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Distribution of Candida species in different clinical samples, their biofilm formation, and drug susceptibility patterns

Sumati Hogade[1], Ahuti Pandya [2], Tejaswini H[3]

M.D. Microbiologist, Professor, Dept of Microbiology, J.N.Medical college, KAHER (KLE Academy of Higher Education and Research), Belagavi. ORCID ID no. 0000-0002-1215-8003, Phone no –9448866944, Email ID –sumatihogade@gmail.com

[2]M.D. Microbiologist, Senior Resident, Department of Microbiology, J.N.Medical college, KAHER (KLE Academy of Higher Education and Research),Belagavi. ORCID ID no. 0009-0004-1741-1874, Phone no – 8949514633, Email ID – ahutipandya05@gmail.com

[3]MSc Microbiologist, Department of Microbiology, J.N.Medical college, KAHER (KLE Academy of Higher Education and Research),Belagavi. Email ID-tejaswinihiremath6@gmail.com

Corresponding Author- Sumati Hogade,M.D. Microbiologist, Professor, Dept of Microbiology, J.N.Medical college, KAHER (KLE Academy of Higher Education and Research), Belagavi. ORCID ID no. 0000-00021215-8003, Phone no –9448866944, Email ID-sumatihogade@gmail.com. Address- Professor, Dept of Micromiology, J.N.Medical college, Belagavi. Pin code-590010.

ABSTRACT

Introduction:A big worry nowadays is the rise in drug resistance to antifungal medications brought on by Candida species, which cancause a variety of clinical conditions, from mucocutaneous infections to invasive diseases that can be fatal. Biofilm production is the primary cause of most diseases caused by Candida spp. In order to effectively treat Candida infections, it is important to identify Candida, evaluate its susceptibility to antifungals, and determine its capacity to build biofilms.

Aim and objective: To speciate Candida isolates from various clinical specimens and to detect biofilm formation by phenotypic methods and to do antifungal susceptibility.

Methods:Total 70 candida isolates were isolated and speciated as per conventional methods. Antifungal susceptibility testing was performed using disc diffusion testing in compliance with CLSI guidelines. Three phenotypic methods—the Tube technique (TM), the Microtitre plate method (MTP), and the Congo Red Agar (CRA) method—are used to detect biofilm.

Results:C. glabrata accounted for 53% of the total isolates, with C. tropicalis

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(20%), C. parapsilosis (11.4%), C. albicans (5.7%). Out of 70 isolates. 58 candiida spp. were biofilm producers by MTP. The resistance of biofilm-
forming candida isolates to antifungals was greater. Amphotericin B (34%) was more effective against Candida species.

Conclusions:The most common species that caused different types of Candida infections was Candida non-albicans. Amphotericin B was the most efficacious
medication. Congo red agar demonstrated superior sensitivity, but the tube method demonstrated great specificity.

Keywords: Candidaspp, Antifungal drugs, Biofilm, Microtitre plate method (MTP), Tube method (TM), Congo Red Agar (CRA) method

Introduction

Candida species are the most common cause of fungal infections. They can cause a range of disorders, from mucocutaneous candidiasis (genitourinary, vulvovaginal, and oropharyngeal) that is not life-threatening to invasive illnesses that can be fatal, such as bloodstream candidiasis [1]. The majority of candidiasis cases were thought to be caused by the most prevalent species, Candida albicans, until recently. However, over the last several decades, a number of studies have shown a growing trend away from most Candida albicans and towards non-albicans Candida species (NAC), such as Candida tropicalis, Candida glabrata, and Candida krusei[2–4]. The primary source of fungal opportunistic infection has reportedly been identified as NAC species [5, 6]. The development of azole resistance, the need to treat fungal infections primarily caused by NAC species, differences in drug susceptibility profiles among yeast isolates, and the frequent isolation of emerging yeasts (i.e., NAC species) in clinical samples led to the development of accurate species identification and in vitro susceptibility testing techniques [7].Candida species are pathogenic because of a few virulence factors, including the ability to evade host defences, adhesion and biofilm formation (on host tissues and/or medical devices), and the production of tissue-damaging hydrolytic enzymes like hemolysins, phospholipases, and proteases [8]. One specific feature of Candida species' pathogenicity is their capacity to form biofilms, which protects them from external stimuli such as host immune system defences and antifungal drugs [9].Biofilms are communities of

