Candida albicans IMPLICATED IN NEONATAL SEPSIS ISOLATED FROM NEONATAL UNIT OF EKITI STATE UNIVERSITY TEACHING HOSPITAL.

BY

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CERTIFICATION

We certify that this research work was performed and presented by MAKANJUOLA, EMMANUEL ADURAGBEMI (MCB/14/2326) and supervised by DR. S.K. OJO.

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DEDICATION

This report is dedicated to God Almighty through his son, our saviour Jesus Christ and the power of the Holy Spirit and also to everyone that are in search for knowledge.

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My utmost gratitude goes to the Almighty God for his immeasurable wisdom, knowledge and understanding he has given to me and also sparing my life up to this moment may his name be praised for ever. Amen. I also appreciate the undying love of my parent Prophet & Pastor (Mrs) Makanjuola and my ever supporting siblings towards me.

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And to my all colleagues, friends who have contributed to the success of this research, may the good lord bless and keep you.

ABSTRACT

Candida albicans is an opportunistic pathogen, which is responsible for a vast range of infections in both immunocompetent and immunocompromised individuals with the ability to access different parts of human body causing diseases and decrease in host defenses. This study is concerned with determining the prevalence of *Candida albicans* implicated in neonatal sepsis. Neonates born or referred to the neonatal intensive care unit (NICU) of EKSUTH who had been diagnosed of sepsis were included in this research. 2ml of blood sample was collected from infants with sepsis, 1ml was pipetted into 9ml Brain Heart Infusion broth and 1ml into 9ml of Fluid thioglycollate medium incubated at 37^0C for 24hours. Blood sample was standardized to 0.5 McFarland standard and 10µl of standardized sample was inoculated onto differential and selective media and incubated at 37^0C for 24hours. *Candida albicans* isolates were confirmed through colony morphology, fungal staining method, Gram staining microscopic examination and Germ tube presumptive test. A total number of 24 blood samples were collected. 3 (12.5%) samples were positive at the first and third successive culture with six (6) distinct isolates of *C. albicans* obtained. In this research, neonate maternal exposure rate to antibiotics before and after birth were 2 (66.6%) out of 3 (100%) and 1 (33.3%) out of 3 (100.0%) respectively. Also, neonate exposure rate to antibiotics was 3 (23.0%) out of 13 (100.0%), 3 (37.5%) out 8 (100.0%) patients were on mechanical ventilator and neonates battling with concomitant sepsis were 3 (25%) out of 12 (100.0%) patients. Pre-disposed infants with candidemia manifested convulsion, respiratory distress, hypothermia, hypothermia and feed intolerance as clinical presentation in this study. This study has also observed that the onset of neonatal sepsis cases was higher in the first week of neonate's life. Therefore, a quick and reliable test is therefore necessary for identification and treatment of any fungal sepsis.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Neonatal Sepsis (NS) is defined as systemic infectious disease occurring in children up to one month of life with clinical symptoms and positive blood cultures (Woldu *et al.,* 2016). It is a leading cause of neonatal deaths in developing countries and an important under estimated problem around the world. It is reported that about 21% to 36% of admitted patients had either bacterial or fungal sepsis and is an important cause of morbidity and mortality during the neonatal period in some studies (Soto *et al.,* 2013; Silva *et al.*, 2014 and Woldu *et al.,* 2016). Candida species is the third most frequent and the leading cause of invasive fungal infections in neonatal intensive care units. According to some studies, it was estimated that Candida infections contribute to nearly 2.4% of early-onset neonatal sepsis (EOS) and 10–12% of Late-onset neonatal sepsis (LOS) (Majeda *et al.*, 2013; Giuseppina *et* al., 2017; Yadav *et al.*, 2017 and Ashwani *et al.*, 2018).

About 360,346 neonates died from neonatal sepsis and other infections in 2011 worldwide (Pont-Thibodeau *et al.,* 2014). Fungal sepsis (candidemia) with incidences between 0.3% and 6.7% have been reported in some studies with mortality rate of 18-35% and great morbidity of 57.2% (Majeda *et al.*, 2013; Silva *et al.*, 2014; Giuseppina *et* al., 2017; Ashwani *et* al., 2018 and Shettigar and Shettigar, 2018). Several adverse neurodevelopmental impairments are exhibited in infants with neonatal sepsis and these includes cerebral palsy, lower mental and psychomotor development index scores, visual impairment and impaired growth (Silva *et al.*, 2014; Zea-Vera and Ochoa, 2015).

1.2 Statement of the problem

Neonatal sepsis has been documented to be a major cause of mortality and morbidity in neonates with candidemia especially in developing countries.

1.3 Justification of the study

This study was conducted owing to the insufficient reports and negligence on the threatening neonatal candidemia infection within the study location, which will serve as an information base and source of knowledge for those in the health sector and policy maker.

1.4 Aim of the Study

The goal of this study is to determine the prevalence of *Candida albicans* as the predominant organism of neonatal candidemia in Neonatal Intensive Care Unit of Ekiti State University Teaching Hospital (EKSUTH).

1.5 Objectives of the study

1. To isolate *Candida albicans* present in blood samples collected at the neonatal intensive care unit, Ekiti State University Teaching Hospital (EKSUTH).
2. To enumerate and determine the occurrence rate of *Candida albicans* in the collected blood samples.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Incidence of neonatal Sepsis

Neonatal sepsis which is known to occur during the first 28 days of life, have been estimated to cause 26% of all death of neonates worldwide (Awoniyi *et al.*, 2009; Jabiri *et al.*, 2016). Fungal sepsis is characterized by candidemia and clinical symptoms caused by microorganisms and their toxic products (Silva *et al.*, 2014 and Mohd *et al.*, 2018). Gram negative bacteria are the commonest causes of neonatal Sepsis, and C. albicans was most often isolated from extremely low birth weight neonates as compared to non-albicans Candida as reported by (Spiliopoulou *et al.*, 2012; Ullah *et* al., 2012 and Ashwani *et al.*, 2018) but infection with non-albicans Candida have increased in recent years (Giuseppina *et* al., 2017).

Candida species, the third most frequent and the leading cause of invasive fungal infections in neonatal intensive care units. According to some studies, it was estimated that Candida infections contribute to nearly 2.4% of early-onset neonatal sepsis (EOS) and 10–12% of Late-onset neonatal sepsis (LOS) (Majeda *et al.*, 2013; Giuseppina *et* al., 2017; Yadav *et al.*, 2017 and Ashwani *et al.*, 2018). Neonatal sepsis encompasses various systemic infections of the newborn, such as septicemia, meningitis, pneumonia, arthritis, osteomyelitis and candidemia (candidemia was defined as an isolation of any pathogenic species of *Candida* from at least one blood culture specimen from a patient with signs and symptoms of infection) etc. (Gebremedhin *et al.*, 2016 and Shettigar and Shettigar, 2018).

