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# Speciation, Biofilm production and Antifungal Susceptibility of Non-albicans Candida species isolated from various clinical samples at a Tertiary care hospital in Hyderabad.

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***Abstract:***
***Background****:Emerging Non-albicans candida species among hospitalised patients are a major threat. Biofilm producing species are associated with chronic and recurrent infections and resistance to antifungal agents. Hence, this study was undertaken to detect biofilm production and antifungal susceptibility pattern of these Non-albicans Candida species.*
***Materials and Methods****:46 Non-albicans candida species isolated from Jan to June 2022 from various clinical samples were further speciated by colony morphology on cornmeal agar and colour production on chrom agar. Biofilm production was detected by Congo red agar and Microtitre plate methods. Broth microdilution method was used to evaluate the antifungal susceptibilities of these isolates.*
***Results****: Among 46 isolates, 20 (43.47%) were C.tropicalis, 14(30.43%) were C.parapsilosis, 7(15.21%) were C.glabrata, 5(10.86%) were C.krusei. Biofilm producers were 15(32.60%) by Congo red agar method and 18(39.13%) by Microtitre plate method. 93.47% of the isolates were sensitive & 6.52% were resistant to Caspofungin. 54.34% of the isolates were sensitive, 19.56% were intermediate sensitive & 26.08% were resistant to Voriconazole.*
***Conclusion:*** *Non-albicans candida species cannot be overlooked as a mere contaminant or non-pathogenic commensals. Biofilm formation is a key virulence factor for survival of these species. As Non-albicans candida species differ widely in susceptibility to routinely used antifungal drugs, antifungal susceptibility testing plays an important role in evaluating therapies.*
***Key Word****: Non-albicans Candida species, Biofilm production, Antifungal susceptibility.*

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## I. Introduction

The incidence of mycotic infections have progressively increased over the last few years[1]. Candida species though commensal organisms that normally colonise mucosal surfaces in an asymptomatic manner can become one of the most significant causes of disabling and lethal infection[1]. Candida species have been recognised as the fourth commonest cause of nosocomial invasive infections[2].Candida albicans is the predominant cause of candidiasis. However, a shift towards Non-albicans candida species has been recently observed[1].

One of the most important factors contributing to virulence of these species is the Biofilm formation which can act as a reservoir of agents, allow coinfection with other pathogens, promote persistence of infection and increase mortality[4]. Biofilm formation is associated with chronic and recurrent human infections which are highly resistant to antimicrobial agents[5].

Antifungal drug therapies are selected empirically, but invitro susceptibility testing is essential for guiding or altering the therapy. The study of Antifungal resistance remains an area of significant importance owing to the rise of drug resistant isolates[6].The resistance of C.albicans, C.tropicalis and C.glabrata to Fluconazole is increased compared to other drugs due to indiscriminate use of Fluconazole for long periods[19]. C.krusei and C.glabrata are intrinsically resistant to Fluconazole. All these factors have led to increase in mortality and morbidity rates in patients with fungal infections warranting rapid identification and antifungal susceptibility testing at the earliest.

Hence, the present study was conducted to isolate Non-albicans candida species from various clinical samples followed by biofilm detection and antifungal susceptibility testing.

## II. Materials And Methods

The present cross-sectional study was conducted in the Microbiology department at Osmania General Hospital, Hyderabad, Telangana for a period of 6 months from January to June 2022. Patient's demographic details like age, sex & other risk factors like Diabetes mellitus & H/O any urinary tract catheterisation were recorded. Candida isolates which showed Gram positive budding yeast cells with or without pseudohyphae& negative for Germ tube test -were included in the study.46 Non-albicans candida isolates obtained from various clinical samples were inoculated onto Sabouraud's Dextrose Agar with Gentamicin & incubated at 37ºC for 24-48 hrs. Periodic subculturing was done every 1 month to maintain viability & purity.

***SPECIATION -***

***CHROM agar method -***Simultaneously, all Non-albicans candida isolates were inoculated onto Candida Chrom agar and incubated at 37 ℃ for 24 - 48 hrs and species were identified by the colour of the colonies on Chrom agar.