microorganisms that live on biotic and abiotic surfaces in an intricate three-dimensional structure. The extracellular matrix (ECM) encases these creatures [10]. Biofilms can form on the plastic surfaces of indwelling devices as well as mucosal surfaces. Biofilms exhibit genetic resistance to two antifungal drugs: amphotericin B (AMB) and fluconazole (FLU) [11]. When it comes to their morphology, the properties of the extracellular matrix (ECM), and their capacity to give antifungal resistance, each species of

Candida differs in how they produce biofilms [12]. This unpredictability makes it more difficult to identify a workable approach to address the hazards posed by Candida biofilms as a distinct issue. Actually, there is a pressing need to identify appropriate therapeutic techniques that may be able to treat patients more effectively as a result of the emergence of these fungal diseases.In this study, all candida isolates are speciated and screened for biofilm detection by phenotypic methods like Microtitre plate method (MTP), Tube method (TM), and Congo Red Agar (CRA) method and compare these phenotypic method of biofilm

detection.

Methods:his study includes 70 isolates of candida from different clinical samples that were received by the department of Microbiology over the course of a year, from August 2012 to August 2013. Following approval from the Institutional Ethics Committee for Human Research (IECHR) and the acquisition of a permission letter, the study was carried out. A germ tube test, urea hydrolysis test, Grame staining, macroscopic inspection, and colony appearance were all used to assess growths on Sabouraud dextrose agar (SDA). Dalmou plate method, growth on CROME agar, Sugar fermentation and assimilation test is performed to speciate Candida species.Using disc diffusion testing, antifungal susceptibility testing was carried out in accordance with the Clinical and Laboratory Standards Institute (CLSI) criteria (document M44-A). The three phenotypic methods of Congo Red Agar (CRA), Tube technique (TM), and

Microtitre plate method (MTP) were utilised to detect biofilm in all 70 candida isolates. (Figure 1 and 2)

Data analysis and interpretation- All data from the investigation were coded and analyzed using SPSS version20. Descriptive statistics such as frequency and percentage of Candida species, Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of Tube method (TM), and Congo Red Agar (CRA) methodwere calculated.

Results:

Seventy species of Candida were identified after isolation from various clinical specimens.

The distribution of Candida isolates by gender revealed that 49% (34) of them were male and 51% (36) were female.The majority of the isolates were obtained from the 0–20-year-old age group comprising 38 out of 70 isolates (54.3%), followed by the age group 21–40 years old (15, 21.4%), age group 41-60 yr (11, 15.7%), age group 61-80yr (4, 5.7%) and age group 81100yr (2,2.9 %)

Out of 70 clinical isolates of candida, C. glabrata accounted for 53% of the isolates, with C. tropicalis (20%), C. parapsilosis (11.4%), C. albicans (5.7%), C. guilliermondii (5.7%), C. krusei (3%) and C. kefyr (1.4%) following closely behind. Sixty-six of the seventy isolates came from blood samples (51.4%), eight from vaginal swabs (11.4%), three from pus samples (4.3%), four from sputum (5.7%), three from catheter tips (4.3%), and eleven from urine samples (15.7%). (Table 1).

Three of the four C. albicans tested positive for both CRA and MTP, two tested positive for TM. 37 C. glabrata were tested; 33 tested positive by MTP, 25 by TM, and 35 by CRA. One C. krusei specimen tested positive using all three techniques. Of the fourteen C. tropicalalis, eight tested positive by TM, twelve by CRA, and fourteen by MTP. There was only one isolated C. kefyr, and it tested negative by CRA and positive by MTP and TM. Six of the eight C.

parapsilosis samples tested positive by MTP, three by TM, and two by CRA. Table 2 shows that of the four C.guilliermondi, three tested positive by TM and all tested positive by both MTP and CRA. (Table 2)