2.2 Types of neonatal sepsis

Neonatal sepsis can be subdivided into early-onset neonatal sepsis and late-onset neonatal sepsis (Soto *et al.*, 2013).

2.2.1 *Early Onset Neonatal Sepsis*

Early-onset neonatal sepsis (EONS) refers to the presence of a confirmed infection in the blood or cerebrospinal fluid (CSF) of patients younger than 72 hours of life (Reyes *et al.*, 2015). EONS can be acquired vertically from the pregnant woman before or during delivery. In this case, microorganisms present in the genital tract of the mothers are of great importance (Soto *et al.*, 2013). Also, Chorioamnionitis, maternal intrapartum fever, prematurity, prolonged rupture of membranes and inadequate intrapartum antibiotic prophylaxis increase its risk (Zea-Vera and Ochoa, 2015).

2.2.2 *Late Onset Neonatal Sepsis*

LONS occurs at 4-90 days of life and is acquired from the care giving environment. The incidence ranges from 1.87 to 5.42 per 1,000 live births (Soto *et al.*, 2013). The main risk factors associated with LONS are prematurity, central venous catheterization (duration > 10 days), nasal canula, gastrointestinal tract pathology, exposure to antibiotics, and prolonged hospitalization (Reyes *et al.*, 2015).

2.3 Causative organisms of Neonatal Sepsis

The main pathogen associated with LONS is Coagulase Negative Staphylococcus CoNS which is responsible for half of episodes. Other important etiologic agents are *E. coli*, *Klebsiella sp* and *Candida sp* in which together they cause one-third of episodes. Less common causes of late-onset sepsis include *S. aureus*, *Enterococcus sp* and *Pseudomonas aeruginosa* (Ullah *et* al., 2012; Zea-

Vera and Ochoa, 2015). Daynai *et* al. (2013) and Ashwani *et al.* (2018) reported that about 90 % and above of systemic Candida infections are caused by *C. albicans, C. parapsilosis, C. guilliermondii, C. glabrata, C. dubliniensis, C. tropicalis* and *C. krusei.*

In developing countries, most pathogens isolated in the hospital setting before 72 h of life are similar to those isolated afterward; it is likely that highly unclean delivery practices lead to infections with nosocomial agents very early in life. (Spiliopoulou *et al.*, 2012; Dong and Speer, 2014 and Zea-Vera and Ochoa, 2015).

2.3.1 Candida albicans

C. albicans is said to be an opportunistic pathogen, with the ability to access different parts of human body causing diseases and decrease in host defenses (Nasution, 2013). It is also a commensal of mucosal surfaces of the oral and vaginal cavities and the digestive tract (Anaul *et al.*, 2012; and Nasution, 2013). *C. albicans* as a pathogen, is responsible for a wide range of infections in both immunocompetent and immunocompromised individuals (Anaul *et al.*, 2012).

Candida albicans is a Gram-Positive Yeast appearing as oval, bud shape, blastoconidia, size 2-3 x 4-6 µm with budded cells resembling an elongated hypha (pseudo hyphae) (Nasution, 2013). The yeast colony is white to cream colored, smooth, glabrous and yeast-like in appearance (Tri Wibawa, 2016). *Candida albicans* is a dimorphic fungus that as ability to switch from a unicellular to a hyphal mode of growth (Tri Wibawa, 2016). The various environmental conditions, such as temperature, pH or serum availability etc. are known to trigger dimorphism. It is thought that it is

this change which allows the organism to invade tissues and, therefore, contributes to the virulence of this micro-organism (Anaul *et al.*, 2012).

Candida albicans is the most common fungal causative agent for urinary tract infection and in oral fungal infection contributes near 50% of all candidiasis cases (Tri Wibawa, 2016). Anaul *et al.* 2012 described Candidiasis caused by *C. albicans* to be divided into superficial (such as oral and vaginal thrush and chronic mucocutaneous candidiasis) and deep-seated (such as *Candida* due myocarditis and acute disseminated *Candida* septicemia) and represent a major clinical problem. According to various studies, *C. albicans* has been the species most often associated with neonatal infections but recently there has been a changing pattern in the isolates recovered from neonates with invasive candidiasis (Asticcioli *et al.*, 2007). *C. albicans* infections are considered as a serious and life threatening public health challenge with high clinical and socioeconomic importance and as a prevalent agents of nosocomial infections (Anaul *et al.*, 2012).

2.3.1.1 Pathogenesis and virulent factors of *Candida albicans*

Candidemia is defined as an isolation of any pathogenic species of *Candida* from at least one blood culture specimen from a patient with signs and symptoms of infection (Shettigar and Shettigar, 2018). A defect in the immune system is an important factor that determine whether *Candida albicans* will continue as a commensal of the parts of an individual body or not (Nasution, 2013). Neonates are initially colonized by *Candida albicans* through vertical transmission during birth or horizontally by parents or caregivers on (Asticcioli *et al.*, 2007). *C. albicans* has various complex mechanisms such as adhesion and invasion, changes of shape morphology from yeast into hyphae form, biofilm formation and its ability to avoid host immune cells.

There are some factors that aid the virulence of *Candida albicans* and these includes; adherence of the fungi to the host's surfaces, production of hydrolytic enzymes, dimorphism, galvanotropism and thigmotropism, phenotypic switching, biofilm formation, and evasion to the host immune response (Tri Wibawa, 2016).

- *Adherence*

The first and important step of *C. albicans* colonization and infection in the oral and other surfaces in human is ADHERENCE (Nasution, 2013 and Tri Wibawa, 2016). *Candida albicans* cell wall plays some important roles in it adherence to host cells and to various tissues and inanimate surfaces. For example, buccal and vaginal epithelial cells, corneocytes, cultured cells (HeLa and HEp-2) surface, as well as biomaterial surface (Nasution, 2013 and Tri Wibawa, 2016).

The cell wall is an essential and highly dynamic fungal structure that has been implicated in several physiological processes such as the maintenance of cellular morphology and osmotic protection of the cell through its rigidity. *Candida albicans* cell wall is made up of substances that are essential for virulence such as derivatives mannoprotein that have immunosuppressive properties to enhance the defense of *C. albicans* against host immune defenses due to its absence in mammalian cells and this fact makes it an ideally attractive target in antifungal research (Nasution, 2013 and Tri Wibawa, 2016).

