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RESEARCH ARTICLE
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INCIDENCE OF BIOFILM FORMATION IN VARIOUS CANDIDA SPECIES ISOLATED FROM CLINICAL SAMPLES AT GOVERNMENT MEDICAL COLLEGE, KOTA.

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Abstract
Candida species are typical skin and mucosal dwellers. Given the rise in these infections during the past ten years, the significance of epidemiological monitoring of yeasts implicated in pathogenic processes is undeniable. Clinical samples were obtained and cultured from the respiratory tract (sputum, bronchial wash, tracheal secretions), saliva, blood, urine, middle ear discharge, vitreous fluid, corneal ulcer, and plastic devices (endotracheal tube, catheter tip, suction tip). The isolated species of Candida were identified. From 270 different clinical sources, 100 isolates of Candida species were found. The most isolated species was Candida albicans (39.5 %), however the most common species was Candida non-albicans (60.5%). C. albicans (20.58%) and C. krusei (38.23%) were commonly isolated in blood cultures. The most common species on the mucosal surface was Candida albicans (63.27%). Hospitalized patients were more likely to have urinary tract infections brought on by yeasts, with C. krusei (50.0%) and C. albicans (25.0%) being the most often isolated. The pathogenecity is influenced by a number of virulence factors, including biofilm, proteinase, phospholipase, etc. Clinical decision-making benefits from early discovery of Candida virulence factors. Therefore, our goal has been to show how biofilm forms using the technique suggested by Branchini et al. (1994). The Staib et al. (1965) approach was used to determine the amount of proteinase generated by Candida. The phospholipase assay was performed using the Samaranayake et al. (2005) method. Conclusions: The findings points to the possibility that the pathogenic potential of the isolates may be reflected in the ability of Candida species to form biofilm. Strong slime production was demonstrated by C. tropicalis and C. krusei. Compared to Candida albicans, the non-Candida albicans produced more proteinase. In this investigation, Candida albicans produced more phospholipase than non-Candida albicans.

Keywords-Candida species, biofilm, Infection, fungi

Introduction

The skin and mucosa are typically home to Candida species. Given the rise in these infections over the past ten years, as well as the changes in species causing candidiasis and empirical antifungal treatment, the significance of epidemiological monitoring of yeasts implicated in pathogenic processes is undeniable.[1] Other Candida species have also become clinically significant

opportunistic pathogens, even though Candida albicans is the organism most frequently linked to serious fungal infections. To aid in colonization, invasion, and pathogenesis, the majority of pathogens, including Candida species, have evolved a potent arsenal of suspected virulence factors and particular tactics. The type of infection, the location and stage of the infection, and the type of host response can all affect the virulence factors that Candida species express to cause infections. The generation of acid proteinase, phospholipase, and biofilm formation are the primary virulence agents. While phenotypic switching or platelet coating may be employed to avoid the immune system, enzymes aid adherence once contact is established by breaking down cell membranes and extracellular proteins, allowing the yeast to penetrate the host. The development of clinical infection depends on biofilms, which are organized microbial populations connected to and enclosed in an exopolymeric material matrix [2]. A crucial part of the pathophysiology of candidiasis is played by the outermost layers of Candida cells, which are necessary for adhesion to the host surface.[3] Protection from the environment, nutrition availability, metabolic cooperation, and the acquisition of new genetic features are all benefits of creating a biofilm. During infection, pathogenic species of Candida release aspartyl proteinases in vivo.[4] When the organism is cultivated with foreign protein—typically bovine serum albumin—as the nitrogen source, the enzymes are secreted in vitro. The synthesis of proteases is thought to improve the organism's capacity to infiltrate and colonize host tissues as well as to elude the host immune system.[5] Phospholipase enzymes are linked to adhesion, penetration, and host cell membrane degradation. When microorganisms invade host cells, the outer cell membrane is penetrated and damaged. According to preliminary evidence, the primary processes for fungal virulence appear to be direct host cell injury and lysis.