***Cornmeal agar method -***Few colonies of all the isolates from culture were picked up and inoculated onto cornmeal agar as parallel lines about 1 cm apart & then criss-crossed several times. A coverslip held with forceps, heated over flame & allowed to cool, then placed over surface and plate was incubated at 25-30℃ for 48-72 hrs. Then, observed under 40x magnification of light microscope. Hyphae and pseudohyphae with blastospores and terminal chlamydospores were noted. Non-albicans candida species were identified based on their colony morphology.

***BIOFILM PRODUCTION –***

***Congo red agar method -***

Congo red agar plates were inoculated with colonies from each isolate and incubated aerobically for 48 hrs at 37°C. Black coloured colonies with dry crystalline consistency were interpreted as positive biofilm producing strains and negative results were indicated by white or very light pink-colored colonies. Candida parapsilosis (ATCC 22019 - non-biofilm producer) was used as negative control.

***Microtitre plate method -***

Biofilm formation was performed on a sterile 96-well microtiter plate.A colony of each isolate was inoculated into tubes containing 2 ml of Brain heart infusion broth (BHIB) and incubated at 37°C for 24 hours. All the broth cultures were diluted at a ratio of 1:20 using fresh BHIB, and 200 µl was placed into microtiter plates and incubated at 37°C for 24 hours. After the completion of incubation, microtiter plates were emptied, rinsed with distilled water three times, and then inverted to blot. Each well was then filled with 200 µl of 1% crystal violet and incubated for 15 mins. After incubation, theplates were again rinsed three times with distilled water. Then 200 µl of ethanol: acetone mixture (80:20 w/v) was added to each well and were read at 450 nm using an ELISA reader and OD was recorded for each well. Sterile BHIB without microorganisms was used as the negative control. The cut-off value was determined by arithmetically averaging the OD of the wells containing sterile BHIB and by adding a standard deviation of +2. Samples with an OD higher than the cut-off value were considered positive, whereas those with the lower optical density than the cut-off wereconsidered negative.

***ANTIFUNGAL SUSCEPTIBILITY –***

***Microbroth dilution method –***

All Non-albicans candida isolates were subjected to antifungal susceptibility testing by Microbroth dilution method.

***Medium used -*** RPMI (Rosewell park memorial institute medium) 1640 medium with glutamine, without bicarbonate in MOPS (3N-Morpholino propane sulphonic acid).

***Inoculum preparation -*** Inoculum was prepared by picking 5 colonies from 24 hrs old culture Candida species & colonies were suspended in 5 ml of 0.145 mol/L sterile saline.Resulting suspension was vortexed for 15 secs &cell density was adjusted with spectrophotometer by adding sufficient sterile saline to increase the transmittance to 0.5 Macfarlands at 530 nm wavelength. This yielded a yeast stock suspension of $1 \times 10^6$ to $5 \times 10^6$ cells/ml. A working suspension was made by 1: 100 dilution followed by 1:20 dilution of stock suspension with RPMI 1640 broth medium which results in $0.5 \times 10^3$ to $2.5 \times 10^3$ cells/ml.

***Antifungal stock solution -*** Sterile distilled water was used to dissolve Caspofungin powder and DMSO (dimethyl sulfoxide) was used for dissolving Voriconazole powder. The antifungal stock suspension was prepared in the range of 16 µg - 0.0313 µg/ml for Caspofungin and Voriconazole.

***Procedure -***Disposable 96 well microtitre plates were used. Sterility control and growth controls were also included.100 µl of 2x concentration of inoculum was added to each well followed by 100 µl of 2x concentration of the drug were added to the corresponding wells (row 1 - highest concentration to row 10 - lowest

concentration). The microtitre plates were incubated at 37 ℃ for 24 to 48 hrs . The MIC (minimum inhibitory concentration) end point was observed and interpreted as per CLSI M27-A3 guidelines. Candida parapsilosis ATCC 22019 strain was used for quality control in all the runs.

## III. Results

During the study period of 6 months from January 2022– June 2022, 46 Non-albicans candida species were isolated. All the isolates were subjected to speciation by Chrom agar and Cornmeal agar methods. The species which did not produce satisfactory colour production by Chrom agar were further confirmed by observing the colony morphology on Cornmeal agar.

Among these isolates, the most common species were C.tropicalis (43.47%), followed by C.parapsilosis (30.43%), C.glabrata (15.21%) and C.krusei (10.86%). Maximum number of Non-albicans candida species were seen in the age group of 51-60 yrs followed by 41-50 yrs. 30(65.21%) were isolated from males and 16 (34.78%) were isolated from females with male to female ratio of 1.8:1. Maximum number were isolated from urine (85%) followed by sputum (11%), suction tip (2%) and blood (2%).Underlying risk factors were catheterisation (54.34%),Diabetes mellitus (50%) and Diabetes along with catheterisation (23.91%).