Adhesion of *C. albicans* depends on the expression of various antigens that acts as adhesion molecules on the surface epithelial of the host cells. Adhesion molecules acting as mediator were classified as proteins serum (serum albumin and transferrin, fibrinogen, C3D complement

fragments and iC3b complement fragment); extracellular matrix proteins (laminin, fibronectin, entactin, vitronectin, collagen); mannan adhesins and other binding proteins (Asticcioli *et al.*, 2007; Nasution,.2013). *ALA1, ALS1, Hwp1, INT1, MMT1, PMT1, PMT6* and *Als1p* are candidate genes putatively considered as encoding adhesins (Tri Wibawa, 2016).

Morphogenetic transformation often occurs after adhesion of *C. albicans* from blastopore into chlamydospora form. The hyphae of this fungus possesses a higher virulence capacity that the spore form because; first, it was bigger and more difficult for phagocytose by macrophage and requires other mechanisms for hyphae elimination on infected host tissues (Naustion, 2013). Second, increase in the amount of infectious elements due to the presence of multiple blastoconidia point on the filament. Also the ability to switch between different morphologies in response to a variety of environmental stimuli might have an important consequence for its survival in different conditions. For example, during human infections or growth on media containing blood serum at 37◦C, hyphal cells are produced from budding yeast cells, the morphology of which could facilitate deep penetration of this pathogen into epithelia, endothelia, and human tissues (Mary *et al.*, 2016 and Nasution, 2013).

- *Production of hydrolytic enzymes*

Hydrolytic enzymes secreted by *Candida albicans* have been discovered to facilitate its commensally and pathogenic characteristics such as adherence to host tissue and inert particles, rupture of host cell membranes, invasion of mucosal surfaces and blood vessels, and evasion of the host's immune response (Tri Wibawa, 2016). There are three major enzymes produced by *C. albicans*: *Secreted aspartyl proteinases* SAP, phospholipases, and haemolysins.

- *Secreted aspartyl proteinases* SAP

Aspartyl proteases are a set of enzymes well characterized in C. albicans that comprise of 10 *SAP* genes (*SAP1, SAP2, SAP3, SAP4, SAP5, SAP6, SAP7, SAP8, SAP9*, and *SAP10) of* 35- 50 kDa in size and responsible for all of the extracellular proteolytic activity of *C. albicans* (Tri Wibawa, 2016). Saps functions for the yeast cells includes nutrient provision, to aid penetration and invasion, and to evade host immune responses. They are known to degrades proteins related to structural and immunologic defences, such as collagen, keratin, mucin, antibodies, complement, and cytokines, during tissue invasion. For example, Sap2p seems to be important for invasion through the endothelial barrier and also represents the most important contribution to virulence in the rat vaginitis model and Sap4p-6p seem to contribute to the survival of C. albicans in macrophage phagocytosis (Mary *et al.*, 2016; Tri Wibawa, 2016). Saps expression by *C. albicans* is regulated by several factors, such as nutritional condition, pH, temperature, and growth phase of the yeast (Tri Wibawa, 2016).

- *Phospholipases*

Tri Wibawa (2016) reported that Phospholipases hydrolyse glycerophos-pholipids, which are major components of mammalian cell membranes. It cleaves fatty acids from phospholipids which in turn destabilizing the membranes. There are seven phospholipase genes have been identified i.e. *PLA, PLB1, PLB2, PLC1, PLC2, PLC3* and *PLD1* (Mary *et al.*, 2016).

- *Hemolysin*

Candida albicans secretes hemolysins enzyme, which is an important putative virulence factor of the genus Candida with a hemolytic capacity to acquire iron from host tissues which it uses for

metabolism, growth and invasion during host infection (Tri Wibawa, 2016). In the human being, iron is found in several proteins, including hemoglobin located in the erythrocytes (Nasution, 2013). *Candida albicans* capable to bind to erythrocytes destroy the erythrocytes with the aid of hemolysins enzyme, but, however, the mechanism of this hemolytic reaction remain unclear (Nasution, 2013. and Tri Wibawa, 2016). About 98.5% of *C. albicans have* hemolytic activity (alpha, beta, and gamma hemolysis) that can be observed readily on blood agar media when culturing the yeast in aerobic condition (Tri Wibawa, 2016).

- *Dimorphism*

The transition of *C. albicans* between yeast and hyphal forms is termed as dimorphism (Tri Wibawa, 2016). The ability of *Candida albicans* to switch between different morphologies in response to a variety of environmental stimuli might have an important consequence for its survival in different conditions and this observation suggested that morphological switching ability plays an important role in virulence. (Mary *et al.*, 2016). This characteristic also contributes to numerous nature of its infection stages, such as adherence to epithelial and endothelial cells, intercellular invasion, iron acquisition from intracellular host sources, biofilm formation, as well as escape from phagocytes and immune evasion (Nasution, 2013; Tri Wibawa, 2016).

The hyphal form has been reported to be more invasive than the yeast form (Mary *et al.*, 2016 and Tri Wibawa, 2016). Whereas, the yeast form is proposed as the form primarily involved in dissemination of the fungus and another group found that transition of *C. albicans* morphology to yeast form may not be the only factor regulate dissemination from the gastrointestinal tract to the

other organs in invasive *C. albicans* infection (Mary *et al.*, 2016 and Tri Wibawa, 2016). Both morphologies have their own function to support its virulence (Tri Wibawa, 2016).

- *Phenotypic switching*

White-opaque phenotypic switching is a type of phenotypic switching carried out by small proportion of *C. albicans* isolates, which are homozygous at the mating type locus (*MTL, a/a or* α/ α) and are able to switch between two distinct cell morphologies: white and opaque (Tri Wibawa, 2016). white and opaque phenotypes show different cellular and colony appearances, gene expression profiles, mating ability and virulence. The white cells appear round and bright under microscope, while opaque cells appear darker, polymorphic and oval (Nasution, 2013). Opaque cells are better colonizers of the skin and are less virulent than white cells in a mouse model of disseminated candidiasis (Mary *et al.*, 2016 and Tri Wibawa, 2016). There are certain environmental conditions that drive *C. albicans* phenotypic switch from one phase to the other although white-opaque switching occurs at a low frequency (Tri Wibawa, 2016). Although opaque cells are less frequently cause systemic infection than white cells, they have better optimization for colonization, such as on the skin (Mary *et al.*, 2016 and Tri Wibawa, 2016).

- *Biofilm formation*

Tri Wibawa. (2016) described Biofilm as a structure made of microbes' consortium supported with extracellular matrix which attach to the surface of living matter or inanimate structure and this feature is common to *C. albicans*. There are two types of *C. albicans* cells involve in the biofilm formation: small yeast-form cells (Blastospores), and long tubular hyphal cells (Mary *et al.*, 2016). The two cell types have their specific role in biofilm formation (Nasution, 2013 and Tri Wibawa, 2016). *C. albicans* is said to be notorious because of its deleterious consequences, such as leads to

antifungal resistance, give an asylum to the yeast because of ability to make evasion against immune surveillance, and act as perfect reservoir for source of infection, as well as several advantages in the fungal's perspective: protection from the environment, resistance of physical and chemical stress, metabolic cooperation, and a community-based regulation of gene expression. Indeed, biofilm formation represents one of the putative virulence factors contributing to the pathogenesis of candidiasis (Mary *et al.*, 2016 and Tri Wibawa, 2016).

