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Medical microbiology: Detection of biofilm production in candida species from blood stream patients

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***Abstract*---**Candida species are the fourth most common cause of bloodstream infections and are the leading cause of invasive fungal infections among hospitalized patients [1]. Candidemia is a life-threatening fungal infection associated with a mortality rate of 38%; [2].The spectrum of candidemia has changed with the emergence of non- albicans Candida (NCAC) species, a strain with the threat of increased mortality especially in immunocompromised and severely ill patients [4]. It is very important to identify Candida to the species level to optimize the selection of the antifungal agent. More importantly, intrinsic and emerging resistance represents a major challenge for empirical, therapeutic and prophylactic strategies. Candida species have also been isolated from the respiratory tract, mouth, skin, ear and eye. Candida is a true opportunistic pathogen that under certain circumstances is able to invade tissues normally resistant to infection.

***Keywords*---**biofilm production, candida species, blood stream patients.

Introduction

Candida species are the fourth most common cause of bloodstream infections and are the leading cause of invasive fungal infections among hospitalized patients [1]. Candidemia is a life-threatening fungal infection associated with a mortality rate of 38%; [2].The spectrum of candidemia has changed with the emergence of non-albicans Candida (NCAC) species, a strain with the threat of increased mortality especially in immunocompromised and severely ill patients [4]. It is very important to identify Candida to the species level to optimize the selection of the antifungal agent. More importantly, intrinsic and emerging resistance represents a major challenge for empirical, therapeutic and prophylactic strategies. Candida species have also been isolated from the respiratory tract, mouth, skin, ear and eye. Candida is a true opportunistic pathogen that under certain circumstances is able to invade tissues normally resistant to infection.

Candida albicans remains the most common fungal isolate recovered from blood. Recent reports indicate a trend toward an increasing prevalence of infections caused by species of Candida. Disseminated fungal infection is a significant cause of mortality in hospitalized patients. Candida species account for between 70 and 80% of fungal bloodstream infections and collectively represent the fourth most common group of pathogens responsible for nosocomial bloodstream infection, with a mortality rate of approximately 50%. Bloodstream infection by Candida species (candidemia) is the most frequent clinical manifestation of invasive candidiasis. Candida species are the fourth most widespread cause of hospital-acquired bloodstream infections in the United States [3-4], with a frequency of 1.5 cases per 10,000 patients days . In a comparable European survives, the frequency is slightly lower, at 0.5-0.7 cases per 10,000 patients days [5-8]. Currently, the highest reported incidence of healthcare related candidemia 3.7 cases per 10,000 patients days comes from an eleven-center sentinel observation plan in Brazil [9]. In India the authors reported Prevalence of candidemia was found to be 2.68% with the predominant species being Non- albicans candida. *C. tropicalis* (58.8%) was the most common isolate followed by *C. parapsilosis* (29.4%), *C. krusei* (17.7%) and *C. albicans* (5.9%).

Among the Candida species causing invasive infections, *C.albicans, C.parapsilosis, C.tropicalis* and *C.glabrata* account for about 80 to 90% of fungal isolates. Although *C.albicans* remains the species most commonly isolated, other newly emerging Candida species, such as *C.glabrata, C.krusei and C.lusitaniae* are increasingly common [1] . In case of *C.parapsilosis*, its ability to survive in the hospital environment, i.e, on the hands of healthcare workers, on intravenous devices and in solutions, increases the possibility of its nosocomial transmission and outbreaks in intensive care units [3,4].

Formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. Fundamental of biofilm formation is coordination and communication within microbial cells via signaling [through release of acyhomoserine lactone (AHL)] cell to cell called Quorum sensing [1]. Candida remains the fungal species most commonly associated with biofilm formation being a major virulence factor. Biofilms are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a

substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription.

In the blood, a niche they frequently inhabit as commensals, these yeasts exist predominantly within bio- films, which are spatially organised heterogeneous communities of fungal cells encased in a matrix of extracelluar polymeric substances (EPS).[96,97] Candida biofilms can also develop on surfaces of prosthesis and medical devices, and exhibit resistance to both antifungals and host defenses compared with their free-living planktonic counterparts. This is likely to be the cause of recalcitrant persistence of Candida on inert, inserted surfaces or, superficial mucosae.[97]

Biofilms are also the most common mode of bacterial growth in nature and are also important in clinical infections, especially due to the high antibiotic resistance associated with them[4, 11, 46]. Although antifungal treatment appears to reduce mortality and excess hospitalization, the rate of candidemia-attributable mortality remains high (19% to 49%) [8, 25]. Risk factors for candidemia are associated with modern therapeutics, including broad-spectrum antibiotics, hyperalimentation fluids, cancer chemotherapy, immunosuppressive agents following organ transplantation, and indwelling medical devices [17, 19]. In particular, we wanted to determine whether the formation of biofilms by Candida species, which is intrinsically associated with its infectiousness [3, 32,] influenced the clinical outcomes in patients with candidemia. Thus the present study was conducted to detect the formation of biofilm in blood stream patients.

Review of Literature

Candida spp. are among the most common fungal pathogens. They are capable of initiating infections in both immunocompetent individuals and immunocompromised hosts, but the incidence of infections is more in immunocompromised individuals; candidiasis, hence, is rightly called the “disease of diseased” [2]. Candida spp. are responsible for various clinical manifestations ranging from mucocutaneous overgrowth to life threatening disseminated infections like candidemia [4]. Although Candida albicans is the most prevalent species involved in both mucocutaneous and disseminated infections, the incidence of candidiasis due to non-albicans Candida (NAC) spp. is increasing [4]. Several factors like severe immunosuppression or illness, prematurity, use of broad spectrum antibiotics and empirical use of antimycotic drugs are reported to be associated with this change.