In MTP, strong biofilm producers were 14(20%), 26 (37.14%) were moderate, 22(31.42%) were weak and 8(11.42%) were non biofilm producers. In TM, strong biofilm producers were 9(12.85%), 11 (15.71%) were moderate, 21(30%) were weak and 29(41.42%) were non biofilm producers. In CRA, strong biofilm producers were s(7.14%0), 29 (41.42%) were moderate, 23(32.85%) wereweak and 13(18.57%) were non biofilm producers. (Table 3)

The tube method's sensitivity was 61.90%, specificity was 57.14%, PPV (positive predictive value) was 92.85%, and NPV (negative predictive value) was 14.28%. The Congo red agar test had a sensitivity of 84.12% and a specificity of 28.57%. 91.37% was the PPV, while 16.67% was the NPV. (Table 4)

All 62 biofilm producing candida isolates showed higher resistance to Ketoconazole (64.5%), followed by Itraconazole (61.3%), voriconazole (58.1%), Fluconazole (56.4%), clotrimazole (54.8%) and Amphotericin B (30.6%). (Table 5)

All 8 non-biofilm producing candida isolates showed lower resistance to antifungal drugs as compared to biofilm producing isolates.Ketoconazole, Fluconazole and amphotericin B resistance is shown in 37.5% isolates, Itraconazole and voriconazole 25% resistance seen, Fluconazole 50% resistance seen.(Table 6)

DISCUSSION

Candida species have become the main culprits behind a number of human infections in recent times. Only four (5.7%) of the 66 isolates in this study were Candida albicans;The other isolates were non-Albicans species, such as C. glabrata (53%) to C. tropicalis (20%), C. parapsilosis

(11.4%), C. guilliermondii (5.7%), C. krusei (3%) and C. kefyr (1.4%).Similar trend is seen in other studies like Nimmala P et al. [13], which isolated 79.6% of Candida non-Albicans, and Shukla R et al. [14], which isolated 75% of Candida non-Albicans. Other studies also show similar trends [15.16.17, 18]. Long-term azole use, a brief course of antifungal therapy, and greater use of over-the-counter medicationsmay all have a role in the change in frequency from albicans to NAC [19]. Compared to male patients, female patients in the current study had a larger percentage of Candida isolates (51.4%). The findings of this investigation were comparable to those of Rakesh Kumar Mukhia et al. [20] and Sonu

Panwar et al. [21], who found that female patients had higher candida species isolation rates 54 (54%) and 66 (55%) than male patients 46 (46%) and 54 (45%), respectively.The age group 21–40 years old (21.4%) had the second greatest prevalence of Candida isolation in this investigation, after the 0–20 age group (54.3%).According to the study, the blood included 36 (51.4%) of the most candida isolates, followed by samples from the urine

(15.7%), vaginal swab (11.4%), stool (8%), sputum (5.7%), catheter tip (4.3%), pus sample (4.3%), and miscellaneous sources (2.9%). Comparable to the study conducted by Khadka S et al. [22], the majority of isolates from urine (48%), sputum (42%), blood (2%), catheter tips (4%), high vaginal swabs (2%) and endotracheal tubes (2%), were found in this study. Urine (30.5%) yielded the most isolates in the study conducted by Lata R Patel [23], with sputum (28.9%) and blood (26%).It is imperative to have a dependable technique for detecting Candida biofilms because they have been identified as a virulence factor that contributes to infection linked to a variety of medical devices. Three in vitro screening tests—the MTP, CRA, and TM methods—were used in this investigation to determine if the isolates could produce biofilms. Given that the development of biofilms is a unique characteristic associated with the pathogenicity of Candida, the biofilm positivity rates of 88.6% that we observed in our study were deemed significant. Three (75%) of the four Candida albicans isolates showed evidence