- *Evasion to the host immune responses*

There are several mechanisms that human immune system develops as a response against *C. albicans* and these comprises of the innate and adaptive immune response (Tri Wibawa, 2016). The innate immune response is nonspecific and broad and it is also regards as the first line of host defense against potentially harmful microbes. Examples of innate immunity are, a group of soluble (complement) and cellular (neutrophil, macrophage) components. Whereas, the adaptive immune response recognizes specific antigenic moieties, resulting to the development of a targeted immune response (Tri Wibawa, 2016).

Several mechanisms have been proposed to explain the mechanism of *C. albicans* evade from the host immune response and is considered as virulence factor of the yeast. For example, interference of sIgA begins with epithelial penetration by hyphae and supported by aspartyl proteinase and phospholipase to reducing the production of saliva (Nasution, 2013). Moreover, the effect of pathogenicity of *Candida albicans* and coaggregates with *S. mutants* improving of acid production which will be followed by decreased sIgA and a disadvantage to immune response (Nasution, 2013).

Candida albicans was shown capable to bind thrombocytes via fibrinogen ligands in the blood stream. *Candida albicans* was capable of binding to thrombocytes and this may in turn camouflage them from the immune system during dissemination through the blood stream resulting to a yeast cells being surrounded by a group of thrombocytes (Nasution, 2013 and Tri Wibawa, 2016).

- *Antibiotic resistance*

Candida albicans not only attached into mucosa, but is also able to penetrate. Aspartyl proteinase enzymes will support *Candida albicans* having early stages to penetrate the layers of keratinized-mucocutaneous tissue. When the complement of C3 component receptors blocked by fungal such as the use of antibiotics. Antibiotics will suppress the growth of competitive microorganisms for *Candida albicans*. These conditions make *Candida albicans* easier to form colonies (Nasution, 2013).

According to some studies it was observed that biofilm formation by *C. albicans* contributes to their increased antibiotic resistance abilities and complicates the treatment of *Candida*-infected patients. Low penetration of antibiotic drugs through the biofilm was postulated to be the reason for this high drugs resistance in *C. albicans* but this idea does not hold true as mutants having defective biofilm can also show high-level of drug resistance. For example, *C. albicans* is getting resistant to many antifungal drugs like flucytosine, fluconazole, amphotericin B, and caspofungin that are commonly used to treat fungal infections (Mary *et al.*, 2016 and Nasution, 2013).

2.4 Risk factor of neonatal Sepsis

The risk factors associated to neonatal sepsis are complex and include interaction of maternal-foetal colonization, transplacental immunity and physical and cellular defense mechanisms of the

neonate. Other are attributed principally to infection, birth asphyxia and consequences of premature birth and low birth weight (Jumah and Hassan, 2007; Awoniyi *et al.*, 2009).

There are several factors that have contributed to the rapid increase of neonatal candidemia in the neonatal care unit globally and they are; global increase in prematurity and survival of premature babies, susceptibility of the infant, size of inoculum, low birth weight, use of broad spectrum and/ or multiple antibiotics, central venous catheters, prolonged urinary catheterization, parenteral alimentation and intravenous fat emulsion, colonization with Candida and/or previous episode of muccocutaneous candidiasis immunosuppressive therapy and virulence of the *Candida sp* (Asticcioli *et al.*, 2007; Majeda *et al.*, 2013; Kapila *et al.*, 2016; Tri Wibawa, 2016 and Shettigar and Shettigar, 2018).

2.5 Epidemiology of neonatal Sepsis

The World Health Organization estimated that there are approximately five million neonatal deaths per year of which 98% occur in developing countries (Awoniyi *et al.*, 2009). About 360,346 neonates died from sepsis and other infections in 2011 worldwide (Pont-Thibodeau *et al.*, 2014). In Africa, 17% of neonatal deaths are attributed to neonatal sepsis in Sub-Saharan Africa and some studies have reported neonatal sepsis to be prevalent in a number of countries (Jabiri *et al.*, 2016). In Nigeria, 6.5 cases of neonatal sepsis per 1000 live births; In Kenya, 5.46 cases per 1000 live births have been recorded in Kilifi. A recent study in Ethiopia indicates that neonatal sepsis in the major new born killer accounting for more than one third of neonatal deaths (Berkley *et al.*, 2005; Gebremedhin *et al.*, 2016).

Globally candidemia affects more than250,000 people yearly and is responsible to more than 50,000 deaths with an Incidence rates of 2 - 14 cases per 100,000 persons in population-based studies (Tri Wibawa, 2016). Also based on the surveillance of nosocomial blood stream infections (BSI) report in the USA between April 1995 and June 1996 reported that Candida was the fourth most common causative agent of nosocomial BSI. Fifty-two percent of 379 of candidemia cases were due to *C. albicans*, and it have reported to be one of the leading causes of catheter-related BSI (Tri Wibawa, 2016). Coetzee *et al.* (2017) reported that according to the South African (SA) neonatal data, the mortality rate of neonatal candida infection was found to vary between 24.2% and 40%, and 19.7% and 22.5% for EOS and LOS, respectively, with an overall mortality of 20.8 - 23%. However, mortality due to Gram-negative microorganisms is more common (69.2%-80%) (Coetzee *et al.*, 2017).

2.6 Clinical features of Neonatal Sepsis

The clinical symptoms manifested by neonates with EONS and LONS are non-specific and usually include temperature instability, respiratory problems, apnea, feeding intolerance, Sick looking, Increased pre-feed aspirate, Lethargy, Chest retraction, Seizure, Grunting, Sclerema, Abdominal distension increase abdominal girth by 2 cm, Central cyanosis, Increased respiratory rate >60/min, Hypothermia (axillary temperature 37.50 C), Bradycardia - Heart rate 160/min (Soto *et al.*, 2013 and Arowosegbe *et al.*, 2017).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Study area

The study area was the neonatal intensive care unit (NICU) of Ekiti State University Teaching Hospital, Ekiti State Nigeria.

3.2 Target population

The targeted population for this research are the neonates (age 0-90 days of life that have been diagnosed of neonatal sepsis) born in the selected teaching hospital or referred from other hospitals during the study period.

3.3 Data collection

The parent/guardian of the infected neonate/neonates were presented with consent form before collection of blood sample. This was used to access various information about the neonate and mother.

