatal sepsis is an important cause of morbidity and mortality among neonates in developing countries accounting for 30-50% of total deaths each year (Bang *et al.*, 2005). Clinical symptoms of sepsis are caused by the micro-organism and their toxic product. Sepsis is mostly characterized by bacteraemia (Waheed *et al.*, 2003). Neonatal sepsis is an invasive infection occurring in the first twenty eight (28) days of life. It could be bacterial, viral, or fungal. Early signs are frequently nonspecific and do not distinguish among organisms. These signs could be multiple and include diminished spontaneous activity, less vigorous sucking, apnea, bradycardia, temperature instability, respiratory distress, vomiting, diarrhoea, abdominal distention, jitteriness, seizures and jaundice. Increasing number of premature or low birth weight babies are mostly die within the first 14 days of life (Mclntosh and Stenson, 2008)

2.2 Classifications of Neonatal Sepsis

According to the onset of age, neonatal sepsis is divided into early-onset sepsis (EOS) and late-onset sepsis (LOS). EOS reflects transplacental or, more frequently, ascending infections from

the maternal genital tract, whereas LOS is associated with the postnatal nosocomial or community environment, with the peak incidence reported to be between the 10th and 22nd day of life. (Hammoud *et al.*,2011; Boghossian *et al.*, 2013; Tsai *et al.*, 2014.). Since the early 1980s, epidemiological studies have observed a general reduction in EOS, probably due to advances in obstetric care and the use of prophylactic intrapartum antibiotics to prevent infections caused by Group B Streptococcus(Bizzarro *et al.*, 2005; Shim *et al.*, 2011).

Meanwhile, the incidence of LOS has increased in parallel with the improved survival of premature infants, especially in those with very low birth weight (VLBW), indicating the role of hospitalization and life sustaining medical devices in the pathogenesis of neonatal LOS (Bizzarro *et al.*, 2005 ; Shim *et al.*, 2011). The onset of LOS is most frequently defined at 72 h after birth, (Lahra *et al* ., 2009; Van den *et al.*, 2010; Leal *et al.*, 2012; Al-Taiar *et al.*, 2013; Tröger *et al.*, 2014.). Apart from immaturity, other well-recorded risk factors for LOS include the long-term use of invasive interventions, such as mechanical ventilation and intravascular catheterisation, the failure of early enteral feeding with breast milk, a prolonged duration of parenteral nutrition, hospitalisation, surgery and underlying respiratory and cardiovascular diseases(Stoll *et al.*, 2004; Boghossian *et al.*, 2013;Tröger *et al.*, 2014)

2.3 Epidemiology

The worldwide incidence of Neonatal Sepsis ranges from 1 to 10 per 1000 live births (Afsharpaiman *et al.*, 2012). However, the incidence of Neonatal Sepsis varies significantly between countries, with higher rates reported in developing countries (Vergnano *et al*, 2005). In Africa, the incidence of Neonatal Sepsis varies from 6.5 to 23 per 1000 live births (Vergnano *et al*, 2005). The prevalence reported from previous hospital based studies in Nigeria ranges

between 7.04and 22.9 per thousand live births (Adejuyigbe *et al.*, 2001; Mokuolu *et al.*, 2002) In a study at a tertiary hospital in Johannesburg South Africa, the incidence of Neonatal Sepsis was reported as 10.5 cases per 1000 live births (Motara *et al.*, 2005). In another Sub-Saharan referral hospital in Zimbabwe, the incidence was much higher, reported as 21 cases per 1000 live births (Seale *et al.*, 2009). In developed countries such as the United States (USA) and Australia, the incidence of Neonatal Sepsis ranges from 6 to 9 per 1000 live births (Afsharpaiman *et al.*, 2012). The contribution of EOS and LOS cases to the overall incidence of NNS also differs significantly between different neonatal institutions (Tiskumara *et al.*, 2009|) A South African study, showed that LOS occurred more frequently than EOS. In this study, 93.5% cases of NNS were of late onset (Ballot *et al.*, 2012). Other studies in both developing and developed countries also showed that LOS occurred more frequently than EOS (Tiskumara *et al.*, 2009). A one year prospective study of neonatal infections in eight neonatal units in Asia, showed that 90% (453/408) of the cases of Neonatal Sepsis were of late onset (Tiskumara *et al*, 2009).

2.4 Risk Factors for Neonatal Sepsis

Neonates can be regarded as immune compromised individuals (Jodi and Bonneau, 2003). They have a series of defects in their specific and nonspecific immunity, which predisposes them to infection. Their immune dysfunction is characterized amongst others by decreased phagocytic activity of white cells, decreased cytokine production and impaired immunoglobulin production. Physical barriers to infections such as the skin are weak and thin, and may be easily interrupted. Beside their inherent predisposition to sepsis, there are many other pre and intrapartum obstetric complications associated with an increased risk of infection in newborn infants (Shah *et al.*, 2012) The following are some of the factors associated with neonatal sepsis: *a)* preterm birth and

low birth weight, *b)* prolonged rupture of membranes *c)* maternal group B *Streptococcus* (GBS) colonization d*)* invasive procedures. (Stephanie *et al.*, 2012)

2.4.1 Preterm Birth and Low Birth Weight

Preterm delivery and low birth weight are well-established risk factors for neonatal sepsis. (Stephanie *et al.*, 2012) Preterm infants have a 3 to 10 fold higher incidence of infection when compared to full term infants, with sepsis more common amongst the most premature infants. Downey *et al.* (2010) reports from India showed 50-60% of septic babies are premature babies and those with birth weight less than 1500g are more vulnerable (Rasul *et al.*, 2007). In a cohort of infants admitted to 250 neonatal intensive care units (NICU) in the US, there were 374 infections for every 1000 admissions for infants < 750 g birth weight and only 7 infections per 1000 admissions in infants >2500 g birth weight. (Downey *et al.*, 2010) Possible explanations for the increased risk of infections in preterm infants include: *a)* immune dysfunction, *b)* maternal genital tract infection that causes preterm labour, with an increased risk of vertical transmission to the newborn, *c)* the frequency of intrauterine infection is inversely related to gestational age and *d)* the need for invasive procedures such as endotracheal intubation in premature infants. The ability of the newborn infant to resist infection is highly dependent on the fetus' ability to acquire and utilize maternal immunity (Jodi and Bonneau , 2003).

