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IDENTIFICATION, BIOFILM FORMATION AND ANTIFUNGAL SUSCEPTIBILITY OF *CANDIDA* SPECIES ISOLATES FROM CLINICAL SPECIMENS

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ABSTRACT

Introduction: *Candida* infection is most common infection. *Candida* species is dimorphic commensal fungi and most frequently cause infection in the immunocompromised, ICU, NICU and HIV patients and also sometimes in healthy persons. ***Method:*** in this investigation *Candida* species were isolates from different clinical samples such as- Sputum, Blood, Urine, Stool, Nail scraping and oral thrush from patients who were suffering from suspected *Candida* infection of the hospitalized patients was carried out to determine the *Candida* species by using gram staining, Chrom agar, germ tube formation, cornmeal agar, biofilm formation and antifungal susceptibility. ***Results*:** Total 115 *Candida* species were isolated during this study, in which *C. albicans* were 54%, *C. tropicalis* 25.2%, *C. parapsilopsis* 8.9%, *C. krusei* 7.8%, *C. glabrata* 1.7% and *C. famata* 1.7% from different clinical samples. Antifungal susceptibility test results in 68.6% sensitivity against fluconazole, 25.2% resistance and 6% intermediates. Voriconazole was 82.6% sensitive, 7.8 intermediates and 10.4% resistance; Amphotericine-B 88% sensitive, 6% intermediates and 5.2% resistance; Intraconazole 77.3% sensitive, 9.5% intermediates and 13.5% resistance. ***Conclusion*:** *C. albicans* and *C. tropicalis* have high capability to cause infection in the highest amount and therefore are highest biofilm producer. Amphotericine-B is more frequently used drug against the *Candida* species and it is almost 90% sensitive to over all *Candida* species.

KEYWORDS*: Candida* infection, Biofilm, Antifungal agent, Susceptibility.

INTRODUCTION

Candida is a ubiquitous fungi, and commensal generally present in healthy humans, on skin, nails, and internal organs of the body but they can cause opportunistic infection and disease as and when condition is favorable. Pathogenic *Candida* species are frequently cause infection in the immunocompromised, ICU (Intensive care unit), NICU (Neonatal intensive care unit) and HIV patients and creates different clinical problems. *Candida* is a fourth pathogens leads to bloodstream infection reported in USA approximately 8-10%.[1, 2] In the previous study, during 2013-2014 in India, the majority of *Candida* species are isolates from oral thrush in which approximately 80% patients are associated from the oral thrush infections.[3] It is an opportunistic disease leads to a fungal infection responsible for oral candidiasis and infection of other part of the human body.[4]

Candida species is a dimorphic fungus and *Candida* cells give them the capacity to cause disease by the adherence to epithelial and endothelial cells surface, biofilm formation, germ tube formation pseudohyphae formation with development of the filamentous form, phenotypic variability, and production of toxins and extracellular enzymes constitute important factors for the emergence of infections by *Candida.* Several pathogenicity of *Candida* species are isolates which are responsible for many infection includes blood stream infection, urinary tract infection gastrointestinal infection, skin infection and oral thrush infection in immunocompromised patients and also belong to ICU and NICU and sometime in a healthy person. There are many virulence factors include adhesin, enzymes, toxin, complements, phenotypic switching contributing to these pathogenicity.[5, 1]

Biofilms are defined as surface-associated communities of cells surrounded by an extracellular matrix. Attachment and adhesion between *Candida* species and material or host cell surface has been implicated in formation of biofilm.[6] Biofilm formation was playing an important role in the pathogenicity of *Candida* species by the adherent to the cell surface to the host. Biofilm formation is the most common mode of fungus growth in nature that are leads to a major clinical infection and serious disease in the immunocompromised patients and patients from ICU and NICU.[7]

Due to the opportunistic infection of *Candida* species antibiotic senstivity testing methods are available to determine the drug activities against the *Candida* species or other microorganism.[8] Each antifungal class utilizes to kill or inhibit the growth of fungal pathogens. Generally more number of systematically antifungal agents belongs to azoles

group including fluconazole, voriconazole, intraconazole and polyenes (Amphotericin-B) are used in the treatment of *Candida* infection and serious disease in the immunocompromised ICU, NICU and HIV patients.[9] In previous study fluconazole are more effective to the *Candida* infection.