NAC species among Candida species is increasing: over the two decades to 1990, NAC represented 10-40% of all candidaemias. Although more than 90% of invasive diseases by Candida are caused by 5 species –*C. albicans, C. glabrata, C. tropicalis, C. parapsilosis and C. krusei* the number of reported species continues to grow. [1] Although *C. albicans* still remains the most common cause of candidemia worldwide, there has been an increase in the isolation of non-albicans Candida species. [1] In the few available studies from India, *C. tropicalis* has been the most common species of Candida isolated from blood. [2],

History of Candida Species

C. albicans, is the most widespread yeast pathogen. This organism is responsible for buccal (oral) thrush,[3]a disease which has been recognized for over 2000 years. The renowned Greek physician, Hippocrates (460–~370 BC), mentions oral thrush: aphthae in the mouth.[4] Thrush ('candidiasis', 'candidosis' or 'moniliasis'— French muguet, German Soor) usually refers to an infection by *C.albicans* of the mucous membranes of the mouth (particularly of babies), the throat or the vagina. Many people have non-clinical infections of *C. albicans*, but its pathogenic effects are often experienced after treatment with antibiotics (which eliminate competing bacteria, as described in 1951 [294]; *C.albicans* itself was identified in the nineteenth century, but most research on its biology and that of other pathogenic yeasts, has been done from the second half of the twentieth century onwards.

Classification

C.albicans are the eukaryotic organism. They reproduce sexually. Many species of Candida have been shown to form ascospores: sexual spores formed in a sac-like structure called the ascus. Candida, therefore, is grouped with the kingdom of fungi, phylum of ascomycota, class of ascomycetes, order of saccharomycetales, family of candidaceae and genus of candida.

Habitat

Candida is a true opportunistic pathogen that under certain circumstances is able to invade tissues normally resistant to infection. Candida is a fungus that normally inhabits the mouth, throat, gastrointestinal tract and vagina. Under normal conditions, candida exists within us in a healthy balance and the body's immune system keeps it from spreading. When your immune system is strong, candida yeasts presents no problem. But, if you have a poor and sugary diet, nutritional deficiencies, exposure to toxins and stress and/or take antibiotics or other medications the good bacteria that prevent fungal infections from developing can get knocked out. Candida yeasts then multiply and further weaken the immune system.

Risk Factors for Candidemia

In the past two decades, a variety of factors like the Acquired Immuno-Deficiency Syndrome (AIDS) epidemic, increased number of patients receiving immunosuppressive therapy for transplantation, the increasing use of antimicrobials in the hospital setups and even in the community have played a key role in altering the epidemiology of invasive fungal infections in general and of candidemia in particular. The importance of risk factors analysis cannot be over emphasized for infections like candidemia so that preventive measures and prophylactic therapy can be initiated for patients at risk. Many studies have established independent risk factors for candidemia on the basis of multivariate analyses. The important independent risk factors include use of broad-spectrum antimicrobials, cancer chemotherapy, mucosal colonization by candida species, indwelling vascular catheters like central venous catheters (CVCs), etc.

Exposure to Long Term Antibiotic Therapy

Long-term antibiotic therapy is one of the most extensively studied risk factors. Exposure to multiple and prolonged use of broad spectrum antimicrobials have been found to be independent risk factors for candidemia. [46] The reason for this being, many of the antibiotics like beta-lactams and vancomycin used in the wards and intensive care unit (ICU) settings lead to the depletion of normal bacterial flora resulting in fungal overgrowth. The increasing use of oral vancomycin in the ICUs results in the depletion of anaerobic bacterial flora of the gut.

Intravascular Catheters and Central Venous Catheters

Intravascular catheters are also one of the important risk factors in the acquisition of candidemia. Candida species adhere avidly to materials used in intravascular catheters and provide a potential nidus for infection.[47.]Some species like *C.parapsilosis* are especially implicated in intravascular catheter-related infections in neonates and in the paediatric age group. The role played by intravascular catheters in perpetuating candidemia has implications for its management. Removal of vascular catheters has been advocated as an adjunctive strategy for treating patients with catheter-related candidemia. However, there is some controversy regarding the benefits and risks of removal of vascular catheters in management of candidemia. [47]

Candida Colonization and Candidemia

The source of BSI with candida species has been a subject of considerable debate in the last couple of decades. Two major sources of infection have been proposed- the gastrointestinal tract (endogenous infection) and the skin (exogenous infection). In the past few years, however, there has been ample evidence pointing towards an endogenous, gastrointestinal origin for candidemia. For some species of candida like c. parapsilosis, however, the skin has been identified as the source of infection. This fact is of clinical importance and c. parapsilosis has been found to be increasingly implicated in BSI after placement of intravascular devices. [46]

Candiduria and Candidemia

Candiduria has been found to be a risk factor for candidemia and can sometimes be an indicator of impending sepsis with candida species in patients admitted to hospitals, especially those in ICUs. A few studies have suggested that as many as 10% of all candiduria cases may be associated with candidemia.[47.]In patients with candiduria, the presence of other risk factors like CVCs, surgical intervention or procedures involving the urinary tract, presence of urinary catheters are significantly associated with development of candidemia. [43]

Surgery and Risk of Candidemia

Surgical procedures in general and gastrointestinal surgeries in particular have been associated with an increased risk of candidemia in patients. Surgical

procedures of the gastrointestinal tract might lead to mucosal disruption and cause seeding of the bloodstream by Candida species which colonize the gut. [48]

Diabetes Mellitus

Few studies have found a greater degree of colonization with candida species in diabetic patients compared to control subjects. [49]Isolates of candida species colonizing diabetic patients have also been found to show a greater degree of resistance to antifungals than strains isolated from control subjects.

Ventricular Assist Devices

Invasive candidemia is also common in Ventricular Assist Devices (VADs) and is associated with poor outcomes. In a study from Washington DC, USA, candidemia developed in 6% of the 117 patients undergoing placement of VADs.[49]Another study found candida species to be responsible for 13.6% of all the BSI among VAD recipients and was second only to Staphylococcus species as a cause of BSI.
50

Candidemia In Patients With Multiple Risk Factors

The presence of multiple risk factors in a particular patient exponentially increases the chances of getting candidemia. For example, it has been demonstrated that a patient receiving eight different antimicrobials and who is colonized with candida species has 832 times higher risk of developing candidemia when compared to a similar patient without antimicrobial therapy. [51]However, the frequency with which many of these risk factors are found in patients admitted in ICUs make them less useful in accurately predicting which patient in the ICU setting will develop candidemia. [46]Many workers have tried to develop risk assessment strategies and calculate "candida scores" to predict the true risk of disease in patients admitted in ICUs Candida risk scores might aid physicians in ruling out candidemia and in identifying those at high risk of candidemia early in the hospital stay.