2.4.2 Prolonged Rupture of Membranes

Throughout pregnancy, the fetus is relatively protected from the maternal microbial flora by the chorioamniotic membranes, the placenta and other antibacterial factors in amniotic fluid (Chiesa *et al.*, 2004) Following rupture of membranes, particularly if the rupture is prolonged, bacteria

may ascend and in some cases lead to fetal infection. Prolonged rupture of membranes (PROM) is defined as rupture of membranes prior to the onset of labor for more than 18 hours. It is a major risk factors for chorioamnionitis and neonatal sepsis (Boskabadia *et al.*, 2011) It is associated with a 10-fold increase in the risk of neonatal infection. The aetiology of PROM is multifactorial and has been associated with the following maternal factors: *a)* black race, *b)* cigarette smoking, *c)* low socioeconomic status, *d)* multiparity, *e)* polyhydroamnios and *f)* a history of PROM in previous pregnancies (Boskabadia *et al.*, 2011).

2.4.3 Maternal Group B *Streptococcus* Colonization

In the 1930s GBS was thought to be a normal commensal, in the 1970s invasive Group B streptococcal disease emerged as the leading cause of neonatal morbidity and mortality in the US (Shet and Ferriere, 2004). Maternal colonization with GBS was linked to neonatal morbidity and mortality. Maternal colonization with GBS substantially increases the risk of EOS due to GBS. (Bayer K and Gotoff, 2012) Over the past decades, there has been a significant decline in the incidence of EOS due to GBS.25 A multicenter study in the US showed a decline of 65%; from 1993- 1998 (Schrag *et al.*, 2005). Clinical trials in the mid-1980s already demonstrated that antibiotic prophylaxis during labour to mothers colonized with Group B *streptococci* was highly effective in preventing disease in newborns (Shet and Ferriere, 2004). The decline demonstrated by the US multicenter study; coincided with the active public health and clinical efforts to increase the administration of prophylactic intrapartum antibiotics to mothers at risk of transmitting GBS (Schrag *et al.*, 2005). Other measures to reduce maternal colonization such as vaginal chlorhexidine wipes have been shown not to be effective in preventing vertical transmission GBS and neonatal sepsis (Cutland *et al.*, 2009).

2.4.4 Invasive Procedures

Interventional invasive procedures aimed at providing nutritional or respiratory support for the ailing newborn may provide excellent opportunities for relatively non-virulent pathogens to establish infection and to invade the host. Transient bacteremia may accompany procedures that traumatize the skin and mucosal membranes such as endotracheal intubation and venipuncture. Immunological mechanisms are then activated to eradicate the bacteremia, but if these fail, overt sepsis can occur. The following are some of the procedures that may predispose the newborn infant to sepsis:

Arterial and venous umbilical catheterization

Central venous catheters

Peripheral arterial and venous cannulation

Intubation and assisted ventilation

Bladder catheterization Hyperalimentation (Chiesa *et al.*, 2004).

2.5 Casuative Organisms Causing Neonatal Sepsis

2.5.1 *Staphylococci*

Staphyloccoci are aerobic, gram-positive bacteria that grow in pairs and clusters (McMillan et *al.*, 2006). They are ubiquitous bacteria that colonize and are pathogenic for humans and animals (McMillan *et al.*, 2006). They are resistant to heat and drying, and may be recovered from nonbiologic environment weeks to months after contamination (Kliegman *et al.*, 2007). They are categorized *by* their ability to produce coagulase into coagulase-positive and coagulase-negative *et al Staphylococci* (CoNS) (McMillan *et al.*, 2006; Kliegman *et al.*, 2007). *Staphylococcus aureus* is a coagulase-positive and is a predominant pathogen causin g a variety

of infections (McMillan *et al.*, 2006; Kliegman. *et al.*,2007). CoNS include *Staphylococcus epidermidis*, *Staphylococcus saprophyticus* and *Staphylococcus haemolyticus*. CoNS are pathogens in neonates, compromised hosts and patients with foreign bodies (McMillan *et al.*, 2006; Kliegman *et al.*, 2007). CoNS has emerged as an important cause of neonatorum sepsis, reported in some studies as the most commonly isolated organism in cases of LOS (Motara *et al.*, 2005). *S. aureus* is carried in 20-40% of the adult population. Neonates become colonized early in life (McMillan et al., 2006; Kliegman *et al.*, 2007). In the NICHD study, CoNS (47.9%) was the most commonly isolated organism, followed by *S.aureus* (7.8%), in cases of LOS (McMillan *et al.*, 2006).

2.5.2 *Klebsiella*

Klebsiella are gram negative enteric rods (McMillan *et al.*, 2006; Kliegman *et al.*, 2007). They lack motility and grow as large mucoid colonies on solid media (McMillan *et al.*, 2006). *K. pneumoniae* is the most common pathogen in this genus followed by *K.oxytoca*. *Klebsiella* species are common opportunistic nosocomial pathogens and newborn outbreaks continue to be a frequent occurrence worldwide. Research of recent shows that *Klebsiella* species are amongst the commonest isolated pathogens in cases of neonatorum sepsis NNS in developing countries (Vergnano *et al.*, 2005).