In this study aimed to identify the *Candida* species isolates from different sample such as; sputum, blood, urine, stool, nail scraping and oral thrush and analyzed their identification, biochemical test, biofilm formation and antibiotic susceptibility.

MATRIALS AND METHODS

The present investigation was conducted on the basis of the isolation of *Candida spp*. from clinical specimens who were referred from Sir Sundar Lal Hospital to Institute of Medical science, BHU, from January 2015 to May 2015. During the period of 6 months 115 different samples was collected such as- Sputum, Blood, Urine, Stool, Nail scraping and oral thrush from patients who were suffering from suspected *Candida* infection and their age group was between 0 to above 60 years of males and females.

Identification

For identification of clinical isolates from different samples, isolates were started from gram stained. Samples were also inoculated on Sabouraud Dextrose agar (SDA), Potato Dextrose agar (PDA) and blood agar plates and plates were then incubated at 37ºC for 24-48 hrs. Identification was also done on HiChrom agar culture, Cornmeal agar (HiMedia Laboratories), Germ tube method and SDA with NaCl medium was used to differentiate between *C. albicans* and *C. dublinensis*. For the complete identification, sugar fermentation and sugar assimilation test were performed.[1, 10]

Biofilm formation

Biofilm formation was performed on commercially sterilized, flat-bottomed 96-well microtitration plates. Sabouraud Dextrose broth (SDB) media supplement with glucose 8% was prepared and deposited into each well of microtitration plate containing 180µl. 20µl standardized *Candida* suspension (3×10^7 CFU/ml) was prepared and deposited into each well of microtitration plate. The plates were covered and incubated for 48 hrs. at 35°C in the orbital shaker at 75 rpm. After the adhesion phase, the plates were removed and the wells washed three times with sterile distilled water to remove loosely adherent cells. Adhered cells were stained with crystal violet and dried it for 15 minute and take absorbance in the

microtitration reader at 405 nm.[11] On the basis of their absorbance examined the biofilm structure.

Antifungal susceptibility

Examination of the antifungal susceptibility test of *Candida* species had been done by the disc diffusion method in this study on the Muller Hinton agar (MHA) plates. Commercially prepared antibiotic disc fluconazole (0.25mg), voriconazole (0.001mg), intraconazole (0.010mg) and amphotericin-B (2.5mg) were used issues from HiMedia laboratory, India. Take sterilized media plates containing MHA with 2% glucose/100ml. Prepare suspensions of *Candida* colonies in normal saline. *Candida* suspension was swabbed on MHA plates from sterilized swab stick. Four antibiotic disc, fluconazole, voriconazole, intraconazole and amphotericin-B were applied on swabbed MHA plate. Incubate at 37°C for 24 hrs after that analyze the activity of drugs against *Candida* species.[12]

RESULT AND DISCUSSION

A total 115 sample of *Candida* species were isolated from different clinical samples include Sputum, Blood, Urine, Stool, Nail scraping and oral thrush of the patients. Those were suffering from suspected *Candida* infection and among those patients some are immunocompromised patients which are also suffering from another disease including HIV patients, tuberculosis, arthritis etc. This study shows that *Candida* species are more frequently causing opportunistic infection and major clinical problem in the immunocompromised, ICU, NICU and HIV patients and also sometime in healthy persons. Out of 115 samples, *Candida* species were obtained from different samples including, 39 (33.9%) from sputum, 19 (16.5%) from blood, 27 (23%) from urine, 13 (11.3%) from stool, 9 (7.8%) from nail scraping, 9 (7.8%) from oral thrush. These samples were isolates from ICU, NICU and immunocompromised patients and also HIV patients, who had been suffered from *Candida* infection and some infectious disease. These patients are highly susceptible to causing *Candida* infection which may leads to some serious disease such as; candidiasis, candidimia, oral candidiasis, urinary tract infection, pulmonary candidiasis. The highest range of *Candida* species were obtained from sputum that means those person are suffering from pulmonary candidiasis.