By Congo red agar method, 15/46 (32.60%) isolates produced biofilm among which C.tropicalis was the predominant biofilm producer. 8/20 (40%) C.tropicalis , 3/14 (21.42%) C.parapsilosis , 3/7 (42.85%) C.glabrata and 1/5 (20%) C.krusei were detected as biofilm producers by this method.

By Microtitre plate method, 18/46 (39.13%) of the isolates produced biofilm among which C.tropicalis was the major biofilm producer. 10/20 (50%) C.tropicalis, 4/10 (28.57%) C.parapsilosis, 3/7 (42.85%) C.glabrata and 1/4 (20%) C.krusei were detected as biofilm producers by this method.

ByMicrobroth dilution method, 100% of C.parapsilosis,100% of C.krusei,90% of C.tropicalis and 85.71% of C.glabrata isolates were susceptible to Caspofungin .14.28% of C.glabrata and 10% of C.tropicalis isolates were resistant to Caspofungin. No resistance to Caspofungin was shown by C.parapsilosis and C.krusei.

Whereas, 50% of C.tropicalis, 64.28% of C.parapsilosis,42.85% of C.glabrata and 60% of C.krusei were sensitive to Voriconazole. 15% of C.tropicalis, 21.42% of C.parapsilosis and 42.85% of C.glabrata showed intermediate sensitivity to Voriconazole. 40% of C.krusei , 35% of C.tropicalis, 14.28% of C.glabrata and 14.2% of C.parapsilosis were resistant to Voriconazole.

Among Biofilm producers detected by Microtitre plate method, 33.33% of C.glabrata and 11.11% of C.tropicalis were resistant to Caspofungin and no resistance was found among C.parapsilosis and C.krusei isolates.Whereas,100% of C.krusei, 40% of C.parapsilosis, 33.33% of C.tropicalis and 33.33% of C.glabrata were resistant to Voriconazole.