2.5.3 *Acinetobacter*

Acinetobacter is a gram-negative rod,oxidase-negative and lactose-nonfermenting organism. (McMillan *et al.*, 2006; Kliegman *et al.*, 2007). It belongs to a genus of coccobacillary bacteria in the family Neisseriaceae (McMillan et al., 2006; Kliegman *et al.*, 2007). The most common

isolates of the genus are *A.baumannii*, *A.lwoffi*, *A.haemolyticus*, and *A.johnsonni* (McMillan *et al.*, 2006; Kliegman *et al.*, 2007). Acinetobacter is an uncommon pathogen in healthy persons, but it is seen with increasing frequency in hospitalized and immunocompromised individuals (McMillan *et al.*, 2006; Kliegman *et al.*, 2007.

2.5.4 *Candida*

Candidiasis is the most common fungal infection in the world. Systemic candidiasis is a serious form of nosocomial infection in low birth weight (LBW) infants (Cloherty *et al.*, 2012). *C. albicans* accounts for most human infections (Cloherty *et al.*, 2012). Other candida species that have been reported with increasing frequency are *C.parapsilosis*, *C. krusei*, *C. lusitaniae and C.glabrata.* (Cloherty *et al.*, 2012).

2.5.5 *Streptococcus agalactiae*

Streptococcus agalactiae, group B *Streptococcus* (GBS), is a Gram-positive encapsulated bacterium that belongs to the group of pyogenic streptococci. It is the only Streptococcus species harboring the Lancefield group B cell-wall-specific polysaccharide antigen that is common to all GBS strains. GBS can be subdivided into 10 different serotypes (Ia, Ib, and II to IX) on the basis of type-specific capsular polysaccharides (Edwards *et al.*, 2016). In most cases, GBS is an asymptomatic colonizer of the digestive and genitourinary tracts of healthy human adults. However, it can cause severe invasive infections in neonates and immunocompromised adult patients. The first reports about GBS as a human pathogen were published in the late 1930s, when three fatal cases of puerperal sepsis caused by GBS were described (Fry, 2017). Since the

1960s , GBS has remained a leading cause of life-threatening neonatal infections (Edwards *et al.*, 2016).

In neonatology, there are two distinguishable clinical syndromes; early-onset disease (EOD) is a GBS infection occurring within the first week of life (usually within the first 24 h), and late-onset disease (LOD) is a GBS infection presenting after 7 days of age (7 to 90 days postpartum). EOD is caused by vertical transmission of GBS from a colonized mother to her newborn, through either ascending infection from the genital tract or GBS transmission to the newborn during labor and birth. Numerous studies have shown that up to 30% of pregnant women worldwide are colonized with GBS, and vertical transmission occurs for roughly 50% of colonized mothers. About 1% of colonized newborns develop EOD. EOD occurs mainly after the onset of labor or in connection with ruptured membranes, although infection of the fetus can happen through intact membranes. Bacteraemia without a focus is the most common clinical syndrome, followed by pneumonia and meningitis. Even today the case fatality rate for EOD is estimated to be 2 to 10%, and fatal outcomes are more frequent among premature neonates (Edwards *et al.*, 2016). Because most EOD is acquired through contact of the neonate with GBS during delivery, intrapartum antibiotic prophylaxis (IAP) administered to GBS carriers prevents vertical transmission in the vast majority of cases, and its widespread use has resulted in significant reductions in the incidence of EOD. In contrast to EOD, LOD is most likely acquired after birth, from breast milk or from nosocomial or community sources. Prematurity is the main risk factor for developing LOD and bacteraemia without a focus of infection is the most common presentation. The mortality rate for LOD is lower, but meningitis and subsequent sequelae are more frequently associated with LOD (Edwards *et al.*, 2016). GBS also causes significant

maternal morbidity, including endometritis, chorioamnionitis, bacteremia, and postpartum wound infections. GBS urinary tract infections are associated with miscarriages, preterm births, and low-birth-weight newborns (Edwards *et al.*, 2016). Although GBS seldom causes disease in healthy adults, it is responsible for serious infections in diabetics, elderly individuals, residents in nursing homes, and otherwise immunocompromised patients (Edwards and Baker, 2005).

2.6 Identification of GBS

Almost all clinical GBS isolates produce another cytolytic toxin, the CAMP (Christie, Atkins, Munch-Petersen) factor (Phillips *et al.*, 2005). This factor is not hemolytic per se, although it lyses sheep erythrocytes pretreated with staphylococcal -lysin (sphingomyelinase). This cytolysin is distinct from the GBS -hemolysin and pigment. The CAMP test involves streaking the strain to be tested perpendicular to a streak of a strain of *Staphylococcus aureus* on sheep blood agar. A positive reaction appears as a characteristic arrowhead zone of hemolysis adjacent to the place where the two lines come into proximity (Facklam *et al.*, 2002). However, this test is not 100% specific. Many *S. porcinus* strains, which are sometimes isolated from the genitourinary tracts of female patients (Facklam *et al.*, 2006), and some group A streptococci (GAS) can also produce positive CAMP test reactions (Facklam *et al.*, 2006; Facklam, 2002). The fact that the CAMP factor is present in nonhemolytic nonpigmented GBS strains can be used to generate beta-haemolysis in otherwise nonheamolytic strains, by incorporating the staphylococcal -lysin into blood agar plates. This principle, which is used in GBS Detect (Hardy Diagnostics), facilitates the detection of nonheamolytic GBS on blood agar. The cfb gene that encodes the CAMP factor is present in the vast majority of GBS isolates and is exploited for the molecular identification of GBS (Ke *et al.*, 2000).