3.4 Eligibility criteria

3.4.1 Inclusion criteria

- Neonates of 0-90 days of life with clinically suspected sepsis born or admitted during the study period.
- Infants who have not received antimicrobial treatment 2 hours prior to sample collection
- Infants who had already been exposed to antimicrobial treatment before referred to study location
- Pre-term and Full term babies.

3.4.2 Exclusion criteria

- Congenital malformations
- Severe birth asphyxia

3.5 Sampling technique

Samples were collected according to Ullah *et al.* (2012) from all the neonates born in the selected hospital and those admitted to the hospital with suspected sepsis that meet the inclusion and exclusion criteria.

3.6 Sample collection, processing and transportation

3.6.1 Collection of blood sample

A 2ml blood sample was collected from neonates that were diagnosed of sepsis, and 1ml was dispensed into 9ml Brain Heart Infusion Broth (Aerobic culture) and 1ml into 9ml Fluid thioglycollate medium (Anaerobic culture) and refrigerated for preservation before transportation.

3.6.2 Transportation of blood sample

Blood cultures were transported in an ice pack bag containing ice packs to the laboratory and immediately incubated at 37°C for 24hours aerobically and anaerobically.

3.6.3 Standardization of blood culture

A 10µl of broth culture was pipetted into a test tube and standardized with normal saline until it matched with 0.5 McFarland standard using CLSI (2017) standard.

3.6.4 Inoculation of standardized blood sample

A 10µl of standardized sample was pipetted aseptically onto the surface of two plates of Sabouraud dextrose agar (SDA) containing 0.05% streptomycin and streaked properly with an inoculating loop. One of the plates was incubated anaerobically in a gas jar and the second plates were incubated aerobically at 37^0C for 24hours. After 24hours of incubation, the plates were examined for fungi growth.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic characteristic of total blood samples collected during this study

A total number of 24 blood samples were collected from the neonatal care unit of Ekiti State University Teaching Hospital, Ekiti State (EKSUTH) confirmed with sepsis development. Out of the 24 (100%) blood samples confirmed with sepsis, only 3(12.5%) were positive for *C. albicans* for the first and third successive culture and these samples were from patients referred to the tertiary hospital.

4.2 Various place of birth of the Patients

Out of the 3 (12.5%) blood samples that were positive for *Candia albicans* from the referred patients, 1 (33.3%) came from neonate born in the traditional home and 2 (50%) from the neonates born at home.

4.3 Risk factors for candidemia observed in this study

Risk factors deduced from the data on the consent form of the research revealed pre-natal and post-natal exposure rate of neonate maternal to antibiotics to be 2 (66.6%) out of 3 (100%) and 1 (33.3%) out of 3 (100.0%) and were respectively positive for *Candida albicans*. It also revealed that 3 (23.0%) out of 13 (100.0%) neonates that were exposed to antibiotics after birth were positive for *Candida albicans* infection. 3 (37.5%) out 8 (100.0%) patients that was given mechanical ventilator and 3 (25%) out of 12 (100.0%) patients battling with concomitant bacterial sepsis were candidemia positive. See table 3 for more details

Table1: Demographic characteristic of neonates and mother under study.

Characteristics	Number of cases		percent (%)	
	With C	Total	With C	Total
Gestational age (weeks)				
<28	3	24	12.5%	100.0%
28-37	-	-	-	-
>37	-	-	-	-
Full term	1	17	5.8%	100.0%
Pre-term (VLBW)	2	7	28.5%	100.0%
Mode of Delivery				
Vaginal	3	24	12.5%	100.0%
Caesarean	-	-	-	-
Non-referral	-	-	-	-
Referral	3	24	12.5%	100.0%

Key words **C:** Candidemia **-:** Nil

Non-referral: patients born at the tertiary hospital.

Referrals: patients referred to the tertiary hospital.

VLBW: Very low birth weight.

Table 2: Various place of birth of the Patients (Neonates)

Places	Number of cases		percent (%)	
	Total	With C	Total	With C
Eksuth	-	-	-	-
General Hospital	5	-	100.0%	-
Private Hospital	8	-	100.0%	-
Traditional Home	3	1	100.0%	33.3%
Church	2	-	100.0%	-
Home	4	2	100.0%	50.0%
Others	2	-	100.0%	-

Key words C: *Candida albicans* -: Nil

Table 3: Risk factors for Candidemia observed in this study

Characteristics	Number of cases		percent (%)	
	Total	With C	Total	With C
Antibiotics (A)				
Neonates Exposed to A	13	3	100.0%	23.0%
Not exposed to A	11	-	100.0%	-
Maternal exposure to A				
Pre-natal	3	2	100.0%	66.6%
Post-natal	3	1	100.0%	33.3%
Parenteral nutrition	24	3	100.0%	12.5%
Mechanical ventilation (>5 days)	8	3	100.0%	25.0%
Concomitant bacterial sepsis	24	3	100.0%	12.5%

Key words A: Antibiotics C: *Candida albicans* -: Nil

4.4 Clinical presentations observed in cases of neonatal candidemia

Among the out-born patients confirmed for candidemia, 1 (4.1%) out of 8 (33.3%) had Hypothermia (Low temperature), 2 (33.3%) out of 6 (100.0%) had Hyperthermia (High temperature), 3 (100.0%) out of 3 (100.0%) had Convulsion and 3 (37.5%) out of 8 (100.0%) developed respiratory distress and were placed on a mechanical ventilator. Table 4 shows the clinical presentations of candidemia in neonates.

4.5 *Candida albicans* isolated from the first and third successive culture

Six isolates were obtained from the 3 positive blood samples for candidemia after the first and third successive aerobic and anaerobic culture at 37^0C for 24 hours.

Table 4: Clinical presentations observed in cases of Neonatal candidemia

Clinical features	Number of cases		percent (%)	
	Total	With C	Total	With C
Feed intolerance	-	-	-	-
Hypothermia	8	1	100.0%	12.5%
Hyperthermia	6	2	100.0%	33.3%
Convulsion	3	3	100.0%	100.0%
Respiratory distress	8	3	100.0%	37.5%

Key words **C:** *Candida albicans* **-:** Nil

Table 5: *Candida albicans* isolates obtained from blood samples

First Culture		Third Culture	
Isolate I.D Morphological Characteristics		IsolateI.D Morphological characteristics	
S21A a white,smooth,glabrous,convex ,yeast		$S21^2A$	same as S21A
S22AN	same as S21A	$S22^2AN$	same as S21A
S24A	same as S21A	$S24^2AN$	same as S21A

Key words

A: Aerobic **An:** Anaerobic **S:** Sample

4.6 Identification of fungi isolates

4.6.1 *Growth patterns on SDA*

The yeast colony appeared white to cream coloured, smooth, glabrous, pasty, convex colonies and yeast-like in appearance which may become wrinkled on further incubation.