Risk Factors For Candidemia In Patients Admitted In Surgical ICUs

Among patients admitted in ICUs, those admitted in surgical ICUs (SICUs) are considered to be at a greater risk for developing candidemia.[48]According to a large prospective multicentre study to evaluate risk factors for development of candidemia in SICU patients (NEMIS SICU study), there was a wide variation in infection rates between institutions with the highest rates of candidemia in urban hospitals caring for trauma patients.[46]The risk factors independently associated with development of candidemia in this study were prior surgery, acute renal failure and parenteral nutrition. Many authors have suggested that an elevated APACHE II score can help identify ICU patients who can have a higher risk of developing candidemia, although some studies suggest there is no relationship between the two. [44]Heitner et al compared the risk factors for candidemia in patients admitted in medicine ICUs (MICUs) to those admitted in SICUs and found significant differences between the two. [52]SICU patients had a longer

duration of antibiotic therapy and received a larger number of antibiotics than patients admitted in MICU. SICU patients were also more likely to have other risk factors like TPN and CVCs.[52]

Among the non-albicans candida species, *C. glabrata* has emerged as an important opportunistic pathogen worldwide. It is the second most common yeast isolated as part of normal flora and its role as a pathogen has only been recognized in the past few decades. Trick et al reported a considerable increase in the isolation rate of *c. glabrata* from BSI in U.S ICUs. [52] In a 8-year long study from Michigan, USA, *C. glabrata* was found to be responsible for 17% of 609 fungemic episodes. [53] *C. glabrata* fungemia is seen more often in older adults and is comparatively uncommon in neonates and in the paediatric age group. [53]

Biofilm

Microbial biofilm is defined as 'a structured consortium of microbial cells surrounded by a self-produced polymer matrix', and both monospecies and polyspecies biofilms exist. Biofilms may adhere to surfaces or be situated in the tissue or in secretions and components from the host may be found in biofilms. In 2002, Donlan and Costerton offered the most salient description of a biofilm. They stated that biofilm is "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription."[2]

Candida species are frequently found in the normal microbiota of humans, which facilitates their encounter with most implanted biomaterials and host surfaces. *C. albicans* remains the fungal species most commonly associated with biofilm formation (28,29, 56), and the increase in candida infections in the last decades has almost paralleled the increase and widespread use of a broad range of medical implant devices, mainly in populations with impaired host defenses. The formation of candida biofilms carries important clinical repercussions because of their increased resistance to antifungal therapy and the ability of cells within biofilms to withstand host immune defenses.

Biofilm Formation

Formation of a biofilm is a complex process that follows several distinct phases, beginning with adsorption on to the surface of a conditioning film derived from bacterial and host molecules, which forms immediately following eruption. This adsorption is followed by passive transport of bacteria mediated by weak long-range forces of attraction. Covalent and hydrogen bonds create strong, short-range forces that result in irreversible attachment. The primary colonizers form a biofilm by autoaggregation (attraction between same species) and coaggregation (attraction between different species). Coaggregation[4] results in a functional organization of bacteria and formation of different morphologic structures such as Corncobs and Rosettes. The microenvironment now changes from aerobic/capnophilic to facultative anaerobic. The attached bacteria multiply and secrete an extracellular matrix, which results in a mature mixed-population

biofilm. After one day, the term Biofilm is fully deserved because organization takes place within it. Transmission occurs from other sites, leading to incorporation of new members into the biofilm and the formation of a climax community. The thickness of the plaque increases slowly with time, increasing to 20 to 30 µm after three days.

Methods for Detection Of Biofilm Formation (1).Tissue Culture Plate Method (TCP) [98]

SE disks were placed in 12-well tissue culture plates and incubated in fetal bovine serum (FBS) for 24 h at 37°C on a rocker table (pretreatment phase) (10, 11). The rocker table was used to provide quasi-linear medium flow over the surface of the disks. The disks were then moved to new plates and washed with PBS to remove residual FBS. To ensure uniform biofilm formation on disks, immersed them in a candida cell suspension. Three milliliters of standardized cell suspension, containing 10^7 blastospores/ml, was added to the wells and the disks were incubated for 90 min at 37°C on a rocker table (adhesion phase). The disks were gently agitated and transferred to new plates to ensure the removal of nonadherent cells. The disks were then immersed in YNB medium with 50 mM glucose and incubated for 48 h at 37°C on a rocker table (biofilm formation phase). For controls, disks were processed in identical fashion, except that no candida cells were added. All assays were carried out in quadruplicate and on different days.

Tube Method (TM) [99]

A loopful of test organisms were inoculated in 10 ml of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times.

Congo Red Agar Method (CRA)[99]

CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, 10 g/L and Congo Red indicator 8 g/L. First Congo Red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 minutes) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55°C.[5] CRA plates were inoculated with test organisms and incubated at 37°C for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production.[5] The experiment was performed in triplicate and repeated three times.

Air Liquid Interface Coverslip Assay[100]

Biofilms were grown by subculturing 18 h cultures to a starting OD_{600} of 0.2 and immediately diluting them 1:100 in BHI supplemented with 0.4% glucose. A 5 ml aliquot of each strain was added to each of the 3 wells previously coated with human plasma. Biofilms were allowed to shake at 100 rpm at 37°C for 18 h. To harvest the biofilms, the media was aspirated off and the biomass was washed twice with 5 ml of sterile water. The biomass was allowed to air dry and stained for quantification. To assess biomass, biofilms were stained with 500 µl of 0.1% crystal violet for 10 min at room temperature. Excess dye was removed and biofilms were washed with 5 ml of sterile water. Before solubilization, coverslips were imaged on a Chemi Gel Doc 2000 imaging station (Bio-Rad Life Sciences, Hercules, CA). Biofilms were air-dried and the biomass was dissolved in 500 µl 33% acetic acid. A 100 µl aliquot was transferred to a 96 well plate and measured on an Infinite M200 Tecan plate reader at an optical density (OD) of 635. Biomass was calculated by averaging three coverslips per strain, and at least seven separate experiments were performed with similar results.