2.7 Antibiotic Resistance Profile of *Streptococcus spp* in Neonatal Sepsis

Antimicrobial resistance (AMR) has become a significant threat to the prevention and treatment of bacterial infections globally (WHO, 2012). Importantly, in low- and middle-income countries, the potential for AMR to lead to increased morbidity and mortality may be greater given the higher burden of bacterial illness in low-income countries, delayed presentation, weaker access to diagnostics (particularly microbiology) and the reduced availability of second-line antibiotics (WHO, 2014). One critical aspect to the global response to AMR is surveillance. However, according to a 2014 report by the World Health Organization (WHO), the WHO Africa region has one of the largest gaps in data on the prevalence of AMR (WHO, 2014). As a consequence of limited laboratory capacity and surveillance networks. An external quality assessment reported several deficits in antimicrobial susceptibility testing in many African countries (Frean *et al.*, 2012). With limited information available on AMR, health departments and humanitarian actors providing health care in this region lack practical information on how AMR may compromise first-line empirical treatments of common bacterial infections. Recent efforts to define the map of AMR in sub-Saharan Africa are not easily translatable into action. For example, a 2014WHO report compiled existing data on AMR focusing on certain bacteria–antimicrobial drug combinations thought to be of public health importance (WHO, 2014). However, physicians and other prescribers, particularly in the absence of microbiology, recognize and manage clinical syndromes rather than specific bacteria. For the treatment of syndromes, a better understanding of the epidemiology of the most prevalent bacterial infections of public health importance may allow improved decision-making on empirical (first-line) antibiotic strategies.

There are several reports of multi resistant bacteria causing neonatal sepsis, and the trend shows increase in resistance to antibiotics commonly used in developing countries (Vergnano *et al.*, 2005 In developing countries, antibiotic resistance of community-acquired infections has increased significantly in the past 20 years (Thaver *et al.*, 2009). *Klebsiella sp.* resistance to gentamicin is 60–72%, to amikacin is 43% and to third-generation cephalosporins is 57–66%. *Escherichia coli* resistance to gentamicin is 13–48%, to amikacin is 15% and to third-generation cephalosporins is 19–64%. A study revealed that most of the commonly used antibiotics has high resistance of bacteria. Bacteria were highly resistant to ampicillin and amoxicillin. Overall resistance rates for ampicillin and amoxicillin were 77.7% and 81.5% respectively .

Both ampicillin and amoxicillin have been found having comparatively less resistance to *Streptococci,* i.e., resistance in 14.28% and 20.8% of cases respectively. It was discovered that *Streptococci* was 100% sensitive to ampicillin. Both gram-positive and gram-negative bacteria have been found resistant against 3rd generation cephalosporins. Cefotaxime and ceftriaxone had high resistance and ceftazidime had relatively less resistance. Many other studies have also described the emerging pattern of resistance against cefotaxime and ceftriaxone, and relatively low resistance to ceftazidime (Shaw *et al.*, 2007; Awoniyi *et al.*, 2009).

Furthermore, provision of empiric treatment brings up antibiotic resistance and stewardship issues (Seale *et al.*, 2009). Reports from different countries revealed the reduced susceptibility to penicillin, and the increased rate of macrolide resistance GBS isolates for the last few decades (Schrag *et al.*, 2000). A 2005-2007 Surveillance in Argentina showed the presence of GBS isolates resistance (in minimum inhibitory concentration; MIC range µg /L) to ciprofloxacin (32-64 µg/L), levofloxacin (16-32 µg/L), ofloxacin (32-64 µg/L), and norfloxacin (32-64 µg/L), and

all were susceptible to penicillin (0.06 μg/L) (Garland *et al.*, 2011). Of the 1160 GBS isolates in Australia, 6.4% demonstrated erythromycin resistance and 4.2% to clindamycin (Garland *et al.*, 2011). Another study in USA revealed that all the neonatal GBS were susceptible to penicillin, vancomycin, chloramphenicol, and cefotaxime. Its resistance rates to erythromycin were 20.2%, and 6.9% to clindamycin (Lin *et al.*, 2000). Another study in France revealed 38.2% erythromycin and 25.6% clindamycin resistance neonatal GBS (Hays *et al.*, 2010)

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

A total of 24 blood samples were obtained from neonates at the neonatal unit of Ekiti State Teaching Hospital, Ekiti State. Before obtaining samples from neonates, approval of parents' consent through filling of consent forms by parents were employed. A total volume 1ml each of blood sample was dispensed in culture bottles containing brain heart infusion broth and fluid thioglycolate medium for aerobic and anaerobic incubation respectively which were transported in an ice pack bag containing ice packs to the laboratory .

3.2 Sample Processing

Blood cultures obtained were incubated aerobically and anaerobically for 24hrs at 37°C followed by observation for bubbles, coagulation and haemolysis. 10µl blood culture was standardised using 0.5McFarland turbidity standards, followed by inoculation of standardised inoculum on Blood agar aerobically and anaerobically while inoculation was done aerobically on Mannitol Salt Agar. Pure culture was obtained by subculturing on nutrient agar plate. Processing of same blood culture after three days with daily records of blood culture changes following same sample processing as previous.

3.3 Plating and Isolation of Bacteria

Exactly 10 µl of appropriately diluted sample was plated on Mannitol salt and Blood agar using the pour plate technique. The inoculated plates were allowed to set, inverted and incubated at 37°C for 24 hours. After the incubation period, the colonies were studied and representative of the respective types were isolated and purified by streaking repeatedly on fresh nutrient agar.

Pure cultures were then stored on nutrient agar slant in the refrigerator at -80^{0}C until required for further use.

3.4 Identification of Bacterial Isolates

The bacterial isolates were identified according to the scheme of Bergey's Manual of Determinative Bacteriology

3.5 Morphological Characteristics of Bacterial Isolates

Each of the bacterial isolate was grown on nutrient agar plate and examined for their growth pattern such as elevation, shape, opacity, edge, surface and size. The isolates were also grown in nutrient broth and examined for their growth pattern such as turbidity, amount of growth, surface growth, sedimentation and deposit

3.6 Antibiotics Sensitivity Test

The bacteria isolated were sub -cultured from stock culture into sterile nutrient broth and incubated at 37°C for 24 hours. The broth culture of the isolated organism was standardised in 0.5McFarland and 10µl dispensed using a micro pippette on Mueller Hinton agar (MHA) plates and streaked with the aid of a sterile inoculating loop. The antibiotic sensitivity test disc (Gram positive disc: Rapid Labs IVD CM-12-8PR100 was used for Gram positive bacteria) was picked and placed on the surface of the MHA plates using a pair of sterile forceps on the inoculated MHA plates. The MHA plates were then incubated at 37°C for 24 hours and observed for clear zone of inhibition or resistance to the antibiotics. ceftazidime (30µg) cefuroxime (30µg) gentamicin (10µg) ofloxacin (5µg) ceftriaxone (30µg) erythromycin (5µg), cloxacillin (5µg) amoxicillin (30µg) bacitracin (B). This was carried out following the scheme of CLSI (2017).