4.6.2 Growth pattern *Yeast extract agar*

An inoculum was picked aseptically from the SDA plate and streaked on the surface of the set agar and incubated at 37°c for 24 hours. After incubation, a white to cream yeast-like glabrous convex colonies was observed in the culture plate (appendix).

4.6.3 Growth pattern on blood agar

Sheep blood of 5% was added into a sterile nutrient agar before pouring into Petri-dish. The agar was allowed to set and aseptically, the mycelium of the fungus (*C. albicans*) was stabbed on the surface of the agar and incubated at 37°C for 24 hours. The plate was examined for growth pattern and morphology after incubation. The growth pattern is similar to that on the sabouraud dextrose agar (appendix).

4.7 Morphological characteristics of different microscopic confirmatory test for *Candida albicans* performed in this study.

Microscopic confirmatory test of *C. albicans* performed in this study includes wet mount examination, lacto-phenol cotton blue staining method, Gram staining reaction and Germ tube presumptive test (see appendix for more details.

Table 6: Morphological characteristics of different microscopic confirmatory test for *Candida albicans*.

Microscopic test	Morphological characteristics
Wet mount (X10 and X40)	appears as oval shaped yeast in clusters and chains.
Lacto-phenol cotton blues staining	appears as blue oval shaped yeast in chains and some singly.
Gram staining method	appears as purple budded yeast cell single and in chains.
Germ tube presumptive test	appears as a yeast developing a cylindrical outgrowth arising from the blastospore with no constriction at the point of attachment.

CHAPTER FIVE

5.0 DISCUSSION

Neonatal sepsis is a life threatening systemic infectious disease which frequently affects infants in neonatal intensive care units (NICUs) all around the world (6.3 in 1,000 admissions), mainly in pre-term and low birth weight (LBW) neonates (Silva *et al.*, 2014). According to previous studies, it was reported that 21% to 36% of admitted patients had either bacterial or fungal sepsis with Candidal infection maintaining third most common cause of late onset sepsis in NICU patients and accounts for 9-13% of blood stream infection (BSI) in neonates and associated mortality rate as high as 20-34% (Shettigar and Shettigar, 2018 and Silva *et al.*, 2014). There are some underlying conditions and risk factors that complements the vulnerability of neonatal candidemia and these includes immature immune system and invasive procedure during the stay in NICU, prematurity, very low birth weight, prolonged mechanical ventilation, total parenteral nutrition, use of broad spectrum antibiotic therapy and prolonged hospitalization (Awoniyi *et al.*, 2009; Majeda *et al.*, 2013; Silva *et al.*, 2014; Kapila *et al.*, 2016; Shettigar and Shettigar, 2018).

Out of the 24 (100.0%) samples collected 3 (12.5%) samples were positive for candidemia for the first and third successive culture on differential and selective media. *Candida albicans* is the only Candida specie isolated from the 3 positive samples and this was ascertained from all the result obtained from the colony morphology, fungal staining, Gram staining microscopic examination and Germ tube presumptive test (i.e. 100% prevalence rate).

The prevalence rate of candidemia in this study is 3 (12.5%) and it is contrastable with the prevalence rate 91.7% reported by Mokhtar *et al.* (2014) in Egypt, 69% (Spiliopoulou; *et al.*, 2012), 43.8% (Silva *et al.*, 2014), 39% (Ashwani *et al.*, 2018), 35.18% (Shettigar and Shettigar,

2018), 34.14% (Giuseppina *et al.*, 2017), 27.1% (Daynia *et al.*, 2013), and 26.5% (Mohd *et al.*, 2018). The reason for the differences in the prevalence rate depends on the varied distribution of *Candida* spp. according to the different geographical areas (Giuseppina *et al.*, 2017). *Candida albicans* was the dominant species in Europe with proportions ranging from 47 to 100% and in North and South America with proportions ranging from 40 to 69.2% (Giuseppina *et al.*, 2017), higher rate of premature babies admitted (Mohd *et al.*, 2018), and (abuse of antifungal drugs) self -medication, long-term treatments and repeated Candida infection (Ashwani *et al.*, 2018). Other factors that may contribute to the low prevalence rate may include differences in health care practices or methodology used, social habits of the community, the study design, the environment, the standard of personal hygiene, education and including differences in the examined population (Giuseppina *et al.*, 2017).

In this study, the demographic characteristic of total neonatal blood sample collected revealed highest incidence rate of candidemia was observed among neonates with gestational age (<28 weeks). This incidence rate was in agreement with that reported in South Africa (Daynia *et* al., 2013), in Hyderabad, South Indian (Shettigar and Shettigar, 2018) and in Porto, Portugal (Silva *et al.*, 2014) and not in agreement with some reports of previous studies done which account for mean gestation age of 30.10±2.15 weeks (Ashwani *et al.*, 2018) and 30 weeks or earlier (Giuseppina *et al.*, 2017). This is because all the cases were majorly Early onset neonatal sepsis. Also, the immaturity nature of the immune system of these neonates have been one of the intriguing factor that poses them early infections and diseases.

Out of the 24 blood samples collected, 3 blood samples were positive for candidemia in which one of the neonates positive for candidemia died within 24hours of life. All the patients admitted to the neonatal intensive care unit during the period of this study were premature and of very low birth weight (VLBW). And this is in support with the work carried out by Giuseppina *et al.* (2017). Neonates become colonized with *Candida* spp. either from their mothers, during birth, or from colonized hospital personnel during their stay in the NICU and this is because their immune system is undeveloped to fight against any infections or diseases (Sara *et al.*, 2007). All the 3 cases positive for candidemia were born through vaginal delivery. Some of the previous research carried out reported that *Candida spp* initially colonize infants by vertical transmission during birth or horizontally by parents or caregivers (Sara *et al.*, 2007).

The place of birth of neonates can be said to be one of the intriguing factor of neonatal sepsis. In this study, two-third (2/3) of the birth cases came from the traditional home and just a case from the birth at home. In an ideal hospital setting, the theater set aside for infants' deliveries is always clean and the atmosphere sterile and free from all kind of infections. The in-flow and out-flow of air are kept under appropriate control unlike the traditional home in the local setting, always untidy and the instruments used are unkempt and unsterilized. In this setting, the neonate born are susceptible to various kinds of infections and pathogenic microbes either from the mother, heavily colonized care giver, water and instruments used and the environments. The birth at home also posed an infant to many infections and diseases but in reduced form when compare to that of the traditional home.