Flow Cells (Biofilm Development in Flow Cells Condition) [101]

The flow cell reactor is used in this work. A pure culture of P. fluorescens is grown in a 1L glass vessel (fermenter 1), aerated and agitated, continuously fed with a sterile nutrient solution consisting of 5 g glucose L-1, 2.5 g peptone L-1 and 1.25 g yeast extract L-1, in phosphate buffer at pH 7. This culture was used to continuously inoculate a 3 L vessel (fermenter 2) containing sterile water. The fermenter was fed with the medium described above and the bacterial culture was diluted with filtrated tap water in order to obtain a suspension with 6x107 cells mL-1 and 20 mg L-1 of glucose. Biofilms were formed on the stainless steel plates placed inside two similar flow cell reactors. One of them was used to promote laminar flows (Re=2000, u=0.21 ms-1) and the other one turbulent flows (Re=5500, u=0.56 ms-1).

Other methods also used for the formation of biofim are :-

- Atomic Force Microscopy (AFM)
- Confocal raman
- Molecular characterization of biofilm using 16s rDNA
- Insitu hybridization with16s r DNA

Treatment

Treatment of Candida infections depends on a number of factors:

- The anatomic site of the infection.
- The patient's underlying illness and immune status.
- The patient's risk factors for disease.
- The specific species of Candida responsible for infection.
- The susceptibility of the infecting strain to antifungal drugs.

Candidemia is the most common manifestations of invasive candidiasis. Candida in a blood culture should never be viewed as a contaminant and should always prompt a search for the source of the bloodstream infection. For many patients, candidemia is a manifestation of invasive candidiasis that could have originated in a variety of organs, whereas for others, candidemia originated from an infected indwelling intravenous catheter [1]. *C. glabrata* has been considered a relatively non-pathogenic member of the normal flora of human mucosal tissues. With the increased use of immunosuppressive drugs persons infected with human immunodeficiency virus are at particular risk of infection with *C. glabrata*. At the present time approximately 15-20% of cases of invasive candidiasis are caused by *C. glabrata.* Strains of *C. glabrata* are frequently resistant to antifungal drugs. *C. krusei* is known as an important fungal pathogen for patients with hematological malignancies and for transplant patients. This organism is of medical importance because of an intrinsic resistance to some of the antifungal drugs Ketoconazole and Fluconazole. *C. krusei* is also poorly susceptible to many other antifungal medications. *C. parapsilosis* is a common non-albicans species isolated from blood cultures. The frequency of bloodstream infection with this species has increased in recent years. Fortunately, candidemia due to this species is associated with a lower mortality rate than bloodstream infections caused by other candida species. *C. parapsilosis* is known for its ability to form biofilms on implanted medical devices, such as vascular catheters. This organism persists in the hospital environment and may be spread to patients by medical personnel. *C. tropicalis* is an important cause of candidemia in patients with leukemia and in those who have undergone bone marrow transplantation. Some strains have inherent resistance to Amphotericin B, the newer Azole drugs, and to Echinocandins. *C. dubliniensis* is associated with oral infection of diabetics and AIDS patients. Occasionally, *C. dubliniensis* had been recovered from systemic infections.

Aims and Objectives

- To isolate Candida species from blood stream samples.
- To study the biofilm formation of Candida species isolated.

Material and Methods

Study Design

A cross sectional prospective study.

Sample Size

90 strains of Candida spp. were collected from blood stream patients.

Place of Study

The present study was conducted in the Department of Microbiology, Santosh Medical College.

Inclusion Criteria

Candida spp isolated from In patients and OPD with high clinical suspicion of fungemia.

Exclusion Criteria

Duplicates isolates from same patients were excluded from the study.

Sample Collection

Strains were collected from the patients who visited OPD and Inpatients from Santosh Medical College & Hospital from blood stream infected patients.

Identification of Candida

Culture

- With a swab, the sample was inoculated into Sabouraud's Dextrose Agar tube .
- Fungal culture tubes were incubated at 37° C and were observed for 24-48 hours.
- Examine regularly for one week and then discarded.

Composition of SDA [82]

Ingredients	Gms / Litre
Dextrose	40.000
Mycological, peptone	10.000
Agar	15.000
Distilled water	1000ml

Final pH (at 25°C) 5.6±0.2 **Formula adjusted, standardized to suit performance parameters Suspend 65 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 and allow to cool and poured into sterile petridishes . These were put in plastic sleeves and refrigerated for 4-6 weeks.

Identification of Candida Species

Identification of candida isolates were done on the basis of macroscopic appearance, colony morphology on SDA, microscopic appearance, Germ tube formation and growth on Crome agar, differential growth on SDA at 37^0C and 42^0C . Germ tube formation test was also done . For further speciation subculture was done on Hi Chrome agar media.

Macroscopic Examination

Fungal culture tube were observed for :

- Color
- Texture
- Growth rate
- Reversed side of the colony were observed for :
- Color
- Pigment

Colony Morphology on SDA

Obverse side – creamy smooth and pasty colonies Reverse side – no color and pigment

Fig- 1 Candida Species on SDA Agar

Microscopic Examination

Preparation of Smear

With a sterile cooled loop, place a drop of sterile water or saline solution on the slide. Sterilize and cool the loop again and pick up a very small sample of a colony and gently stir into the drop of water/saline on the slide to create an emulsion. The smear was then subjected to gram staining.

Gram Staining[80]

Procedure[80]

1. Prepare and heat-fix smears.
2. Flood the crystal violet for one minute.

3. Pour off excess dye and wash gently in tap water and drain the slide against a paper towel.
4. Expose the smears to Gram's iodine for one minute.
5. Wash with tap water and drain carefully. (Do not blot.)
6. Wash with 95% alcohol for 30 seconds.
7. Wash with tap water at the end of the 30 seconds to stop the decolorization.
8. Counterstain with 0.25% safranin for 30 seconds.
9. Wash, drain, blot, and examine under oil.