CHAPTER FOUR

4.0 RESULTS

4.1 Morphological Characteristics of Bacterial Isolates

Table 4.1 shows the morphological and cultural characteristics of isolates obtained, the isolates were all positive to Gram staining and appeared cocci occurring in chains. Isolates appeared creamy on nutrient agar with slightly raised elevation, dry surfaces and having an edge which was entire.

Table 4.1: MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF BACTERIAL ISOLATES

SAMPLE NO	GRAM REACTION	COLOUR	ELEVATION	SURFACE	EDGE
S4 ANA MAC	Purple cocci in chains	Cream	Slightly raised	Dry	Entire
S4 ANA MSA1	Purple cocci in chains	Cream	Slightly raised	Dry	Entire
S4 ANA MSA2	Purple cocci in chains	Cream	Slightly raised	Dry	Entire
$S13^2$ AER BA	Purple cocci in chains	Cream	Slightly raised	Dry	Entire
$S13^2$ AER MSA	Purple cocci in chains	Cream	Slightly raised	Dry	Entire
$S16^2$ AER BA	Purple cocci in chains	Cream	Slightly raised	Dry	Entire
S7 AER BA	Purple cocci in	Cream	Slightly raised	Dry	Entire
S14 AER BA	Purple cocci in chains	Cream	Slightly raised	Dry	Entire
S15 AER BA	Purple cocci in chains	Cream	Slightly raised	Dry	Entire
$S23^2$ AER BA	Purple cocci in chains	Cream	Slightly raised	Dry	Entire
$S23^2$ ANA BA	Purple cocci in chains	Cream	Slightly raised	Dry	Entire

S24^2 AER BA	Purple cocci in chains	Cream	Slightly raised	Dry	Entire

KEY: **S**= SAMPLE NO **ANA**= ANAEROBIC **AER**= AEROBIC **BA**=BLOOD AGAR **MSA**= MANNITOL SALT AGAR **MAC**= MCCONKEY **S^2**= SECOND CULTURE at Day Three

4.2 Biochemical Characteristics of Bacterial Isolates

The result in table 4.2a and table 4.2b shows the biochemical characterisation of isolates obtained at day one and three respectively. Table 4.2a shows S4 ANA MAC, S4 ANA MSA1, S4 ANA MSA2, S4 ANA MSA2, $S13^2$ AER BA, $S13^2$ AER MSA and $S16^2$ AER BA were all negative to all biochemical test except CAMP test. Table 4.2b shows that S7 AER BA, S8 AER BA, S14 AER BA, S15 AER BA, $S23^2$ AER BA, $S23^2$ ANA BA, $S24^2$ AER BA and $S24^2$ ANA BA were only positive cetrimide, DNASE, coagulase and methyl red.

Table 4.2a BIOCHEMICAL CHARACTERISTICS OF BACTERIAL ISOLATES AT DAY 1

ISOLATES NUMBER	OXI	CAT	URE	TIS	MR	VP	DSE	COA	CIT	CET	CMP
S4 ANA MAC	-	-	-	-	-	-	-	-	-	-	+
S4 ANA MSA2	-	-	-	-	-	-	-	-	-	-	+
S13^2 AER BA	-	-	-	-	-	-	-	-	-	-	+
S13^2 AER MSA	-	-	-	-	-	-	-	-	-	-	+
S16^2 AER BA	-	-	-	-	-	-	-	-	-	-	+
S7 AER BA	-	-	-	-	+	-	+	+	-	+	-
S8 AERBA	-	-	-	-	+	-	+	+	-	+	-
S14 AER BA	-	-	-	-	+	-	+	+	-	+	-
S15 AER BA	-	+	+	-	+	-	+	+	+	+	+
S23^2 ANA BA	-	+	+	-	+	-	+	+	+	+	+
S24^2 AER BA	-	+	+	-	+	-	+	+	+	+	+
S24^2 ANA BA	-	+	+	-	+	-	+	+	+	+	+

KEYS: OXI= **OXIDASE**, CAT= **CATALASE**, URE= **UREASE**, TIS= **TRIPPLE IRON SUGAR** MR = **METHYL RED**, VP= **VOGES-PROSKAUER**, DSE= **DNASE**, COA=

CAOGULASE, CIT= **CITRATE** CET= **CETRIMIDE,** CMP= **CAMP,** S^2= **SECOND CULTURE at Day Three**

Table 4.2b BIOCHEMICAL CHARACTERISTICS OF BACTERIAL ISOLATES AT DAY 3

ISOLATES NUMBER	OXI	CAT	URE	TIS	MR	VP	DSE	COA	CIT	CET	CMP
S4 ANA MAC	-	+	+	-	+	-	+	+	+	+	+
S4 ANA MSA1	-	+	+	-	+	-	+	+	+	+	+
S4 ANA MSA2	-	+	+	-	+	-	+	+	+	+	+
S13^2 AER BA	-	+	+	-	+	-	+	+	+	+	+
S13^2 AER MSA	-	+	+	-	+	-	+	+	+	+	+
S16^2 AER BA	-	+	+	-	+	-	+	+	+	+	+
S7 AER BA	-	+	+	-	+	-	+	+	+	+	+
S8 AER BA	-	+	+	-	+	-	+	+	+	+	+
S15 AER BA	-	-	-	-	+	-	+	+	-	+	-
S23^2 ANA BA	-	-	-	-	+	-	+	+	-	+	-
S24^2 AER BA	-	-	-	-	+	-	+	+	-	+	-
S24^2 ANA BA	-	-	-	-	+	-	+	+	-	+	-