MATERIALS AND METHODS

Patients receiving care in Kota government hospital and assisted living facilities provided 270 distinct clinical samples. Before collection, the patients had never been exposed to antifungal medications. 112 samples were taken from the respiratory tract (sputum, bronchial wash, tracheal secretion) and saliva, 130 from blood, 12 from urine, 2 from middle ear discharge, 1 from vitreous fluid, 1 from corneal ulcer, and 9 from plastic devices (endotracheal tube, catheter tip, suction tip).

In 10% KOH, every respiratory specimen and exudate was analyzed. The smears were also analyzed and stained with gram stain. As the primary isolation medium, Sabouraud's dextrose agar was used to inoculate the samples. SDA biphasic medium containing gentamicin and chloramphenicol was utilized for blood samples. For a week, or longer if necessary, the culture media was incubated at 37°C.

By evaluating the production of germ tubes, pellicles, assimilation, and sugar fermentation, the species was identified. To demonstrate the presence of chlamydospores, they were cultivated on cornmeal agar. The species was identified via culture on candid chrom agar.

Using a technique suggested by Pfaller MA et al., biofilm development was assessed for each isolate as well as the standard strains. [6] Ten milliliters of Sabouraud's liquid medium supplemented with glucose (final concentration of 8%) were added to a tube containing a loopful of organisms from the SDA plate. Following a 24-hour incubation period at 37°C, the tubes' walls were stained with safranin and the broth was aspirated out. A score of zero (0+), weak positive (1+), moderate positive (2+), or strong positive (3+) was assigned to biofilm formation.

The significantly modified Hendry AT et al. method [7] was used to detect the Candida proteinase using bovine serum albumin medium (dextrose 2%, KH2PO4 0.1%, MgSO4 0.05%, agar 2% mixed after cooling to 50°C with 1% bovine serum albumin solution). Inoculating 10 µl aliquots of the yeast suspension (about 108 yeast cells/ml) into the wells punched onto the medium's surface allowed for the detection of proteinase activity. For two days, the plates were incubated at 37°C. Following incubation, the plates were dyed with 1.25% amidoblack and fixed with 20% trichloracetic acid. 15% acetic acid was used for decolorization. Degradation of the protein was shown by opaqueness of the agar, which corresponded to a zone of proteolysis surrounding the wells that could not be stained with amidoblack. Proteinase production was measured using the diameter of the unstained zones

surrounding the well. The ratio of the well's diameter to the proteolytic unstained zone's diameter was used to calculate the proteinase activity (Pz). There was no proteinase activity in the strain when Pz = 1. Therefore, low Pz indicates high enzyme synthesis. Phospholipase was estimated using a slightly modified version of Samaranayake et al.'s approach [8]. 13.0 g Sabouraud dextrose agar (SDA), 11.7 g NaCl, 0.111 g CaCl2, and 10% sterile egg yolk made up the egg yolk medium. Twenty milliliters of the supernatant were added to the sterilized medium after the egg yolk was centrifuged at 500 g for ten minutes at room temperature. By inoculating 10 µl aliquots of the yeast suspension (about 108 yeast cells/ml) into the wells punched onto the surface of the egg yolk medium, extracellular phospholipase activity was found. Following a 48-hour incubation period at 37°C, the diameter of the precipitation zone surrounding the well was measured. The Pz value, or phospholipase activity, was calculated. There was no phospholipase activity in the strain when Pz = 1. Therefore, low Pz indicates high enzyme synthesis.

Results

Of the 100 isolates, 39 were Candida albicans, 7 were Candida glabrata, 4 were Candida guilliermondi, 2 were Candida kefyr, 31 were Candida krusei, 4 were Candida parapsilosis, and 8 were Candida tropicalis. Table 1 displays the distribution of Candida species in various clinical samples.