**Figure 1-**

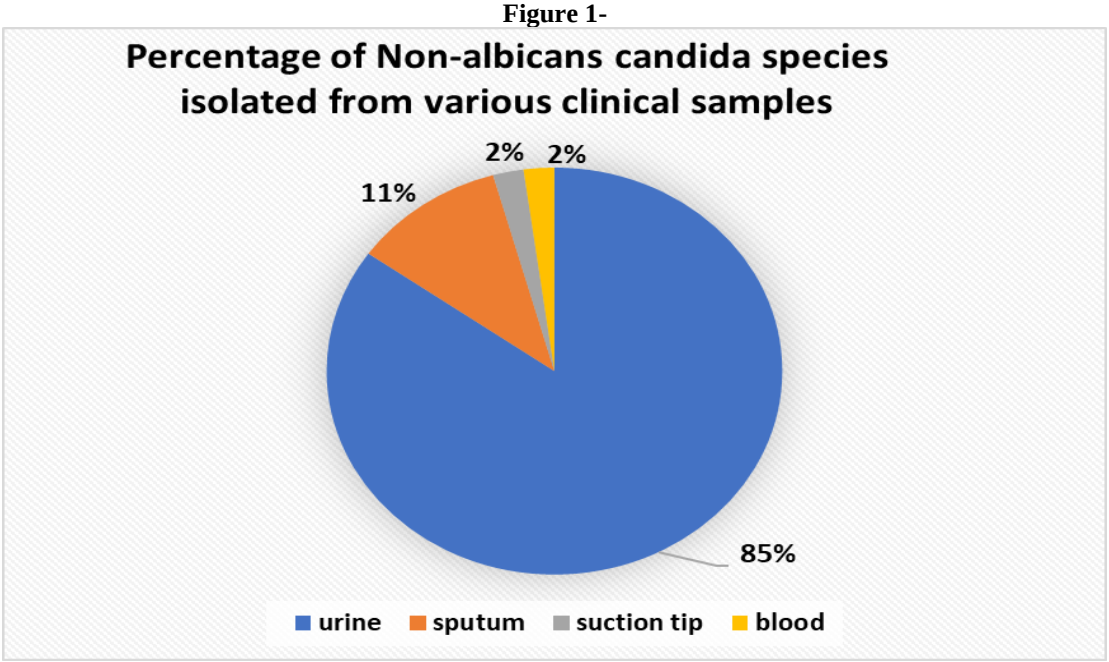


**Figure 2 -**

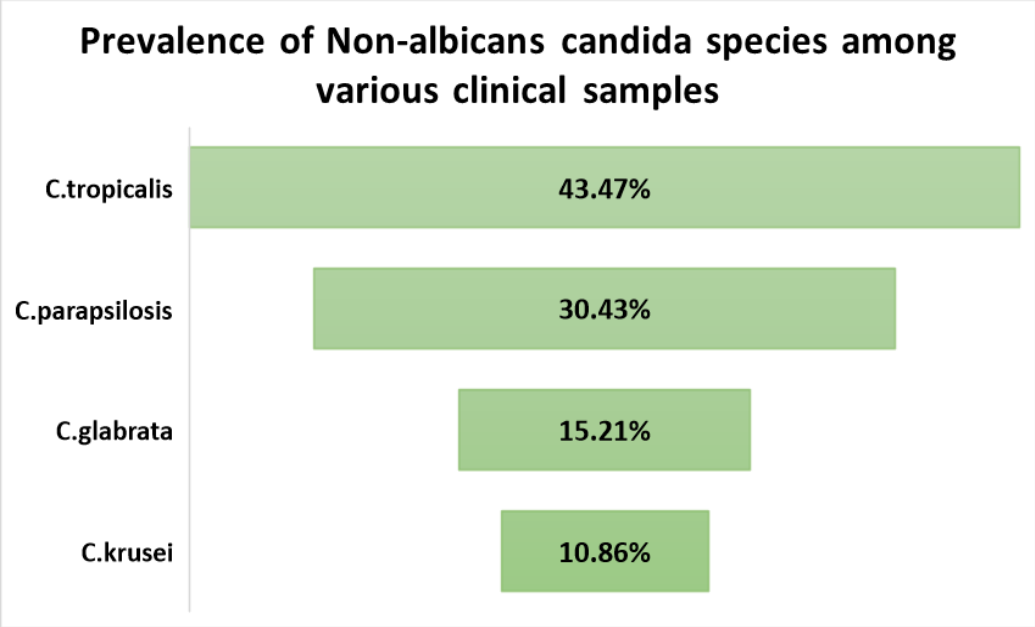
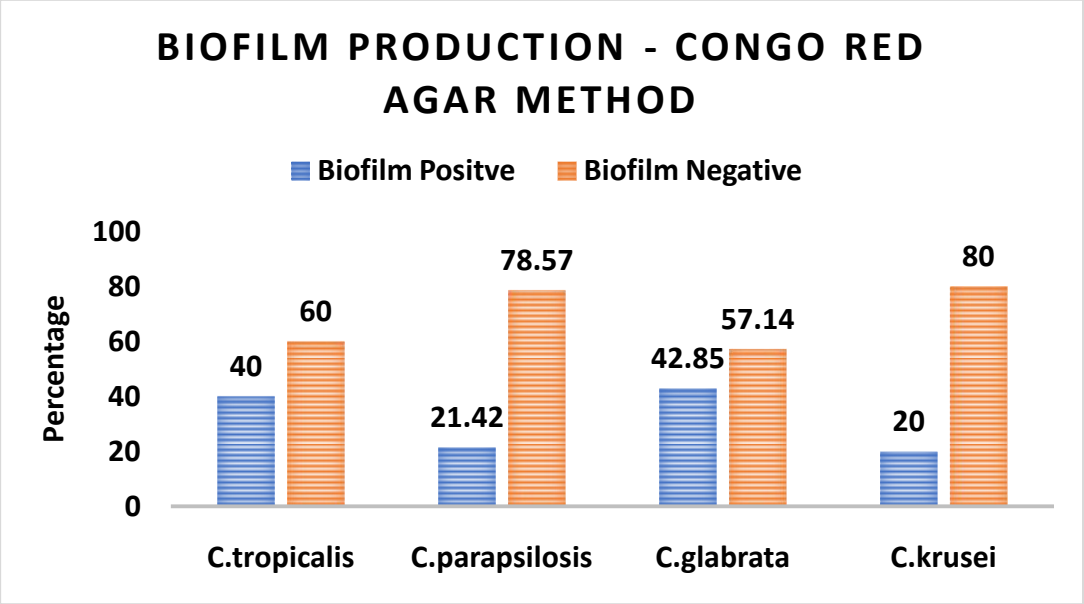