Table-1: *Candida* species isolates from different clinical samples

S. No	*Candida* spp.	Sputum	Blood	Urine	Stool	Nail	Oral thrush	Total spp.
1.	*C. albicans*	23 (20%)	11 (9.5%)	15 (13.04%)	7 (6.1%)	4 (3.4%)	3 (2.6%)	63 (54%)
2.	*C. tropicalis*	9 (7.8%)	2 (1.7%)	8 (6.9%)	5 (4.3%)	2 (1.7%)	3 (2.6%)	29 (25.2%)
3.	*C. parapsilopsis*	3 (2.6%)	3 (2.6%)	1 (0.8%)	00	1 (0.8%)	2 (1.7%)	10 (8.9%)
4.	*C. krusei*	2 (1.7%)	2 (1.7%)	2 (1.7%)	1 (0.8%)	1 (0.8%)	1 (0.8%)	9 (7.8%)
5.	*C. glabrata*	1 (0.8%)	00	00	1 (0.8%)	00	00	2 (1.7%)
6.	*C. famata*	00	1 (0.8%)	1 (0.8%)	00	00	00	2 (1.7%)

The inspections were undertaken to this study that *C. albicans* was more frequently associated with sputum, blood, urine, stool, nail and oral thrush that are able to cause infection that may leads to complicated and serious disease into the immunocompromised, ICU, NICU and HIV patients and also sometime in healthy persons. As shown in table-1 that *C. albicans* was 54%, *C. tropicalis* 25.2%, *C. parapsilopsis* 8.9%, *C. krusei* 7.8%, *C. glabrata* 1.7% and *C. famata* 1.7% isolates from different clinical samples**.** *C. tropicalis* is another more infrequently able to cause clinical problems in a high amount.

Table-2: Identification of *Candida* species (on the basis of HiChrom agar culture, Cornmeal agar, Germ tube method, SDA with NaCl based identification and their result has been shown in Table**)**

S.no.	Isolates	Species	CHROMagar plates	Corn meal plates	Germ tubes	SDA + NaCl	Size
1.	62	*C. albicans*	Apple green colour colonies	Pseudohyphae & Chlamydospores	+	+	4-6µm
2.	30	*C. tropcalis*	Dark blue colour colonies	Pseudohyphae & Blastospores	Pseudo GT +	NA	4-5 µm
3.	10	*C. parsilopsis*	Cream colonies	Pseudohyphae & Blastospores	-	NA	4-8 µm
4.	9	*C. Krusei*	Rose pink colour colonies	Pseudohyphae & Blastospores	-	NA	2.6µm-
5.	2	*C. glabrata*	Pale pink to violet colonies	Pseudohyphae & Blastospores	-	NA	2-4 µm
6.	2	*C. famata*	White to light colour colonies	Pseudohyphae	-	NA	-

Note- NA= not applicable

Comparative study of *C. albicans* and *non-albicans*:

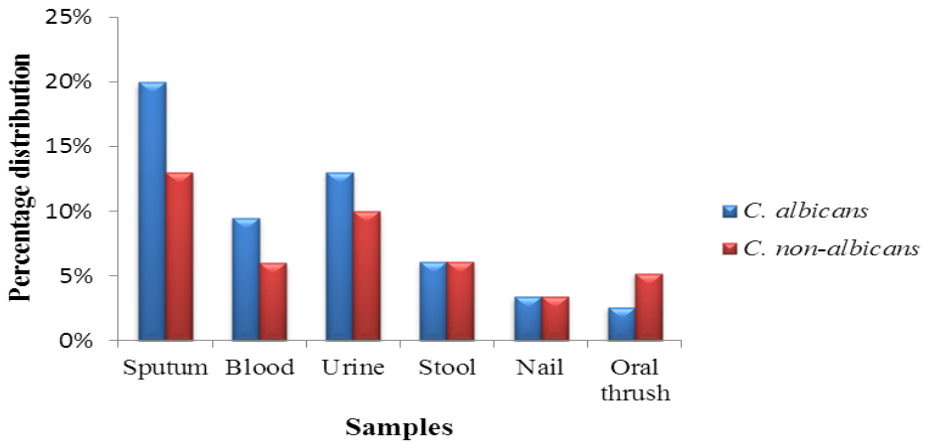

Fig-1: This graph is showing the Comparative study of *C. albicans* and *non-albicans*.