KEYS: OXI= **OXIDASE**, CAT= **CATALASE**, URE= **UREASE**, TIS= **TRIPPLE IRON SUGAR**, MR = **METHYL RED**, VP= **VOGES-PROSKAUER**, DSE= **DNASE**, COA= **CAOGULASE**, CIT= **CITRATE** CET= **CETRIMIDE**, CMP= **CAMP**, S^2= **SECOND CULTURE at Day Three**

4.3 Organisms Implicated at Successive Culture Stages

The result obtained shows in table 4.3 that certain organisms were implicated at each successive stage. *Streptococcus agalactiae* was implicated in the first culture of sample S4 ANA MAC while Staphylococcus spp was predominantly isolated in the second culture. The samples have different organisms predominantly isolated at different successive stages of culture. This table also shows that a total number of 14 *Streptococcuss spp*. strains were isolated, where 6 (42.8 %) were *Streptococcus agalactiae* and 8 (57.1%) were *Streptococcus pyogenes*

Table 4.3 ORGANISMS IMPLICATED AT SUCCESSIVE CULTURE STAGES

SAMPLE NO	FIRST CULTURE (D1)	SECOND CULTURE (D3)
S4 ANA MAC	*Streptococcus agalactiae*	*Staphylococcuss spp*
S4 ANA MSA1	*Streptococcus agalactiae*	*Staphylococcuss spp*
S4 ANA MSA2	*Streptococcus agalactiae*	*Staphylococcuss spp*
S13 AER BA	*Streptococcus agalactiae*	*Staphylococcuss spp*
S13 AER MSA	*Streptoccus agalactiae*	*Staphylococcuss spp*
S16 AER BA	*Streptoccus agalactiae*	*Staphylococcuss spp*
S7 AER BA	*Streptococcus pyogenes*	*Staphylococcuss spp*
S8 AER BA	*Streptococcus pyogenes*	*Staphylococcuss spp*
S14 AER BA	*Streptococcus pyogenes*	*Staphylococcuss spp*
S15 AER BA	*Streptococcus pyogenes*	*Staphylococcuss spp*
S23 AER BA	*Staphylococcuss spp*	*Streptococcus pyogenes*
S23 ANA BA	*Staphylococcuss spp*	*Streptococcus pyogenes*
S24 AER BA	*Staphylococcuss spp*	*Streptococcus pyogenes*
S24 ANA BA	*Staphylococcuss spp*	*Streptococcus pyogenes*

KEY: D1= DAY ONE, D3=DAY THREE

4.4 Antibiotic Susceptibility of Isolated *Streptococcus* spp.

Result obtained shows the antibiotics susceptibility pattern of isolated organisms from sample S4 ANA MAC showing total resistance to all antibiotics used. Isolated organisms from S4 ANA MSA1, S4 ANA MSA2, S4 ANA MSA2, $S13^2$ AER BA and $S13^2$ AER MSA showed different susceptibility and resistance to the antibiotics used. Tables 4.3 shows their susceptibility and resistance.

Table 4.4 ANTIBIOTIC SUSCEPTIBILITY PATTERN OF ISOLATED *Streptococcus* spp.

ISOLATES	ANTIBIOTIC SUSCEPTIBILITY PATTERN								
	CAZ	CRX	GEN	CTR	ERY	CXC	OFL	AUG	B
S4 ANA MAC	10R	9R	10R	20R	7R	9R	7R	7R	8R
S4 ANA MSA1	6R	6R	25S	6R	10R	22R	35S	6R	11R
S4 ANA MSA2	22S	16R	25S	24S	7R	6R	27S	24S	6R
$S13^2$ AER BA	25S	25S	16R	6R	20R	6R	25S	20R	20R
$S13^2$ AER MSA	6R	6R	20R	25R	25S	25S	20R	15R	6R
$S16^2$ AER BA	6R	6R	6R	15R	18R	25S	6R	6R	6R
S7 AER BA	17R	23R	23R	9R	6R	6R	15R	6R	6R
S8 AER MSA	6R	33S	33S	25S	6R	6R	29R	31S	32S
S14 AER BA	8R	6R	25S	20R	6R	20R	13R	6R	6R
S15 AER BA	8R	25S	25S	25S	6R	6R	25S	25S	25S
$S23^2$ AER BA	6R	6R	17R	6R	25S	15R	22R	6R	6R
$S23^2$ ANA BA	6R	6R	13R	6R	15R	6R	27S	6R	6R
$S24^2$ AER BA	6R	6R	6R	6R	6R	6R	6R	30S	6R

KEYS: CAZ = Ceftazidime (30µg), CRX = Cefuroxime(30µg), GEN = Gentamicin (10µg),

CTR = Ceftriaxone (30µg), ERY = Erythromycin (5µg), CXC = Cloxacillin (5µg),

OFL = Ofloxacin (5µg), AUG = Amoxlalin (30µg) Bacitracin (B)

S = Susceptibility, R = Resistance

4.5 Percentage susceptibility of *Streptococcus* spp.

Result obtained in table 4.5 shows that 5 strains of 6(14) *Streptococcus agalactiae strains* was 83.3% resistance and 1 strains of 6(14) was 16.6% susceptible to cefuroxime and varying resistance and susceptibility percentage to other antibiotics. 8 strains of 8(14) *Streptoccoccus pyogenes* showed 100% resistance to Ceftazidime, and 0 strains of 8(14) other strains likewise showed varying reistance and susceptibility to antibiotics as represented in table 4.5

Table 4.5 PERCENATGE SUSCEPTIBILITY OF *Streptococcus* spp.