**Figure 3 -**



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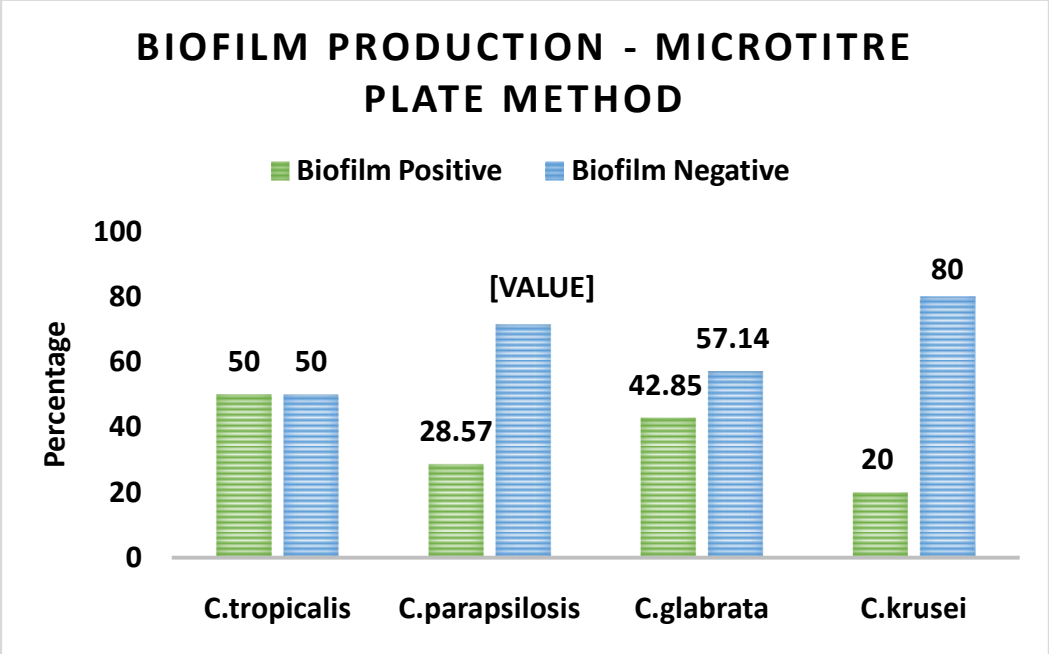
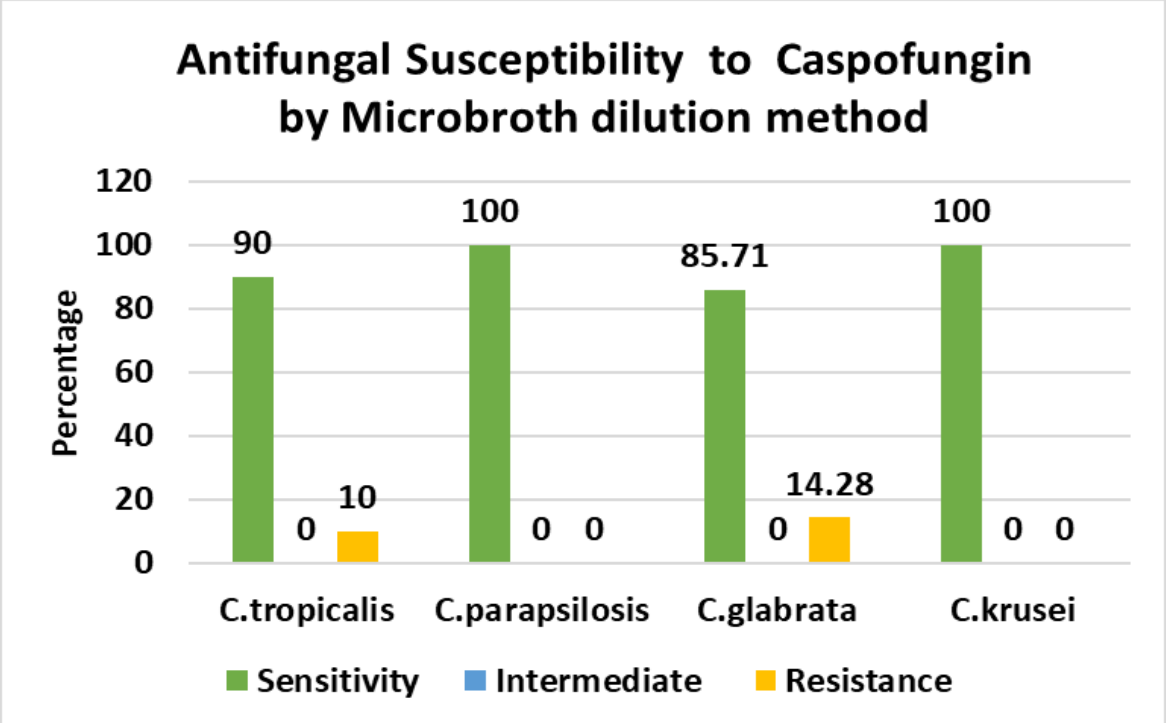