of biofilm formation. Ninety-nine (89.4%) of the 66 non-Candida albican isolates produced biofilm. Marak MB et al.'s study [24] reveals that of the 41 isolates of Candida albicans, 21 (51.2%) strains were positive for producing biofilm, and 28 (57.14%) isolates of non-Candida albicans produced biofilm. The tube method's sensitivity and specificity were 61.90% and 57.14%, respectively. According to Khatri S et al. [25], TM has a 91.8% sensitivity and a 100% specificity. About 63-66% of TM positive was found in a few other studies [26, 27]. The Congo red agar test had a sensitivity of 84.12% and a specificity of 28.57%. Thus, our investigation demonstrates that the Congo Red Agar method had superior sensitivity but the Tube method demonstrated high specificity. Nonetheless, we discovered that, in comparison to non-albicans Candida species, C. glabrata had a higher proportion of biofilm positive (56.06%). This is in contradiction to the findings of other researchers, such as Agwan V [28], who reported that C. tropicalis was the highest biofilm producer, followed by C. albicans. For strong biofilm-producing isolates, the tube approach correlates well with the MTP test as well, according to our research. It is not suggested to use the tube method to detect biofilm formation in Candida species, though, because it is difficult to classify weak biofilm-positive isolates and biofilm negative isolates. We conclude that the use of Congo Red in the evaluation of fungal biofilm formation may be limited due to its interaction with the extracellular matrix and cell wall composition. Congo Red binds to chitin and glucan found in the cell wall in addition to the carbohydrates of the extracellular matrix (ECM) produced by the Candida. As such, we are unable to recommend the CRA test as a screening technique in general for the production of biofilms by Candida spp. The microtiter plate method can be adjusted for different biofilm development experiments and has been determined in accordance with established methodologies. This test yields a quantifiable answer and is quick, effective, dependable, and repeatable.Of the 70 isolates of candida found in our investigation, 62 produced biofilm and had a greater resistance to ketoconazole (64.51%), voriconazoles (58.06%), fluconazole

(56.4%), clotrimazole (54.83%), and amphotericin B (30.64%). Comparable to a study by Lamsal et al. [29] that shows the majority of C. albicans isolates were susceptible to amphotericin B, with 76.19% of C. albicans having a MIC of ≤1 µg/mL and the remaining 23.81% having a MIC > 1 µg/mL, the majority of Candida species that form biofilms are sensitive to amphotericin (69.4%) in our study.In a study conducted by P. M. Punithavathy, [30] biofilm forming cells showed increased resistance to fluconazole.The majority of patients, particularly those in underdeveloped nations, find it difficult to acquire amphotericin B due to its expensive cost, despite the fact that it is still the medicine of choice for the majority of fatal and widespread fungal infections.

Conclusion

According to our research, the most prevalent species that causes different types of Candida infections is Candida non-albicans. The ability of Candida species to form biofilms, which protects them from external factors including host immune system defences and antifungal drugs, is one specific trait that makes them dangerous. Amphotericin B, a popular antifungal medication, showed a significant incidence of sensitivity in Candida species that form biofilms as well as those that do not.Periodically monitoring candida species is essential because of the significant geographic variance in their distribution and resistance patterns. This will support antifungal stewardship in addition to helping the clinician promptly initiate directed therapy in the form of suitable antifungal drugs.

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Table 1- Distribution of Candida isolates in different clinical samples

Candida species	**Urine**	**Stool**	**Sputum**	**Catheter Tip**	**Blood**	**Genital Swab**	**Pus**	**Other**	**Total**
C.albicans	-	-	-	-	2	2	-	-	4 (5.7%)
C.glabrata	3	-	4	1	26	3	-	-	37 (53%)
C.krusei	-	1	-	-	-	-	-	1	2 (2.8%)
C.tropicalis	4	1	-	-	4	2	3	-	14 (20%)

C.kefyr	-	-	-	-	1	-	-	-	1 (1.4%)
C.parapsilosis	4	1	-	-	2	-	-	1	8 (11.4%)
C.guilliermondii	-	-	-	2	1	1	-	-	4 (5.7%)
Total	11 (15.7%)	3 (4.3%)	4 (5.7%)	3 (4.3%)	36 (51.4%)	8 (11.4%)	3 (4.3%)	2 (2.9%)	70

Table 2 - Detection of biofilm by MTP, TM, and CRA of the 70 candida species

Candida Species	MTP		TM		CRA	
	NBP	BP	NBP	BP	NBP	BP
C.albicans (n=4)	1	3	2	2	1	3

C.glabrata (n=37)	4	33	12	25	2	35
C.krusei (n=2)	1	1	1	1	1	1
C.tropicalis (n=14)	0	14	6	8	2	12
C.kefyr (n=1)	0	1	0	1	1	0
C.parapsilosis (n=8)	2	6	5	3	6	2
C.guilliermondii (n=4)	0	4	1	3	0	4

Microtitre plate method (MTP), Tube method (TM), and Congo Red Agar (CRA) method,

Non biofilm producing (NBP), Biofilm Producing (BP).