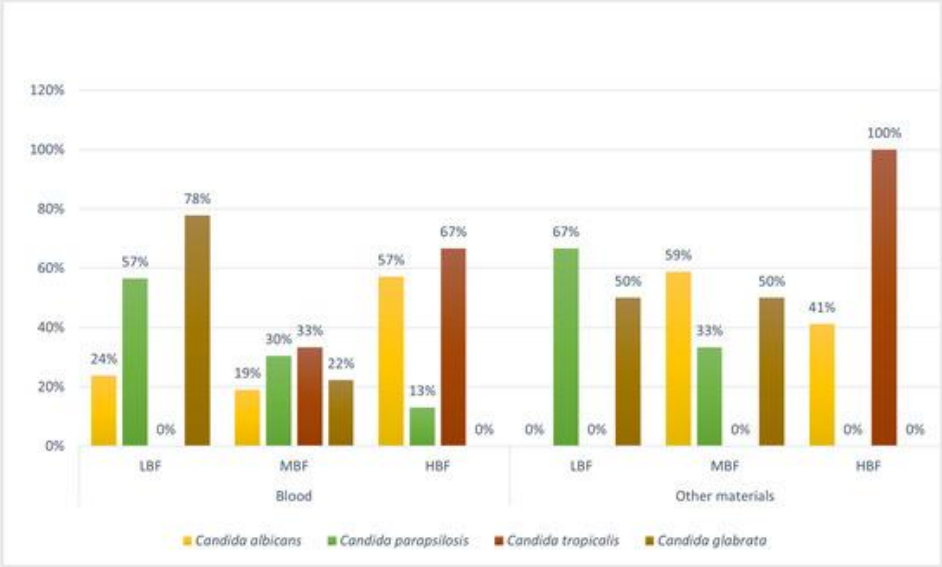
Out of the 100 Candida species isolates that were recovered from the clinical isolates, 73 (73%) of them formed biofilm. Compared to the percentage of all non-albicans Candida species isolates that generated slime (90.32%, 55 of 61; P<0.0001), only 51% (20 of 39) of C. albicans isolates produced biofilm. C. tropicalis and C. krusei exhibited strong biofilm formation. C. albicans was found to produce weak biofilms. In 89 (80.18%) of the isolates, proteinase activity was found. C. albicans (Pz 0.18), C. kefyr (Pz 0.16), and C. guilliermondii (Pz 0.17) produced the most proteinase, whereas C. glabrata (Pz 0.29) produced the least.

Of the isolates, 49 (44.14%) had phospholipase activity. C. guilliermondii produces the most phospholipase (Pz 0.07), followed by C. parapsilosis (Pz 0.08). C. tropicalis is the least productive (Pz 0.27).

Figure 1 illustrates the generation of biofilm, proteinase, and phospholipase by Candida species isolated from clinical specimens.

Table 1: *Candida* species isolated from different clinical samples

Source of clinical isolates	Respiratory tract	Blood	Urine	Plastic devices	Eye	Middle ear discharge	Pus	Total
Positive isolates	42	30	12	9	2	2	3	100
C. albicans	27	6	3	3	0	0	0	39
C. krusei	9	11	6	3	1	0	1	31
C. tropicalis	3	2	1	1	0	0	1	8
C. parapsilosis	0	3	1	0	0	0	0	4
C. guilliermondii	1	2	0	0	0	1	0	4
C. pseudotropicalis	0	2	0	0	0	0	0	2
C. glabrata	0	4	1	0	1	1	0	7
C. stellatoidea	2	0	0	2	0	0	1	5



Percentage values of tertile groupings of high, medium, and low biofilm-producing strains of *Candida* spp. based on material. *C. tropicalis* and *albicans* appear to be the species with the highest number of strains in the HBF category, in contrast to *C. parapsilosis* and *glabrata*, which show high percentages of strains in the LBF category, confirming the following scale of producers: *C. tropicalis* > *C. albicans* > *C. parapsilosis* > *C. glabrata.* LBF: low biofilm forming; MBF: moderate biofilm forming; HBF: high biofilm forming.