**Figure 5 -**



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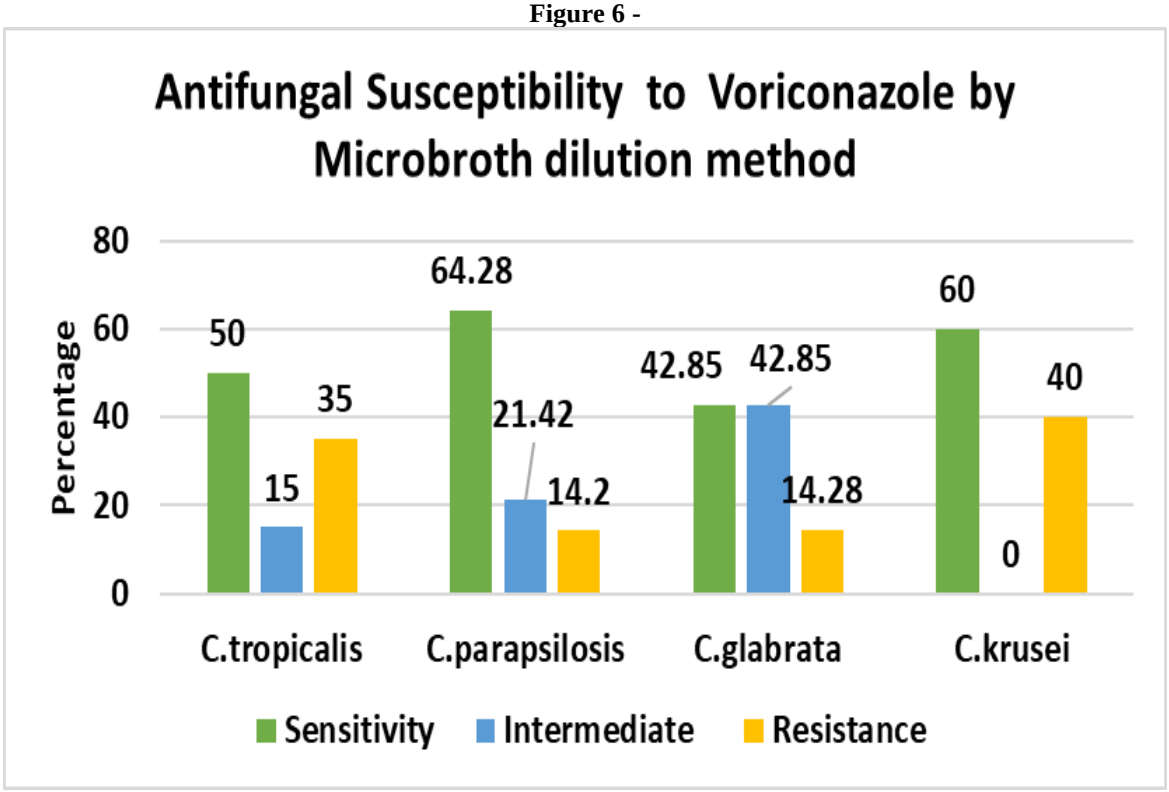