	Streptococcus agalactiae		*Streptococcus pyogenes*	
ANTIBIOTICS	RESISTANCE%	SUSCEPTIBILIT%	RESISTANCE%	SUSCEPTIBILITY%
CAZ	66.6 (4)	33.3 (2)	100 (8)	0(0)
CRX	83.3 (5)	16.6(1)	75 (6)	25(2)
GEN	66.6 (4)	66.6 (2)	62.5 (5)	37.5(3)
CTR	83.3 (5)	16.6 (1)	75 (6)	25 (2)
ERY	83.3(5)	16.6 (1)	100 (8)	0 (0)
CXC	66.6 (4)	33.3 (2)	100 (8)	0 (0)
OFL	50 (3)	50 (3)	62.5 (5)	37.5(3)
AUG	83.3 (5)	16.6 (1)	50 (4)	50 (4)
B	100 (6)	0 (0)	75 (6)	25 (2)

KEYS: CAZ = Ceftazidime (30µg), CRX = Cefuroxime(30µg), GEN = Gentamicin (10µg),

CTR = Ceftriaxone (30µg), ERY = Erythromycin (5µg), CXC = Cloxacillin (5µg),

OFL = Ofloxacin (5µg), AUG = Amoxacilin (30µg) Bacitracin (B)

S = Susceptibility R = Resistance

CHAPTER FIVE

5.0 DISCUSSION

Neonatal sepsis, a life-threatening condition, needs immediate empirical antimicrobial therapy. It is important to choose an antibiotic combination that covers the most common pathogens (Aheri *et al.*, 2011). Blood culture remains the gold standard for diagnosis of neonatal sepsis, despite its low sensitivity which may be due to small volume of blood sample, or empirical antibiotics prior to sampling (Kalathia *et al.*, 2013). It was observed that there was high emergence of multi drug resistance among neonates in this study. A previous study from India stated that multidrug resistant organisms were leading causes of early as well as late onset sepsis (Viswanathan *et al.*, 2012). The worrisome rise in levels of antimicrobial resistance among pathogens retrieved from NICUs highlight the needs for better under-standing of the problem of early onset sepsis and implementing strategies to combat, especially in countries with limited resources (Chaurasia *et al.*, 2016).

Group B Streptococcus 6 (42.8%) and *streptococcus pyogenes* 8 (57.1%) were the prevalent Gram-positive pathogen in this study, which contrasts with the findings in other published literature from low- to middle-income settings, in which the total absence of these pathogen was reported (Shah *et al.*, 2012) Isolation of group B Streptococcus in our study is an indication of missed opportunities for antenatal screening. This prevalence of colonization was in agreement with different studies conducted worldwide such as: Tanzania (8.9%) (Joachim *et al.*, 2009) and Ethiopia (5%) (Schmidt *et al.*, 2012). The discrepancies in prevalence among these countries might be associated with the Global variability of maternal colonization with GBS and *streptococcus pyogenes* (differences in geography and season), the mode of delivery (in which

newborns born by spontaneous vaginal delivery had usually more GBS colonization), and the availability of laboratory facilities and experiences of laboratories to detect GBS and *streptococcus pyogenes*. The regional differences, variability in the sample size, methods employed for *streptococcus* spp. detection, availabilities of laboratory facilities, experiences of laboratory technologists might be possibly explained the disparities. The differences could also be explained by variations of maternal colonization and density of *streptococcus* spp. colony and mode of delivery.

In this study, all the *Streptococcus* spp. strains were resistance to ceftazidime , cefuroxime, gentamicin ceftriaxone, erythromycin, cloxacillin , ofloxacin, amoxacilin and bacitracin were estimated to be 66.6%, 83.3%, 33.3%, 83.3%, 83.3%, 66,6%, 50%, 83.3% and 100%, respectiveley. Highest resistance was recorded with ceftazidime while gentamycin had the lowest resistance. *Streptoccocus pyogenes* strains were resistance to ceftazidime, cefuroxime, gentamicin ceftriaxone, erythromycin, cloxacillin , ofloxacin, amoxacilin and bacitracin were estimated to be 100%, 0%, 62.5%, 75%, 100%, 100%, 100%, 62.5%, 50%, and 75%, respectively. Highest resistance was recorded with ceftazidime, erythromycin and cloxacillin while cefuroxime had the no resistance. Tallur *et al.* (2013) concur with this study that most *Streptoccous spp* isolates were resistant to ceftazidime , cefuroxime, ceftriaxone, erythromycin and cloxacillin. Almost all the isolates in our study were sensitive to either gentamycin 66.6% or amoxacilin 50% and hence a co-prescription of these two antibiotics appears prudent as the initial choice while awaiting for the blood culture reports. In agreement with our findings, a study in Egypt showed that of the GBS strains isolated from the neonates were 29.4% resistance to erythromycin and 17.6% were resistance to ceftazidime (Sultan *et al.*, 2012) Another studies

conducted in different parts of the world such as, in France showed that *Streptococcus pyogenes* strains were 48.2% resistance to ceftazidime 38.2% to erythromycin (Hays *et al.*, 2016). Another report from Tanzania revealed that the neonatal GBS were100% susceptible to penicillin, ampicillin, and ciprofloxacin whereas susceptibility to ceftriaxone and erythromycin were 93.8% and 81.3%respectively (Joachim *et al.*, 2009).

This variation might be explained by the fact that the laboratory facilities and health literacy of the people in our setting are different from other developed countries. Additionally, a study explained that antibiotics currently prevent an estimated 29,000 cases of early onset GBS disease per year. This approach may be challenging in low-income countries where many births take place at home, and laboratory capacity for the screening of *Streptococcus* spp. is limited (Seale *et al.*, 2009).