Discussion

Humans and their surroundings are home to the asexual, diploid, dimorphic fungus Candida. There are comparatively few species of Candida that are harmful to humans. Numerous superficial and deep-seated mycoses, including cutaneous, mucocutaneous, subcutaneous, or systemic candidiasis, can be brought on by these organisms. Candida organisms are commensals; in order for them to function as pathogens, the host's regular defenses must be disrupted. Thus, immunocompromised conditions, diabetes mellitus, and iatrogenic factors such as antibiotic usage, indwelling devices, intravenous medication use, and hyperalimentation fluids are common risk factors for Candida infections. Due to an increase in individuals with impaired immune systems, advanced age, extended antimicrobial and severe cancer chemotherapy, invasive surgery, and organ transplantation, candidiasis has become a concerning opportunistic illness.

As demonstrated by Mujika et al., the current investigation demonstrated the preponderance of non-Candida albicans and the distribution of Candida species in various clinical samples. [1] C. krusei was the most prevalent isolate across all samples. The most common species found in respiratory tract samples was Candida albicans (41.37%). Patients over 60 who had productive coughs most often developed a subsequent Candida infection. 120 blood samples were taken from the dialysis and intensive care units. Non-Candida albicans were the most common species that were isolated from the blood samples. C. krusei was the most prevalent isolate. Microorganisms that enter the intracutaneous wound during or after catheter placement are the primary cause of the majority of catheter-related septicemias. [9–11]

The percentage of these infections caused by species other than Candida albicans is steadily increasing. [12–14] 84 people with diabetes who were older than 60 had their saliva samples taken. C. albicans was found in 23 (74.19%) and non-Candida albicans in 8 (25.8%) of the saliva samples from 31 patients. These patients showed symptoms of a urinary tract infection, and urine samples revealed C. krusei 6 (50.0%) and C. albicans 3 (25.0%). The majority of the isolates from the eye, middle ear discharge, and pus were not Candida albicans species. Patients' plastic devices, such as

catheter tips, suction tips, and endotracheal tubes, were gathered. These cultures mostly produced Candida albicans and Candida krusei.

A population of bacteria and their extracellular polymers adhered to a surface is called a biofilm. [15] A group of bacteria encased in the slime they secrete is called a biofilm. Since the capacity to create biofilms is linked to pathogenecity, it should be regarded as a significant virulence factor during candidiasis. Through their ability to evade host immune responses, endure antifungal therapy, and withstand competitive pressure from other organisms, biofilms may contribute to the maintenance of fungi's roles as commensal and pathogen. As a result, treating illnesses linked to biofilms is challenging. [16] High levels of antibiotic resistance in the related species are similarly linked to the development of biofilms. [17] Isolates of Candida krusei showed the highest frequency of biofilm positive, followed by Candida tropicalis, Candida kefyr, Candida guilliermondii, Candida parapsilosis, Candida glabrata, and Candida albicans. On the other hand, isolates of Candida parapsilosis and Candida glabrata were far less likely to form biofilms than the more pathogenic Candida albicans, according to Hawser and Douglas [18]. In this investigation, the species of Candida was more closely associated with biofilm development than the infection site. When the strains were categorized by the age of the patients and the infection site, there were no appreciable variations in the biofilm production. During infection, pathogenic species of Candida release aspartyl proteinases in vivo. The adhesion, tissue injury, and invasion of host immune responses are caused by secreted aspartic proteinases (Saps). During the infectious process, proteinases perform a variety of specialized tasks, such as breaking down molecules to obtain nutrients, breaking down or warping host cell membranes to promote adhesion and tissue invasion, and breaking down host immune system cells and molecules to prevent or fend off host antimicrobial attack. In this investigation, Candida non albicans 56 (50.45%) had a lower capacity to produce proteinase than Candida albicans 33 (67.34%). The amount of proteinase generated varied significantly between species ($P<0.05$). A diverse collection of enzymes known as "phospholipases" are able to hydrolyze one or more ester linkages in glycerophospholipids. Direct host cell damage and lysis has been suggested as a key mechanism contributing to microbial pathogenicity because phospholipase targets membrane phospholipids and digests these components, resulting in cell lysis [19]. Phospholipase was generated by 23 (46.93%) of the C. albicans isolates and 26 (42%) of the non-Candida albicans isolates. In demonstrating that C. albicans isolated from the blood samples had higher extracellular phospholipase activity, the study's findings are consistent with those of Ibrahim et al. [20].