The Comparative study of *C. albicans* and *non-albicans* revealed that isolates of *C. albicans* was greater than *non-albicans.* Highest number of *C. albicans* isolates was from sputum (20%), blood (9.5%) and urine (13.04%), in comparison to *non-albicans* 13%, 6% and 10% respectively. *But C. albicans* and *non-albicans* isolates were found in equal amount of 6% and 3.4% from stool and nails respectively and majority of *non-albicans* were found in the oral thrush (5.2%) as well as in *C. albicans* i.e., 2.6% isolates. In our study, out of 115 isolates of *Candida* species, *C. albicans* were 54% and non-*albicans* were 46% that was observed from clinical samples. Our study revealed that *C. albicans* were more frequently observed isolates from the hospital population in the immunocompromised, ICU, NICU, HIV patients and sometimes in healthy persons. In the *non-albicans, C. tropicalis* has emerged as an important cause of infection in the immunocompromised patients is about 25.2% in this study.

Biofilm formation

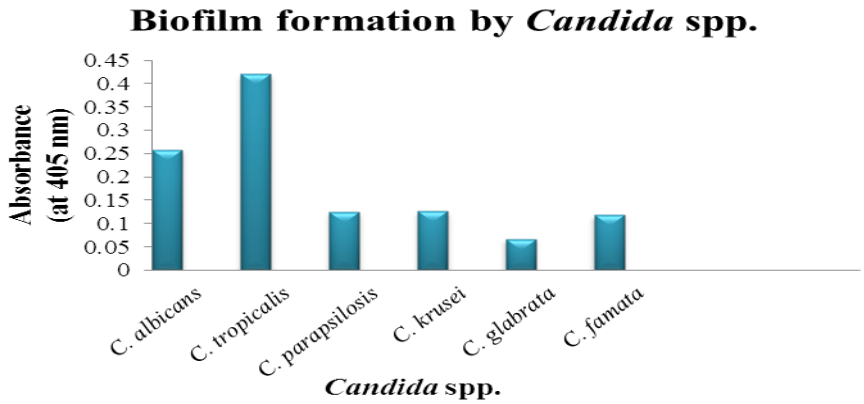

Fig-2: The above graph showing biofilm formation of *Candida* spp.

Biofilm formation plays an important role in the pathogenesis of fungi. In this study we have also demonstrated that all *Candida* species had ability to biofilm formation on microtitration plate to adhere on their surfaces, which were evaluated on the basis of their absorbance into microtitration reader. The mean value was calculated of all biofilm absorbance of *Candida* species then the outcome of the absorbance of *C. albicans* was 0.25, C. *tropicalis*, *C. parapsilopsis, C. krusei, C. glabrata,* and *C. famata* was 0.42, 0.125, 0.126, 0.067, and 0.118 respectively. *C. tropicalis* revealed a high capacity to form biofilm according to their absorbance as show in the graph (Fig-2). The absorbance of *C. albicans* was less than *C. tropicalis*. The above graph shows the lowest absorbance of *C. glabrata* i.e. less biofilm producer as well as *C. tropicalis* is highest biofilm producer and has ability to cause some clinical problems.

These studies were performed on *Candida* cells growing in yeast nitrogen base (YNB) medium, that are supports growth of hyphae, pseudohyphae and blastospores. The hyphal forms of *Candida* species are believed to play an important role in fungal infection by the formation of biofilm into the immunocompromised, ICU, NICU and HIV patients. After 48 hrs, biofilm formed in this medium showed no difference in terms of dry biomass and metabolic activity, compared to biofilm grown in YNB.

Antibiotic susceptibility

In this study, it was observed that *C. albicans* and *C. tropicalis* were causing more infections and sometime many serious diseases in the immunocompromised, ICU, NICU and HIV patients. On analyzing the antifungal susceptibility test we observed that amphotericine-B and fluconazole was more sensitive to the all *Candida* spp. Out of 115 samples, fluconazole was 68.6% sensitive, 25.2% resistance and 6% intermediates. Voriconazole was 82.6% sensitive, 7.8% intermediates and 10.4% resistance. Amphotericine-B 88% sensitive, 6% intermediates and 5.2% resistance. Intraconazole 77.3% sensitive, 9.5% intermediates and 13.5% resistance. These drugs belong to broad spectrum group and more effective to those patients who suffered from suspected *Candida* infection in immunocompromised ICU and NICU and HIV patients.