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

This study shows that *Streptococcus* spp. colonization was highly resistant to multiple broad-spectrum antimicrobials. Resistance varied based on different isolate of *Streptoccocus agalactiae and Streptococcus pyogenes* against antibiotics used frequently in the NICU (Neonatal intensive care unit) as the first line or the second line empirical treatment. It is worrisome that infants admitted from the with early onset sepsis had different resistance and susceptibility index to the multiple antibiotics like ceftazidime, cefuroxime, ceftriaxone, erythromycin, cloxacillin, ofloxacin and bacitracin. It is worthy of note that antibiotics in Nigeria are available over the counter and do not require a physician's prescription. This study calls for global regulations to restrict the use of indiscriminate use of antimicrobials in the community as well as in the hospital setting.

6.2 . RECOMMENDATION

Neonatal sepsis has been a major challenge globally thus requiring certain recommendations generated from this study which are:

1. Government at all levels should be involved in creating massive awareness in taking sides with preventive medicine rather than curative medicine.
2. Government of developing countries should at all cost increase annual budgetary allocation of the health sector and ensure increased provision of diagnostic test kit to various health organisation for quick time diagnosis.
3. Indiscriminate use of drugs and over the counter drug sales should be prohibited to reduce the rate at which etiological agent develop resistance.

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APPENDIX I

Sterilization of Media and Apparatus

Autoclaving

The culture media used for the isolation, cultivation and identification of the bacterial isolates were sterilized by autoclaving at 121^0C for 15 minutes under pressure.

Flaming

The mouth of the conical flasks, test tubes, McCartney bottles and Bijou bottles were sterilized by flaming with a Bunsen burner before and after taking/pouring media into them. Inoculating loops and needles were sterilized using the Bunsen burner flame before and after inoculation. Streaking, inoculation and pouring of molten agar into Petri dishes were done close to the Bunsen burner flame in order to avoid contamination and maintain sterility.

Disinfection

The work tops were swabbed with 70% ethanol before any activities to provide a sterile condition and also after analysis in order to prevent contamination.

Gram Staining

A thin film of each isolates (18-24 hour old) was made on a grease free microscope slide. The smear was heat fixed by passing it lightly through a Bunsen flame. The fixed smear was then flooded with basic crystal violet dye and left for a minute before rinsing off with tap water. The smear was flooded with Gram's iodine solution for a minute and rinsed off with tap water. The smear was decolorized with 70% ethanol and rinsed immediately with tap water. The smear was then counter stained with safranin for 30 seconds, rinsed with tap water, allowed to dry and

examined under oil immersion objective of a microscope. Gram positive cells stained purple. The shape and the arrangement of the cells were also observed.

Biochemical Characteristics of Bacterial Isolates

The following biochemical test were carried out

Catalase Test

A drop of 3% H_2O_2 was placed on a surface of clean and dry glass slide using a sterile inoculating loop. A colony was transferred on it and emulsified. A positive result is the rapid evolution of oxygen (5-10 sec.) as evidence by the presence of bubbles. A negative result shows no bubbles or only a few scattered bubbles.

Citrate Utilization Test

Bacterial colonies were picked with a wire loop and inoculated into slope of Simmon's citrate agar and incubated overnight at 37°C. If the organism has the ability to utilize citrate, the medium changes its colour from green to blue.

Methyl red and Voges-Proskauer Test

Sterile MRVP medium in test tubes were inoculated with 0.1mL of 24 hours old broth culture of each isolates and incubated at 35°C for 5 days. The MRVP broth culture was aseptically divided into two portions labeled MR and VP. 5 drops of methyl red reagent was added to the MR test tubes and examined for colour change, a red colouration denoting a pH of 4.5 or less indicated a positive result while yellow colouration denoted a negative result. 0.5mL of 6% α-naphthol solution and 0.5mL of 16% of potassium hydroxide solution was added to the VP test tubes,

shaken vigorously and left for 5-10 minutes. Development of red colouration indicated a positive reaction while a negative reaction was indicated by no colour change.

Triple sugar iron reaction test

Tubes was poured with TSI agar such that each tube contained both a slant (on the top) and a butt (on the bottom) and inoculated with bacteria. The tubes were then observed to examine the development of black colour.

Coagulase

A drop of coagulase plasma was placed on a clean, dry glass slide. And a drop of distilled water

a drop of distilled water or saline is placed next to the drop of plasma as a control. With a loop With a loop, a portion of the isolated colony was emulsified after placing it into each drop. The solution was mixed well and rocked gently for 5 to 10 seconds. A positive result shows macroscopic clumping in 10 seconds or less in coagulated plasma drop and no clumping in saline or water drop while a negative result shows no clumping in both drop.

Urease Test

Slant of urea agar medium was prepared and inoculated with isolated bacteria on the entire surface of the slant. The tubes were inoculated at 37°C. The slant was observed for a colour change at 24 hours. Urease production was indicated by a bright pink (fuchsia) colour on the slant. Any degree of pink colour development was considered as a positive reaction. Prolonged incubation was avoided as it might result in a false-positive test due to hydrolysis of proteins in the medium. To eliminate protein hydrolysis as the cause of a positive test, a control medium lacking urea was used.

Oxidase

The isolated bacteria was grown on a nutrient agar plate for 24 hours at 37°C.After 24 hours 0.2 ml of 1% α-naphthol followed by 0.3 ml of 1% p-aminodimethylaniline oxalate was added and observed for colour change.

DNASE Test

A sterile loop full of colony from a 24hours culture was picked and inoculated on the DNase plate. Plate was incubated at 37 degree Celsius for 24ours. The surface of the agar was flooded with 1N HCl solution and plate was observed after five minutes

CAMP Test

Using an inoculating loop, a beta-lysin-producing Staphylococcus aureus (ATCC25923) was streaked in a straight line across the center of a blood agar plate. Test organism was streaked in a straight line perpendicular to the *S. aureus* leaving 1cm space between the two streaks. The plate was incubated at 37 degree Celsius in the incubator for 18-24 hours.

Positive result shows enhanced hemolysis is indicated by an arrow head-shaped zone of beta-hemolysis at the junction of the two organisms. Negative result shows no enhancement of hemolysis.

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