CONCLUSION

The frequency of invasive, often life threatening *Candida* infections has increased dramatically largely as a function of medical technology advances. Throughout the world, the dominance of *Candida albicans* has been challenged by the increased prevalence of serious infection caused by *Non albicans Candida* species.

In this study, there was a clear shift in prevalence of infection by *Candida albicans* to those of *Non albicans Candida* species in the various clinical samples isolated and also shows them as strong biofilm producers compared to *C.albicans* species. These data suggest that, biofilm formation as a potential virulence factor might have a higher significance for *non-albicans* Candida species than for *C.albicans* and also that the biofilm structure varies with the different species and strains of candida, the nature of the colonized surface and its localization. The key to successful management lies in early recognizing and diagnosis of species. Colony morphology on corn meal agar and CHROMagar are more reliable and rapid methods for speciation of *Candida* isolates. CHROMagar is superior in case of mixture of species in the same sample.

For Biofilm formation MTP method is gold standard follwed by Tube method and CRA method.

So it has become imperative for us to take timely steps for early speciation and antifungal susceptibility pattern of the clinically significant *Candida* isolates prevalent in our society. This study can be futher improve by identifing antifungal susceptibility pattern of *Candida* isolates so as to prevent development of resistance in candida species.

Further research to improve diagnostic, preventive and therapeutic strategies is necessary to reduce the considerable mortality and morbidity.

References

1. Mohandas V, Ballal M. Distribution of Candida species in different clinical samples and their virulence: biofilm formation, proteinase and phospholipase production: a study on hospitalized patients in southern India. J Glob Infect Dis. 2011 Jan;3(1):4-8. doi: 10.4103/0974-777X.77288. PMID: 21572601; PMCID: PMC3068577.Senet JM. *Candida* adherence phenomena, from commensalism to pathogenicity. Int Microbiol 1998;1:117-22.
2. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J. Adherence and biofilm formation of non-Candida albicans Candida species. Trends Microbiol. 2011 May;19(5):241-7. doi: 10.1016/j.tim.2011.02.003. Epub 2011 Mar 15. PMID: 21411325.Cutler JE. Putative virulence factors of *Candida albicans.* Annu Rev Microbiol 1991;45:187-218.
3. Pfaller MA, Messer SA, Hollis RJ. Variations in DNA subtype, antifungal susceptibility, and slime production among clinical isolates of Candida parapsilosis. Diagn Microbiol Infect Dis. 1995 Jan;21(1):9-14. doi: 10.1016/0732-8893(94)00114-c. PMID: 7789100.Staib F. Serum-proteins as nitrogen source for yeast like fungi. Sabouraudia 1965;4:187-93.
4. Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA. Differential expression of Candida albicans phospholipase B (PLB1) under various environmental and physiological conditions. Microbiology (Reading). 2003 Jan;149(Pt 1):261-7. doi: 10.1099/mic.0.25829-0. PMID: 12576599.Chakrabarti A, Singh K, Das S. Changing face of nosocomial candidaemia. Indian J Med Microbiol 1999;17:160-6.
5. D'Antonio D, Romani F, Pontieri E, Carruba G. Catheter related candidaemia caused by *Candida lipolytica* in a patient receiving allogenic bone marrow transplantation; J Clin Microbiol 2002;40:1381-6.
6. Pfaller MA, Messer SA, Hollis RJ. Variations in DNA subtype, antifungal susceptibility, and slime production among clinical isolates of Candida parapsilosis. Diagn Microbiol Infect Dis. 1995 Jan;21(1):9-14. doi: 10.1016/0732-8893(94)00114-c. PMID: 7789100.Vinitha M, Ballal M. Proteinase and phospholipase as virulence factors in *Candida* isolated from blood. Rev Iberoam Micol 2008;25:208-10.
7. Hendry AT, Bakerspigel A. Factors affecting serum inhibited growth of Candida albicans and Cryptococcus neoformans. Sabouraudia. 1969 Oct;7(3):219-29. PMID: 5385158. Yücesoy M, Karaman M. Candida türlerinin biyofilm üretimi ve antifungal duyarlilik paternleri [Biofilm production and antifungal susceptibility patterns of Candida species]. Mikrobiyol Bul. 2004 Jan-Apr;38(1-2):91-8. Turkish. PMID: 15293907. inhibitory concentration values of antifungal agents. Mem Inst Oswaldo Cruz 2005;100:319-24.
8. Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA. Differential expression of Candida albicans phospholipase B (PLB1) under various environmental and physiological conditions. Microbiology (Reading). 2003 Jan;149(Pt 1):261-7. doi: 10.1099/mic.0.25829-0. PMID: 12576599.
9. Chakrabarti A, Singh K, Das S. Changing face of nosocomial candidaemia. Indian J Med Microbiol 1999;17:160-6.
10. Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA. Comparison of biofilms formed by Candida albicans and Candida parapsilosis on bioprosthetic surfaces. Infect Immun. 2002 Feb;70(2):878-88. doi: 10.1128/IAI.70.2.878-888.2002. PMID: 11796623; PMCID: PMC127692.
11. Astiz ME, Rackow EC, Still JG. et al. Pretreatment of normal humans with monophosphoryl lipid A induces tolerance to endotoxin: A prospective, double-blind, randomized, controlled trial. Crit Care Med. 1995;23:9. [PubMed]