Table-3: Antibiotic susceptibility for Candida species

Candida spp.	No. of Isolates	Zone diameter (mm) defining											
		Fluconazole			Variconazole			Amphotericine-B			Intraconazole		
		S (≥19)	**I** (15-18)	**R** (≤16)	**S** (≥14)	**I** (10-13)	**R** (≤8)	**S** (≥15)	**I** (10-14)	**R** (≤9)	**S** (≥16)	**I** (10-15)	**R** (≤13)
C. albicans	62	43 (69.3%)	3 (4.8%)	16 (25.3%)	53 (84.4%)	3 (4.8%)	6 (9.6%)	56 (90.4%)	3 (4.8%)	3 (4.8%)	51 (82.2%)	3 (4.8%)	8 (12.9%)
C. tropicalis	30	21 (70%)	2 (6.6%)	7 (23.3%)	25 (83.3%)	2 (6.6%)	3 (4.7%)	27 (90%)	1 (3.3%)	2 (6.6%)	24 (80%)	3 (10%)	3 (10%)
C. parapsilosis	10	7 (70%)	1 (10%)	2 (20%)	8 (80%)	1 (10%)	1 (10%)	9 (90%)	1 (10%)	-	6 (60%)	2 (20%)	2 (20%)
C. krusei	9	6 (66.6%)	1 (11.1%)	2 (22.2%)	7 (77.7%)	1 (11.1%)	1 (11.1%)	7 (77.7%)	1 (11.1%)	1 (11.1%)	6 (66.6%)	2 (22.2%)	1 (11.1%)
C. glabrata	2	1 (50%)	-	1 (50%)	1 (50%)	-	1 (50%)	1 (50%)	1 (50%)	-	1 (50%)	1 (50%)	-
C. famata	2	1 (50%)	-	1 (50%)	1 (50%)	1 (50%)	-	1 (50%)	1 (50%)	-	1 (50%)	-	1 (50%)

NOTE- S= sensitive, I= intermediates, R= resistant

Table-3 represents the antifungal susceptibility results of four drugs against all *Candida* species. Out of 62 *C. albicans* isolates, 69.3% was sensitive, 4.8% intermediates and 25.3% was resistance to the fluconazole; whereas 84.4% sensitive, 4.8% intermediates and 9.6% resistance to the voriconazole. For amphotericin-B 90.4% sensitive, 4.8% intermediates and 4.8% resistance; while 82.2% sensitive, 4.8% intermediates and 12.9% resistance to the intraconazole. For 30 isolates of *C. tropicalis* 70% was sensitive, 6.6% intermediates and 23.3% resistance against fluconazole; 83.3% sensitive, 6.6% intermediates and 4.7% resistance to the voriconazole; 90% sensitive, 3.3% intermediates and 6.6% resistance to the amphotericin-B; 80% sensitive, 10% intermediates and 10% resistance to the intraconazole. Similar to this result various studies also revealed that fluconazole shows gradual decreased in the sensitivity against *Candida* species. While, amphotericine-B is more frequently used against the *Candida* species and it is almost 85-90% sensitive to over all *Candida* species in this study.

CONCLUSION

In this study, we obtained many different pathogenicity among *Candida* species isolates such as- *C. albicans, C.tropicalis, C. parapsilosis, C. krusei, C, glabrata, C. famata* and from different clinical samples but in which, some properties related to *C. albicans* and *C. tropicalis* cells give them the capacity to cause major disease infectious species among them. Adherence to cell surface, germ tube formation, biofilm formation with consequent development of the filamentous form, phenotypic variability, and production of toxins and extracellular enzymes constitute important factors for the emergence of infections by *Candida* species. In this investigation we found that *C. albicans* were obtained from sputum in a highest number that shows it causes infection in chest that leads to pulmonary candidiasis and may also cause serious problem and *C. albicans* was also isolates in blood that leads to candidimia. The majority of *C. tropicalis* and *C. albicans* are causing a major infection in urinary tract, and *C. tropicalis* are also a high biofilm producer that leads to many serious diseases in male and female and may also cause vaginal infection. Other many *Candida* spp. were causing a lot of infections and disease in the immunocompromised ICU, NICU and HIV patients also. Amphotericine-B is more frequently used against the *Candida* species and it is almost 85-90% sensitive to over all *Candida* species as well as fluconazole is found to be less sensitive drug against *Candida* species.

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