Interpretation[81]

Gram reaction – Gram positive
Morphology – Yeast cells of approximately 4-8 micrometer seen with / without budding cells.
Arrangement – Presence and absence of pseudohyphae

Quality control

Positive control- Staphylococcus aureus ATCC 25923 Negative control – Escherichia coli ATCC 25922
Gram staining was done by growth on culture media as described earlier.

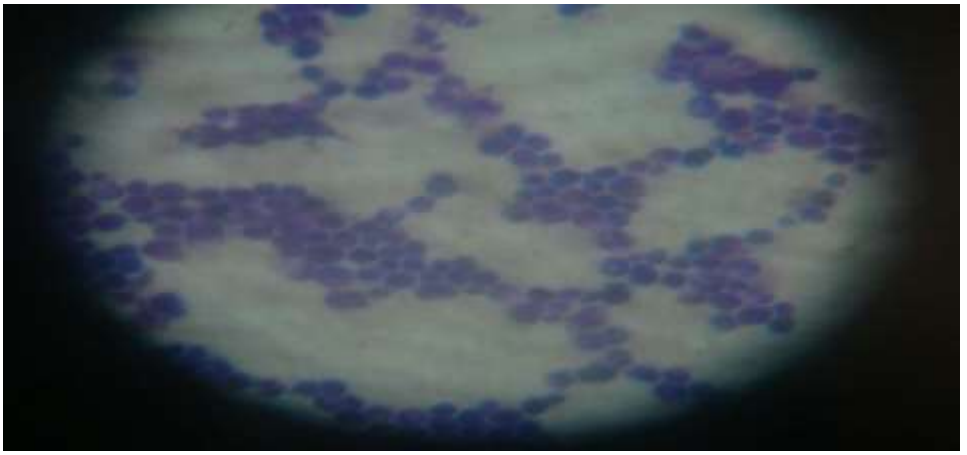


Fig- 2 Gram Staining Of Candida Species

Germ Tube Test[83] Principle

The germ tube is a screening procedure used to differentiate the candida albicans from other yeast . Approximately 95-97 % of candida albicans isolated develop germ tubes when incubated in a proteinaceous media. Germ tubes are short non-septate germinating hyphae. They are ½ the width and 3- 4 times the length of the cell from which they arise. The junction of the germ tube and cell is not constricted. Buds and pseudo-hyphae can be distinguished from germ tubes by the constricted attachment.

Procedure [84]

1. Put 3 drops of serum into a small Vitek tube.
2. Using a Pasteur pipette, touch a colony of yeast and gently emulsify it in the serum. The pipette can be left in the tube.
3. Incubate at 37° C for 2-4 hours but no longer.
4. Transfer a drop of the serum to a slide for examination
5. Coverslip and examine microscopically using 40X objective.

Quality control

Positive- C.albicans (ATCC10231)
Negative- C tropicalis (ATCC13803)

Interpretation [85]

Germ tubes are appendages half the width and 3 to 4 times the length of the yeast cell from which they arise. There is no constriction between the yeast cell and the germination tube

Positive Test: Presence of short lateral filaments (germ tubes)
Negative Test: Yeast cells only.

Fig- 3 Germ Tube Formation Of Candida.

Chrome Agar Candida Media[86]

CHROMagar™ Candida is a selective medium for the isolation and presumptive identification of yeast and filamentous fungi and differentiation of Candida species.

Principle

Specially selected peptones supply the nutrients in CHROMagar Candida. The chromogen mix consists of artificial substrates (chromogens), which release

differently colored compounds upon degradation by specific enzymes. This permits the differentiation of certain species, or the detection of certain groups of organisms, with only a minimum of confirmatory tests. Chloramphenicol inhibits most bacterial contaminants.

Composition

Approximate Formula* Per Liter Purified Water	
Chromopeptone	10.0 g
Glucose	20.0 g
Chromogen Mix	2.0 g
Chloramphenicol	0.5 g
Agar	15.0 g

Adjusted and/or supplemented as required to meet performance criteria.

Procedure

a. Streak the specimen onto a CHROM agar petridish.
b. Incubate plates at 35 ± 2°C in an aerobic atmosphere.
c. Examine plates after 36–48 h for amount of growth and color formation.
d. After proper incubation, read plates against a white background. Plates from specimens containing yeasts will show growth.

Organisms	ATCC™	Recovery	Colony Color
Candida albicans	60193	Fair to heavy growth	Light to Medium Green
Candida albicans	10231	Fair to heavy growth	Light to Medium Green
Candida krusei	34135	Fair to heavy growth	Mauve to Rose Pink
Candida tropicalis	1369	Fair to heavy growth	Dark Blue to Metallic Blue
Candida tropicalis	9968	Fair to heavy growth	Grey Blue
Candida krusei	24408 5	Fair to heavy growth	Pink and Fuzzy
Candida glabrata	15126	Fair to heavy growth	Cream to White

Other species → white to mauve

Fig- 4 Growth of Candida Species on Chrome Agar.

1. Purple –*C.krusei*
2. Purple –*C.krusei*
3. Green – *C.albicans*
4. Metallic blue – *C.tropicalis*

Biofilm Formation[87]
Safranine Tube Method

Procedure

- A loopful of organisms from Sabouraud's Dextrose Agar (SDA) plate was inoculated into tube containing 10 ml Sabouraud's Dextrose broth supplemented with glucose (Final concentration 8%).
- The tubes were then incubated at 37 C for 24 hrs.
- After which the broth was aspirated out and the walls of the tubes were stained with 1% safranine.
- Tubes were then kept still for 7 minutes.
- Safranine then was removed and tubes were examined for biofilm production. Slime production was scored by two observers simultaneously twice each to reduce as much as possible intra and inter observer's difference.