12. Mattei AS, Alves SH, Severo CB, Guazzelli Lda S, Oliveira Fde M, Severo LC. Determination of germ tube, phospholipase, and proteinase production by bloodstream isolates of Candida albicans. Rev Soc Bras Med Trop. 2013 May-Jun;46(3):340-2. doi: 10.1590/0037-8682-0045-2013. PMID: 23856861.
13. Riceto ÉB, Menezes Rde P, Penatti MP, Pedroso Rdos S. Enzymatic and hemolytic activity in different Candida species. Rev Iberoam Micol. 2015 Apr-Jun;32(2):79-82. doi: 10.1016/j.riam.2013.11.003. Epub 2014 Apr 1. PMID: 24704439.
14. Arendrup MC. Candida and candidaemia. Susceptibility and epidemiology. Dan Med J. 2013 Nov;60(11):B4698. PMID: 24192246.
15. Chandra J, Mukherjee PK, Ghannoum MA. In vitro growth and analysis of Candida biofilms. Nat Protoc. 2008;3(12):1909-24. doi: 10.1038/nprot.2008.192. PMID: 19180075.
16. Yücesoy M, Karaman M. Candida türlerinin biyofilm üretimi ve antifungal duyarlilik paternleri [Biofilm production and antifungal susceptibility patterns of Candida species]. Mikrobiyol Bul. 2004 Jan-Apr;38(1-2):91-8. Turkish. PMID: 15293907.
17. K. Hooli, S. Thilakawardana, J. Lara, J-P. Kermoal,S. Pfletschinger, "Flexible Spectrum Use betweenWINNER Radio Access networks", IST Mobile Summit, Greece, June 2006.
18. Mattei AS, Alves SH, Severo CB, Guazzelli Lda S, Oliveira Fde M, Severo LC. Determination of germ tube, phospholipase, and proteinase production by bloodstream isolates of Candida albicans. Rev Soc Bras Med Trop. 2013 May-Jun;46(3):340-2. doi: 10.1590/0037-8682-0045-2013. PMID: 23856861.