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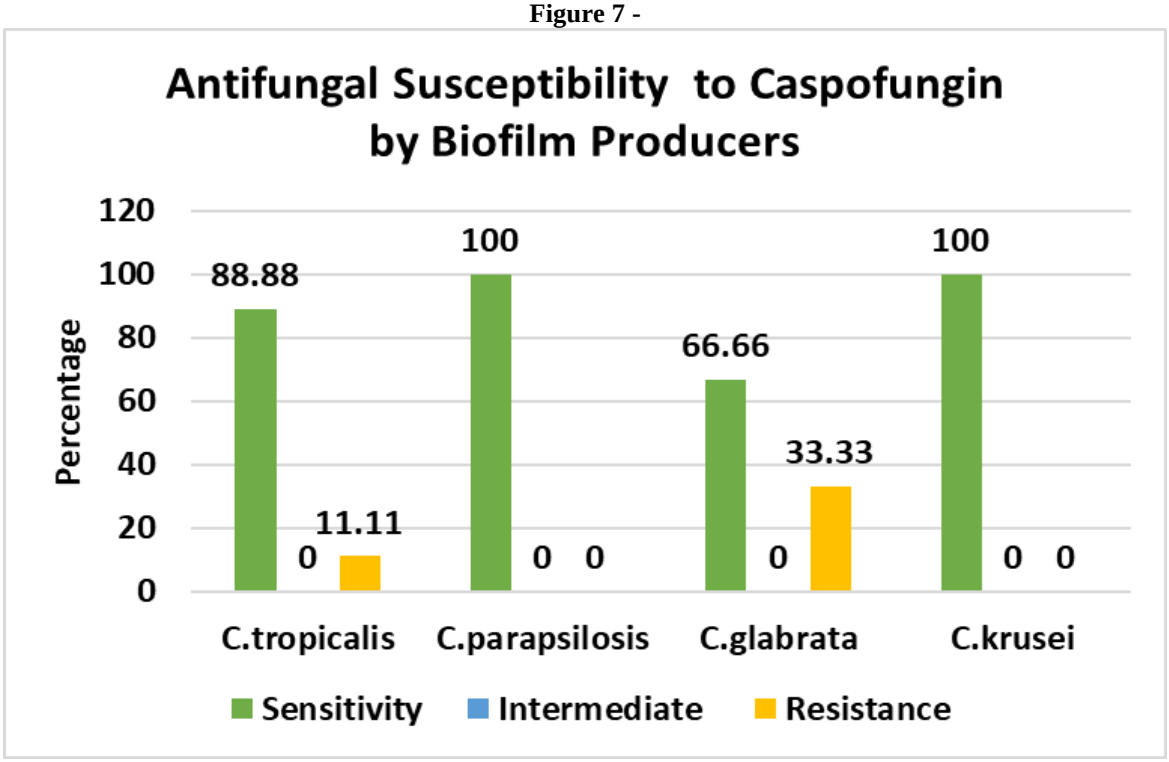

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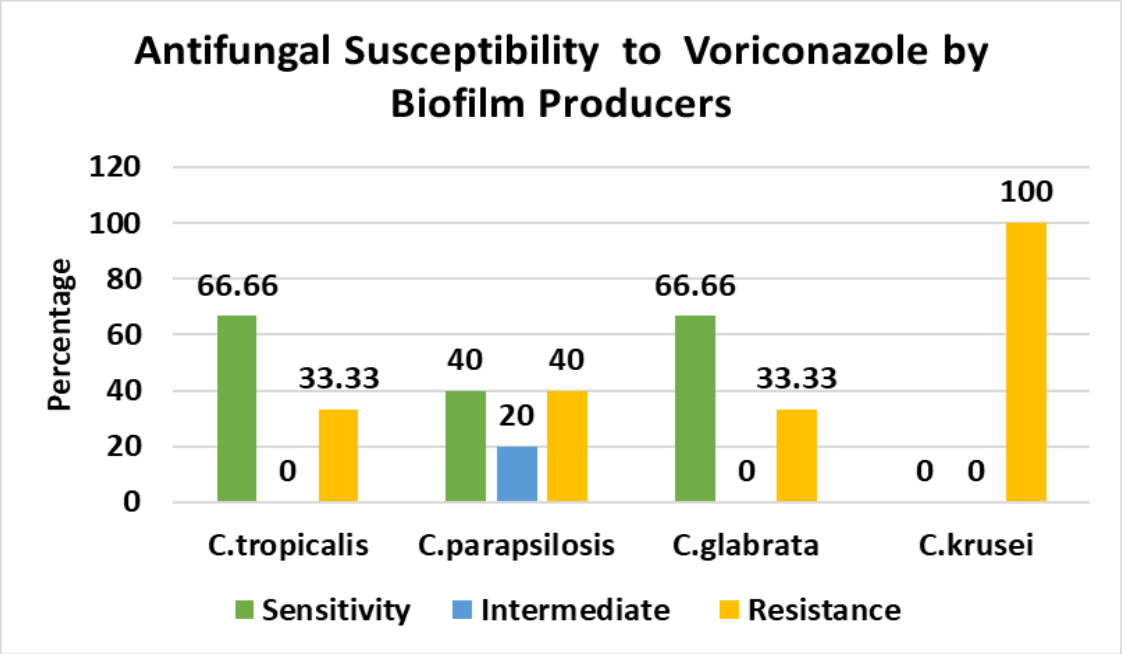