Interpretation

- Weak positive - (1+)
- Moderate positive - (2+)
- Strong positive - (3+)

Results

Of the total Candida strains isolated from BSI patients**,** majority were males (63%) while females were less in number (37%).

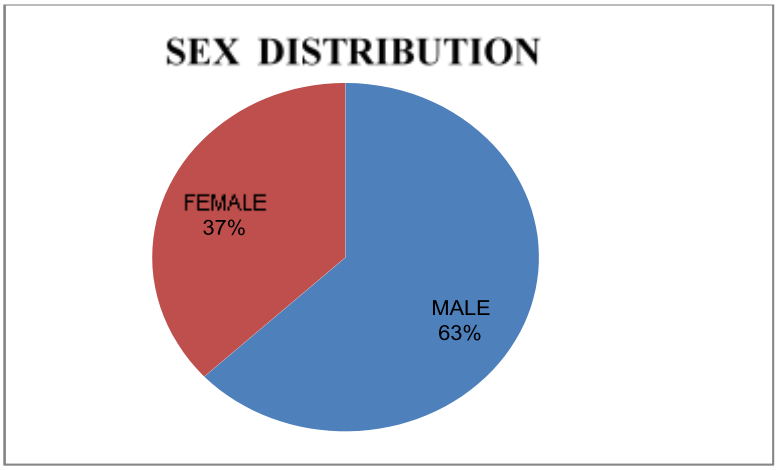
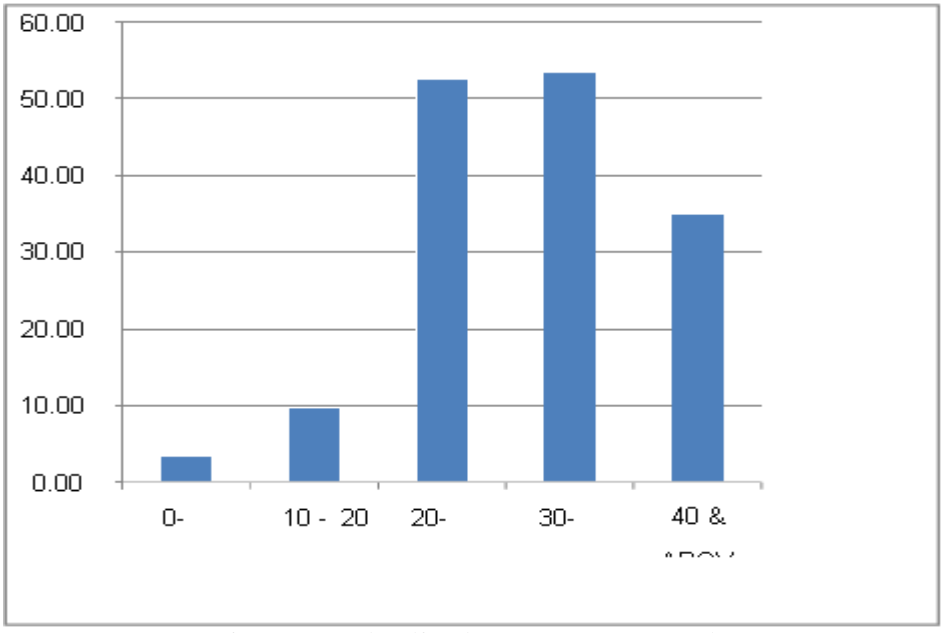


Fig 5- Sex Distribution of Candida Strains.

Age Distribution



Graph 1- Age Distribution Among BSI patients.

Maximum isolation was found in the age group of 30 to 40 years. Species distribution from candida strains isolated from blood stream patients.

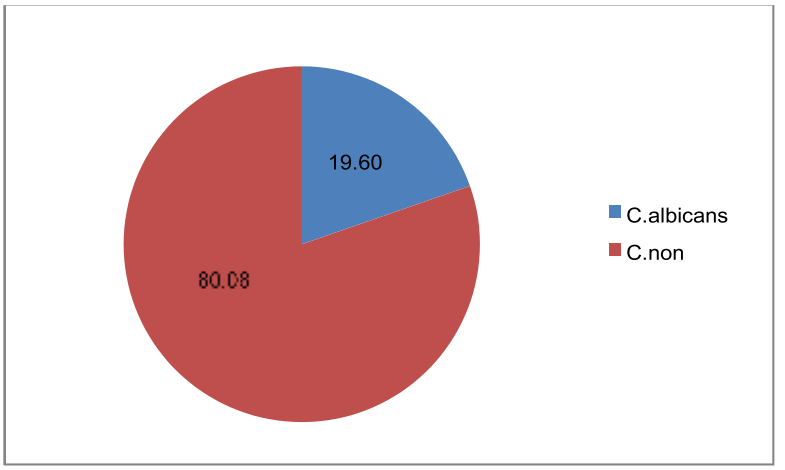


Fig.- 6 Distribution of C.albicans and Non Albicans Candida species Among BSI Patients

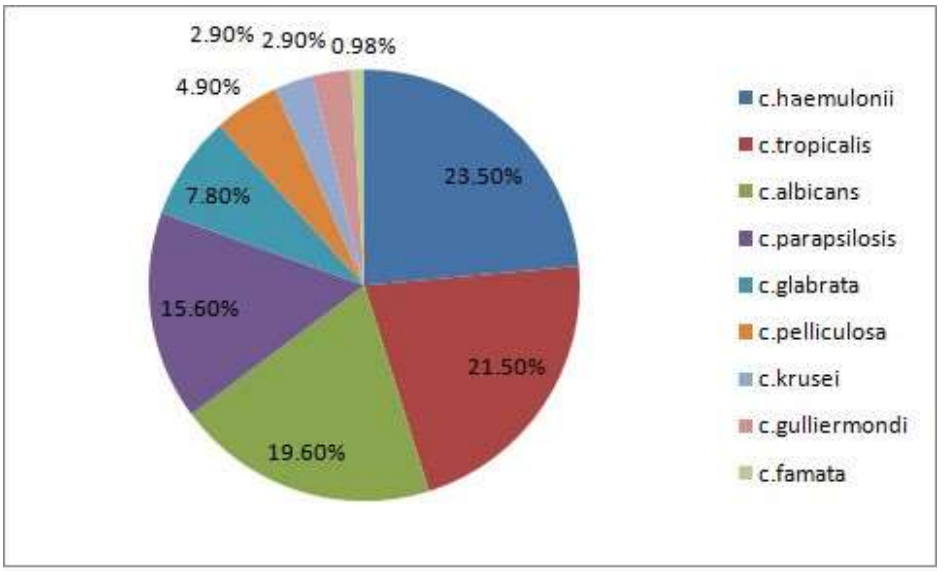


Fig-7 Candida Species Distribution Among BSI Patients.