The risk factors include in the consent form of this study includes neonate exposure to antibiotics, maternal exposure to antibiotics (pre-natal and post-natal), central venous catheter, parenteral nutrition, mechanical ventilation (>5days) and concomitant bacterial sepsis. The 3 infants whose samples were positive for candidemia have been exposed to antibiotics earlier before they were diagnosed of candidemia.

In this study, 37.5% of the neonates were placed on invasive mechanical ventilation (> 5days) before diagnosis of fungal sepsis. This rate is smaller when compared with those of previous study which account for 69.2% in South Africa (Daynia *et al.*, 2013) and 86.7% in Porto, Portugal (Silva *et al.*, 2014). The differences could be as a result of the number of samples collected, differences in study duration (years), methods and methodology applied. Also, 37.5% cases were reported in this study to have concomitant sepsis before they were diagnosed with candidemia.

There were six distinctive *Candida albicans* isolates obtained from the positive blood samples for the first and third successive culture. It was also observed that sample 21 had two *C. albicans* isolates in its aerobic culture, sample 22 had two *Candida albicans isolates* in it anaerobic culture, sample 24 had two *C. albicans* isolates, one in its aerobic culture in the first successive and one in the anaerobic culture in the third successive stage. These isolates were identified based on their growth pattern on Sabouraud dextrose agar, Yeast extract agar, Blood agar. Their morphological characteristics observed was comparable to the morphological characteristics reported in India by Jasim *et al.* (2016), Khanpur kala in India by Yadav *et al.* (2017) as a white to cream coloured, smooth, glabrous, pasty, convex yeast colonies after incubation at 37^0C for 24 hours (see appendix).

5.1 CONCLUSION

The sporadic annual increase in the incidence rate of bacterial and fungi sepsis among pre-term and full term neonates in the neonatal intensive care unit of hospital has resulted into a corresponding increase in infants' admission, morbidity, and mortality in the under-developed countries in West Africa such as Nigeria, Ghana, Sierra Leone etc. In today's research, though there has been increase in identifying causative agents of bacterial sepsis and its solution unlike the fungal sepsis battling with paucity of information. While neonatal sepsis that results to candidemia are mostly difficult to diagnosed and cure unlike the bacterial sepsis and sometimes result to the death of infected infants. This have become an unavoidable burden on the economy of nations in the world especially the developing countries and under-developed countries which require a quick medical action and response.

5.2 RECOMMENDATION

As a result of the challenges nations faced due to neonatal sepsis, thus calling for urgent attention in antibiotic administration through;

1. Proper recommendation and implementation of standard guidelines for treatment of neonatal sepsis (Fungi and Bacteria).
2. Microbiological research should be encouraged in the discovery of new potent antibiotics through research funding by the government and private sectors.
3. Over the counter drug sales should be curbed nationwide so as to curtail the increase in the antimicrobial resistance in etiological agents.

4. Involvement of government of developing countries in encouragement and creation of awareness on preventive medicine rather than curative medicine and provision of free rapid diagnostic test kits available to every health sectors and health organization.
5. Immunization of newly born infants against pathogenic fungi that attacks neonates at their early age should be implemented globally across the nations.

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APPENDIX

BRAIN HEART INFUSION BROTH (BHIB)

Brain heart infusion broth (BHIB) is liquid medium rich in nutrients, suitable for the cultivation of several fastidious strains of bacteria, such as streptococci, meningococci and pneumococci, fungi and yeasts. BHIB is recommended in Standard Methods for water testing and in antimicrobial susceptibility tests.

Composition: Gelatin Peptone (10.00g/l), Beef Heart Infusion (10.00g/l), Calf Brain Infusion (7.50g/l), Sodium Chloride (5.00g/l), Disodium Phosphate (2.50g/l), Dextrose (2.00g/l).

Preparation

Suspend 37 grams of the medium in one litre of distilled water. Mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. Dispense into appropriate containers and sterilize at 121°C for 15 minutes. The prepared medium should be stored at 2-8°C. The colour is amber. For best results, the medium should be used on the same day or, if not, heated in a boiling water bed to expel the dissolved oxygen and left to cool before using.

FLUID THIOGLYCOLLATE MEDIUM

Fluid Thioglycollate medium is used for sterility testing of biologicals and for cultivation of anaerobes, aerobes and microaerophiles.

Composition: Tryptone (15.000g/l), Yeast extract (5.000g/l), Dextrose (Glucose) (5.500g/l) Sodium chloride (2.500g/l), L-Cysteine (0.500g/l), Sodium thioglycollate (0.500g/l), Resazurin sodium (0.001g/l), Agar (0.750g/l), Final pH (at 25°C) 7.1±0.2.

Preparation

Suspend 29.75 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 25°C and store in a cool dark place preferably below 25°C. Note: If more than the upper one-third of the medium has acquired a pink colour, the medium may be restored once by heating in a water bath or in free flowing steam until the pink colour disappears.

SABOURAUD DEXTROSE AGAR

Sabouraud Dextrose Agar is used for the cultivation of yeasts, moulds and aciduric bacteria from clinical and non-clinical samples.

Composition: Dextrose (Glucose) (40.000g/l), Mycological, peptone (10.000g/l), Agar (15.000g/l), Final pH (at 25°C) 5.6±0.2.

Preparation

Suspend 65.0 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates.

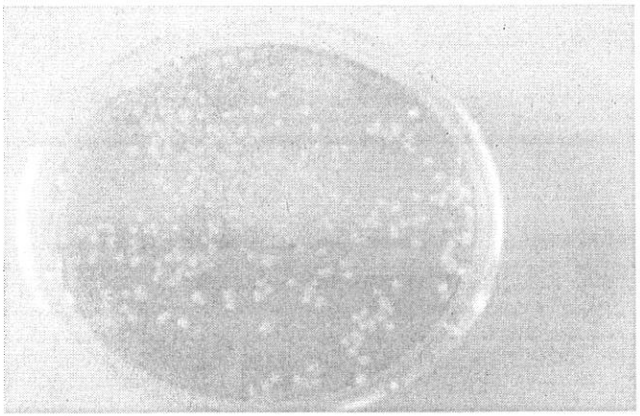

Plate 1: Image of the *Candida albicans* growth on SDA.

BLOOD AGAR

Blood Agar Base is recommended as a base to which blood may be added for use in the isolation and cultivation of fastidious pathogenic microorganisms.

Composition: HM peptone B (10.000g/l), Tryptose (10.000g/l), Sodium chloride (5.000g/l), Agar (15.000g/l), Final pH (at 25°C) 7.3±0.2.

Preparation

Suspend 40.0 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add 5% v/v sterile defibrinated blood. Mix well and pour into sterile Petri plates.

YEAST EXTRACT AGAR

Composition: Peptic digest of animal tissue (5.000g/l), Yeast extract (3.000g/l), Agar (15.000g/l) Final pH (at 25°C) 7.2±0.2.