## IV. Discussion

Biofilms are notoriously difficult to eliminate and serve as a source of recalcitrant infections and their study is highly relevant for public health [20].Increased species diversity and incidence of infections have resulted in the need for an accurate and rapid identification of Candida isolates and for proper patient management and also for the prevention of emergence of drug resistance as various species respond differently to antifungal agents[21].

The present study was conducted at a tertiary care hospital among 46 Non-albicans candida isolates. In this study, C.tropicalis was the predominant Non- albicans candida species isolated and it was similar to the studies done by Rajesh Bansal et al.,[5] and Preethi Chaudhary et al.,[10]

The present study showed maximum number of Non-albicans candida species among 51-60 yrs followed by 41-50 yrs.This was similar to the study done by M.B.Marak et al.,[14]

In this study, maximum number of isolates were obtained from males (65.21%).Similar male preponderance type distribution of Non-albicans candida isolates was found in the study done by N.Pahwa et al.,[27].

Maximum number of NAC species were isolated from urine(85%) followed by sputum (11%), blood (2%) and suction tip (2%) in the present study.This was similar to the studies done by Singh M et al.,[25] and Urvashi Chongtham et al.,[30]

Present study showed risk factors to be Catheterisation (54.34%) and Diabetes mellitus (50%).These findings were similar to the study done by Abhishek kumar Jain et al.,[29]

In the present study, 50% of C.tropicalis ,28.57% of C.parapsilosis, 42.85% of C.glabrata and 20% of C.krusei were detected as Biofilm producers by MTP method. Percentage of biofilm production by C.tropicalis and C.krusei isolates were similar to the study done by Rajesh Bansal et al.,[5] and the percentage of biofilm production by C.parapsilosis was similar to the study done by Tulsidas S et al.,[24]

In this study, 100 % of C.parapsilosis, 100% of C.krusei, 90% of C.tropicalis and 85.71% of C.glabrata were sensitive to Caspofungin. The sensitivity pattern of C.tropicalis, C.parapsilosis, C.glabrata were similar to the study done by Meng Xiao et al.,[26]. However, the sensitivity pattern of C.kruseiwas not similar.

This study showed that 50% of C.tropicalis, 64.28% of C.parapsilosis,42.85% of C.glabrata and 60% of C.krusei were sensitive to Voriconazole.The sensitivity pattern of C.tropicalis was similar to the study done by Meng Xiao et al.,[26].However,the sensitivity pattern of C.parapsilosis,C.glabrata and C.krusei were decreased when compared to the studies done by Meng Xiao et al.,[26] and Oberoi et al.,[17]

Overall, the present study showed that 93.47% and 54.34% of Non-albicans Candida isolates were sensitive to Caspofungin and Voriconazole respectively.The sensitivity pattern to Caspofungin was similar to the study done by Preethi Chaudhary et al.,[10].However, the sensitivity pattern to Voriconazole was much decreased when compared to the study by Preethi Chaudhary et al.,[10].

## V. Conclusion

Isolation of Non-albicans candida species cannot be overlooked as a mere contaminant or non-pathogen. In this study, Microtitre plate method was found to be more sensitive, accurate & reliable for Biofilm detection. Caspofungin still remains the most sensitive antifungal agent compared to azoles. Among biofilm producers, Voriconazole showed decreased sensitivity pattern compared to Caspofungin. Because of the increasing incidence of Non-albicans candida infection along with the emergence of drug resistant phenotypes, speciation is extremely important and helps the clinicians to select appropriate antifungal agents for better treatment outcomes.

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