The most common Candida spp. isolated was*: C. haemulonii* (23.5%), followed by *C. tropicalis* (21.5%), *C. albicans* (19.6%), *C. parapsilosis* (15.6%), *C. glabrata* (7.8%), *C. pelliculosa* (4.9%), *C. krusei* and *C. gulliermondii* (2.9%) and *C. famata* (0.98%). Of the total of 90 strains with Candida species 28.8% showed biofilm production.

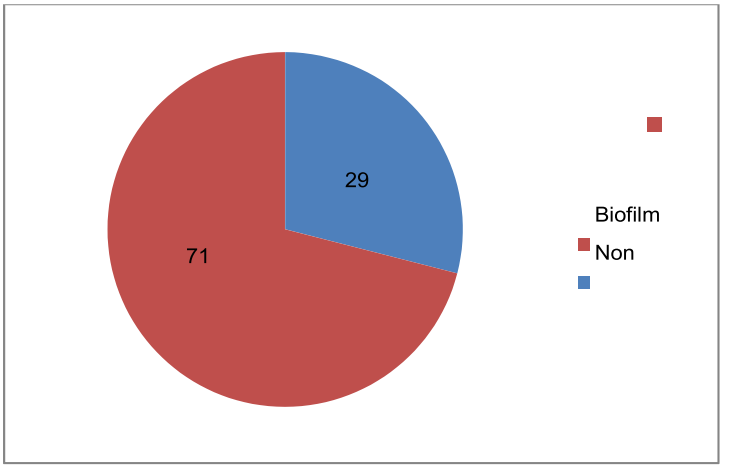


Fig 8- Distribution of Biofilm Formation.

Table.1 Distribution of Biofilm production in Candida spp.

Species	No. of species	Positive	1+	2+	3+	Total	Percentage
C.haemulonii(21)	21	6	3	3	0	6	28.57%
C.tropicalis(21)	21	7	4	3	0	7	33.33%
C.albicans	18	9	6	2	1	9	50%
C.parapsillosis	14	3	2	1	0	3	21.42%
C.glabrata	7	1	1	0	0	1	14.28%
C.pelliculos	4	0	0	0	0	0	0%
C.krusei	2	0	0	0	0	0	0%
C.gulliermondi	2	0	0	0	0	0	0%
C.famata	1	0	0	0	0	0	0%
Total (90)						26	

Only 26(29%) of candida spp. isolates produced biofilm. Strong biofilm production was seen in *C.albicans*, followed by *C.haemulonii* and *C. tropicalis*. Weak biofilm production was seen in *C.famata* followed by,*C.krusei, C.gulliermondi*. whereas moderate biofilm production was seen in *C.parapsillosis* and *C.glabrata* as showed in Table 1.

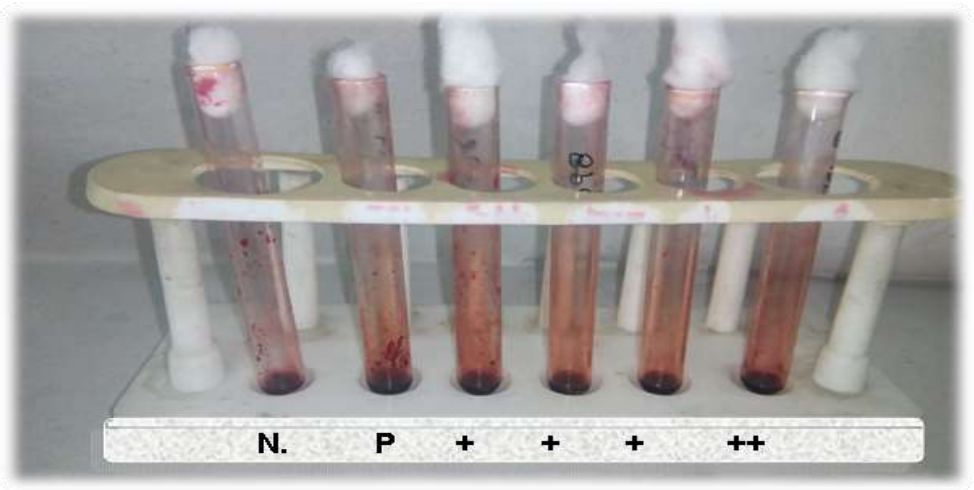


Fig 9 - Biofilm Formation of Candida isolates.

- N.C. – Negative control
- P. – Positive control
  - + - Weak positive
  - ++ - Moderate positive
  - +++ - Strong positive

Discussion

Candida is an asexual, diploid, dimorphic fungus that is present on humans and in their environment . A relatively small number of *Candida* species are pathogenic for humans. These organisms are capable of causing a variety of superficial and deep-seated mycoses such as cutaneous, mucocutaneous, subcutaneous, or systemic candidiasis. *Candida* organisms are commensals; and to act as pathogens, interruption of normal host defenses is necessary. Therefore, general risk factors for *Candida* infections include immunocompromised states,diabetes mellitus, and iatrogenic factors like antibiotic use,indwelling devices, intravenous drug use, and hyperalimentation fluids. Candidemia has emerged as an alarming opportunistic disease as there is an increase in number of patients who are immunocompromised, aged, receiving prolonged antibacterial and aggressive cancer chemotherapy or undergoing invasive surgical procedures and organ transplantation.[19]