Preparation

Suspend 23 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.

GRAM STAINING TECHNIQUE

The Gram stain procedure was originally developed by the Danish physician Hans Christian Gram to differentiate *pneumococci* from *Klebsiella pneumonia*. In brief, the procedure involves the application of a solution of iodine (potassium iodide) to cells previously stained with crystal violet or gentian violet. This procedure produces "purple coloured iodine-dye complexes" in the cytoplasm of bacteria

Composition: Gram iodine, crystal violet, acetone, running water, safranin, glass slide, pipette, inoculating loop

Preparation

The first consideration is the correct preparation of the smear. Make a thin film of the material on a clean glass slide, using a sterile loop or swab for viscous specimens. Air dry, then heat fix the slide by passing it several times through a flame (the slide should not become too hot to touch).

1. Flood slide with crystal (or gentian) violet- 60 seconds. 2. Flood with Gram's iodine - 180 seconds. 3. Carefully decolorize with 95% ethanol until thinnest parts of the smear are colorless. (Wash with water). This third step is the most critical and also the one most affected by technical variations in timing and reagents. 4. Flood with safranin (pink color) (10% Fuchsine) - 60 seconds. (Wash with water). 5. Air dry, or blot with absorbent paper.

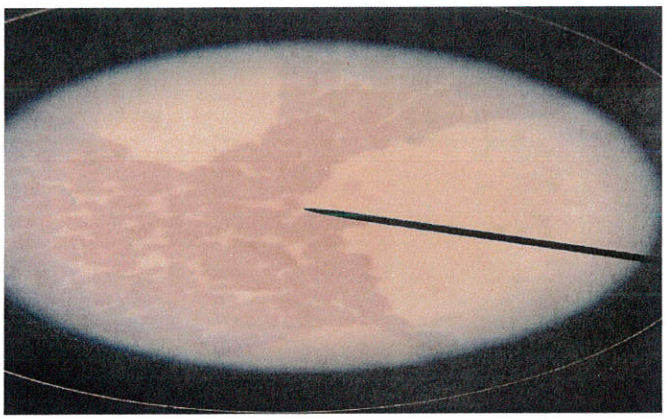

Plate 4: Shows the Gram staining reaction of *C. albicans*.

LACTO-PHENOL COTTON BLUE TECHNIQUE

The lactophenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi and is simple to prepare. The preparation has three components: Lactic acid preserves the fungal structure and clears the tissue while phenol acts as a disinfectant and cotton blue imparts blue colouration to the fungal spores and hyphae(2).

Composition: Phenol crystals (20.000g/l), Cotton blue (0.050g/l), Lactic acid (20.000g/l), Glycerol (20.000g/l), Distilled water 20.000l.

Preparation

1) Place a drop of Lacto phenol Cotton Blue reagent on a clean and dry slide. The stain imparts a blue colouration on hyphae. 2) By using a nichrome inoculating wire, carefully tease the fungal culture, into a thin preparation. 3) Place a coverslip on the preparation. Wait for about 5 minutes. 4)Observe first under microscope with low power for screening in low intensity.

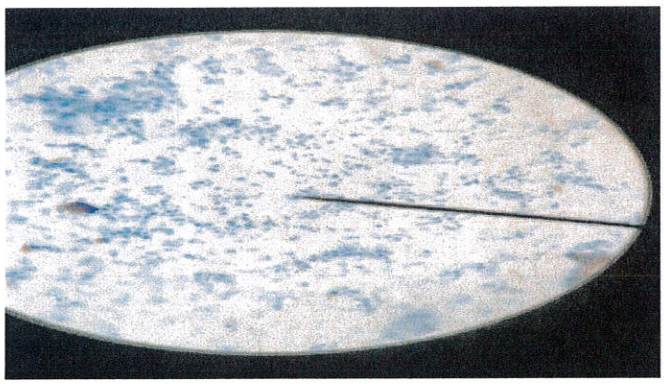

plate 5: Picture of *C. albicans* stained with Lacto-phenol dye view under X40 Objective lens.

WET MOUNT OF *Candida albicans*

A drop of sterile distilled water was placed at the center of a clean grease free glass slide aseptically, and from a 24hours growth culture of *C. albicans*, the mycelium was picked with an inoculating loop and with the dropped distilled water a light smear was made. The smear was allowed to air dry at room temperature. After drying the prepared slide was view at X10 and X40 objective lenses.

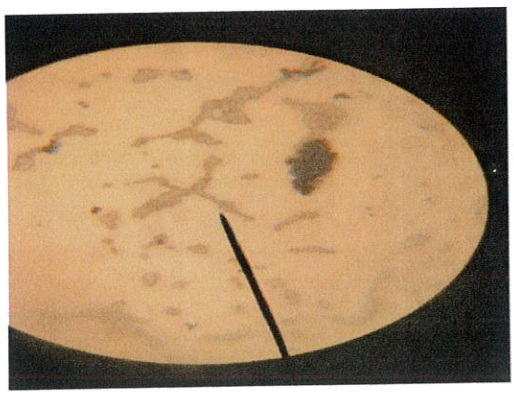

Plate 6

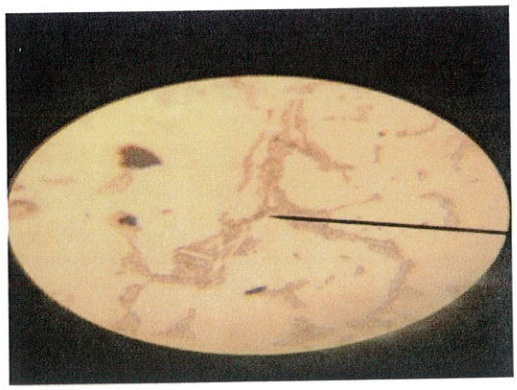

Plate 7

In the diagram above, Figure 3 & 4 represent the picture of *c. albicans* wet mount under X10 and X40 objective lenses.

GERM TUBE TEST

Healthy human blood was collected and allow to fully clot. After clotting the blood sample was centrifuge to separate the serum from other blood components. From a 24 hours' growth culture of *C. albicans*, very small inoculum was picked aseptically with a sterile inoculating loop and was suspended in a test tube containing normal human serum (0.5ml-1ml) by rubbing the loop against the wall of the test tube. The serum becomes little turbid because the pasty colonies have been diluted in the serum. The resulting mixture was incubated for 2-3hrs at 42°c -45°c. after incubation, a drop of mixture was placed aseptically on a clean grease free glass slide and covered with a clean cover slip. The slide was first observed under the low-power objective lens to locate the group of cells and later viewed under X100 objective lens (highest power) of the light microscope.