Table 3 - Comparison of detection of biofilm formation by MTP, TM and CRA method

	Biofilm formation	Screening methods		
Clinical isolates		MTP	TM	CRA
	High	14	9	5
	Moderate	26	11	29
	Weak	22	21	23
	Non biofilm producers	8	29	13

Microtitre plate method (MTP), Tube method (TM), and Congo Red Agar (CRA) method

Table 4 - Statistical evaluation of TM and CRA methods against standard MTP method for detection of biofilm formation in candida species

Methods	Test Characteristics (%)			
	Sensitivity	Specificity	PPV	NPV
TM	61.90%	57.14%	92.85%	14.28%
CRA	84.12%	28.57%	91.37%	16.67%

Positive Predictive Value (PPV), Negative Predictive Value (NPV),Tube method (TM), and Congo Red Agar (CRA) method

Table 5- Antifungal drug sensitivity pattern in candida isolates which are positive for biofilm production.

Candida spp.	No. of isolates	No of Isolates resistant to Antifungal drugs					
		Clotrimazole	Ketoconazole	Voriconazole	Itraconazole	Fluconazole	Amphotericin B
C.albicans	3	2	3	2	2	2	2
C.glabrata	33	19	21	18	20	16	7

C.krusei	1	-	-	-	1	-	1
C.tropicalis	14	9	10	10	10	11	7
C.kefyr	1	-	-	-	-	-	-
C.parapsilosis	6	1	2	3	2	2	1
C.guilliermondii	4	3	4	3	4	4	1
Total	62	34 (54.8%)	40 (64.5%)	36 (58.1%)	38 (61.3%)	35 (56.4%)	19 (30.6%)

Table 6- Antifungal drug sensitivity pattern in candida isolates which are negative for biofilm production

Candida spp.	No. of isolates	No of Isolates resistant to Antifungal drugs					
		Clotrimazole	Ketoconazole	Voriconazole	Itraconazole	Fluconazole	Amphotericin B
C.albicans	1	-	-	-	-	-	-
C.glabrata	4	3	2	1	2	3	3
C.krusei	1	-	-	-	-	-	-

C.parapsilo	2	-	1	1	-	1	-
sis							
Total	8	3 (37.5%)	3 (37.5%)	2 (25%)	2 (25%)	4 (50%)	3 (37.5%)

Figure 1 – Biofilm detection by Congo Red Agar Method (CRA)

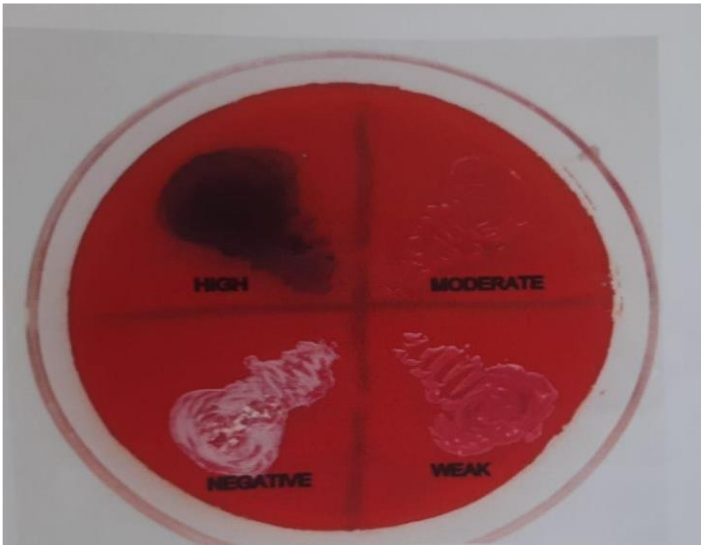

Figure 2- Biofilm detection byTube method (TM)