In the present study 90 Candida strains was collected from blood stream patients [(53)]. Of the 90 strains of Candida, 63% isolates were from male patients and 37% isolates were from female patients, of which 5% found to be in below 10 year age group and 35% in 30-40 age group. In a study by Silva, et al.[23] (2009) s prevalence of Candida carriage was seen in 10% in children and 60% in adult group. The present study showed the distribution of *Candida* species only by percentage found that C albicans gave 19.6% while non-*Candida albicans* gave 80.08% positivity from blood stream isolates. Our study showed maximum isolation of Candida species in *C.haemulonii* 21(23.5%) followed by *C.albicans*

18(19.6%) and *C.tropicalis* 21(21.5%) comprised the highest isolates in BSI while the *C.parapsillosis* 14(15.6%)and

C.glabrata 7(7.8%) comprises the moderate isolates but *C.krusei* 2(2.9%) and *C.famata* 1(0.98%) isolates was the least isolate found. However, in a study done by Saroj Golia et al[42] they reported *C.albicans* (35.7%) to be the most common species followed by *C. tropicalis* (26.7%), *C. parapsilosis* (19.6%), Candida *glabrata* (13.6%), and *C. krusei* [4].

According to Taff HT et al., [3] and Jin Y et al., [7] they reported *C.albicans* was the most common species (505 [49.3%] of 1,068 isolates) followed by *C. parapsilosis*(19.9%)and *C. glabrata* (1.4%). *C. tropicalis*, *C. krusei*and *C. du bliniensis* were identified in 5.1%, 4.3%, and 1.9%, respectively, of the BSI patients. The remaining 42 (4.0%) isolates were caused by *C.guilliermondi* (n = 11), *C. lusitaniae* (n = 7), *C. kefyr* (n = 5), *C. famata* (n = 3), *C. rugosa* (n = 3), and *C.pelliculosa* (n = 3).

In another study, Ruzicka et al.[19] noted that out of 147 isolates of *Candida*, they detected isolation in 79 (53.7%). The most common isolate from samples was *C. krusei*. *C. albicans* (41.37%). The remaining species isolated from the blood samples were *C. tropicalis* (25.5%), followed by *C. parapsilosis* (16.6%), *C. glabrata* (8.8%), *C. pelliculosa* (4.8%), *C. krusei* and *C. gulliermondii* (2.5%).[19] Biofilms may help maintain the role of fungi as commensal and pathogen, by evading host immune mechanisms, resisting antifungal treatment and withstanding the competitive pressure from other organisms. Consequently, biofilm related infections are difficult to treat.[16].[17]

Girishkumar CP et al., [6] studied biofilm production and they found that *C. albicans* gave 42.9 % while *non-Candida albicans* Candida gave 93.1% biofilm positivity from blood stream isolates. In our study biofilm positivity occurred most frequently in isolates of *C.haemuolini* followed by *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. parapsilosis*, *C. glabrata*, *C. famata* and *C. albicans*. In contrast, Hawser et al[18] reported that isolates of *C. parapsilosis* and *C. glabrata* were significantly less likely to produce biofilms than the more pathogenic C. albicans. Biofilm production in this study was more related to the species of Candida than to the site of infection.

In present study only 29% of Candida strains produced biofilm, Candida species isolated producing strong biofilm production was seen in *C.albicans* (50%) , followed by *C.tropicalis* (33.33%), *C.haemulonii* (28.57%) , *C.parapsilosis* (21.42%), Weak biofilm production was seen in *C.glabrata*(14.28%) from BSI samples. (15). In contrast, with Vinitha et al[17] in which a total of 81(73%) out of 111 Candida species isolates obtained from the clinical isolates produced biofilm. Candida species isolates producing slime strong biofilm production was seen in *C. albicans*. Weak biofilm production was seen in *C.krusei* and *C. tropicalis*.

In contrast to our study, Shin et al. (37) (11) showed that isolates of *C. parapsilosis*, *C. pseudotropicalis* and *C. glabrata* made significantly less biofilm than *C. albicans*, which is more pathogenic. Our study found that *C. albicans* isolates consistently produce more biofilm in vitro than non-*C. albicans* isolates

(19). On the other hand, NCAC species, particularly *C. tropicalis* and *C. parapsilosis*, appear to form biofilms readily when grown in SDB medium containing 8% glucose (37).

Fungal biofilm formation is a complex phenomenon distinct from adhesion. It is best studied using pathogenic species grown on relevant bioprosthetic materials under near-physiologic conditions. Study of such systems will reveal the true nature of fungal biofilms and their biology. Demonstration of common biofilm features across different taxa(15) extends the implication of this study beyond fungi to other organized cellular communities. The impact of this information will be widespread, ranging from new environmental microbiology insights to the development of antimicrobials specifically targeted against biofilm-associated infections.

Summary and Conclusion

- In the present study total number of 90 Candida strains were selected.
- Candida strains from males were 63% while from females were 37%.
- Maximum rate of isolation was reported in the age group of patients above 30 years of age and least in 0-10 years of age group.
- Out of 90 Candida strains, Candida albicans were identified in 19.6% - samples while the remaining 80.08% isolates were identified as Non Candida albicans.
- The distribution of Candida species isolated from blood stream patients was maximum with C.haemulonii 21(23.5%) followed by *C.tropicalis* 21 (21.5%), *C.albicans* 18(19.6%), *C.parapsillosis* 14(15.6%) , *C. glabrata* 7(7.8%), *C.famata* 1(0.98%) and *C.krusei* 2(2.9%).
- Of the total 90 strains 29% showed biofilm production while 71% did not show biofilm formation.
- High biofilm producers among Candida species were *C.haemulonii* 21(23.5%) followed by *C.albicans*18(19.6%) and *C. tropicalis* 21(21.5%)while low biofilm producers were *C.krusei* 2(2.9%) and *C.famata* 1(0.98%)
- Thus these species possess a threat in the future due to commonly used antifungal drugs as microorganisms in biofilms.
- As only limited number of Candida isolates could be tested in this study, further clinical studies need to be performed involving more number of isolates.
- A better knowledge of molecular events in Candida biofilm formation could present new strategies to prevent Candidemia.

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