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Detection of Phospholipase Activity in Clinical Isolates of Candida species in a Tertiary Care Hospital

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Abstract: *Candida is a yeast-like fungus and omnipresent in the environment, which makes it one of the most common pathogens for opportunistic infections. Candida usually causes mucosal Candidiasis but may also cause systemic and disseminated infections in debilitated patients. Phospholipase production has been reported to be one of the virulence factors in Candida. This study was carried out to isolate various species of Candida from different clinical specimens and to check their Phospholipase activity. All the clinical specimens suspected of fungal aetiology were analysed and subjected to Microbiological identification of Candida species. Candida species were subjected toPhospholipase activity by Egg Yolk Agar Plate method. A total of 7 varieties of Candida species (C.albicans 25.45%, C. tropicalis 32.72%, C. krusei 12.72%, C. kefyr 12.72%, C. dubliniensis 10.90%, C. glabrata 3.63%,C.utilis 1.81%) were isolated from various clinical samples.Out of total 54 isolates, 29(53.70%)showed extensive Phospholipase activity.*

Keywords: *Candida albicans, Candida-non-albicans,* Phospholipase activity, Candidiasis, Egg Yolk.

Introduction: Yeasts are omnipresent in the environment and some belong to the normal human flora. *Candida* is a yeast like fungus and is one of the normal flora of human body residing mostly in vagina, mouth, intestinal tract, skin and other mucous membranes. It changes in the internal and external environment from a completely harmless saprophyte into a virulent pathogen especially in immunocompromised individuals. Morphologically *Candida* are oval yeast cells, with pseudo-hyphae. When grown in the laboratory, *Candida* appears as large, round, white or cream colonies with a yeasty odour on agar plates. *C. albicans* ferments glucose and maltose producing acid and gas, sucrose with acid and without gas production and fails to ferment lactose which helps it to distinguish *C. albicans* from other *Candida species.*

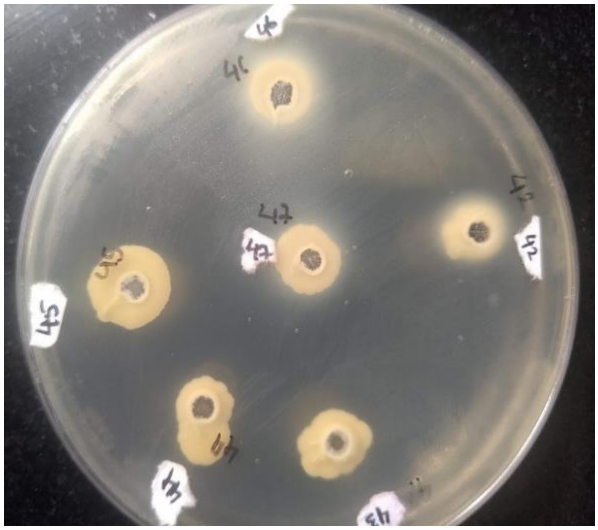
Although most infections of Candidiasis are caused by *C. ablicans*, there has been striking increase in the frequency with *non-albicans Candida species* in last few years as well.[1] There has been an increase in treatment failure because of drug resistance.[2] It can also be attributed to some virulence factors. These putative virulence factors include secretion of proteinase, adherence to host cell surface, germination, and Phospholipases, though not even a single putative factor has been clearly reported with pathogenicity.[3] There are reports showing Phospholipase activity as one of the important virulence factors of *Candida species*.[4] There is study showing co-relation between Phospholipase activity with mucosal invasion **[5].** *Candida* isolates producing greater number of Phospholipases leading to increased mortality in rat models which has been reported**[6].**

Phospholipases contribute to pathogenicity of *bacteria* [7], *protozoa* [8], *and rickettsiae*[9] the role of these membrane damaging enzymes is proposed in the pathogenicity of *C. albicans* as well. Other than Phospholipase, Secreted Aspartyl Proteinases, Germ tube formation, adherence to host tissue and phenotypic switching are also factors contributing to the virulence of *Candida.* Both hydrolytic enzymes cause cellular damage. Different species of *Candida* produce varying degree of Phospholipases; hence speciation of the *Candida* till species level becomes important. *Vidotto et al.* [10] have reported that the correlation between Phospholipase activity and high Germ tube formation can facilitate the mucosa penetration. A comparison between the level of production of Phospholipase of *C. albicans* isolated from cases of infections and commensals [11] has been described.

Therefore, the present study was planned to isolate *Candida spps*. in various clinical specimens and to detect their Phospholipaseactivity

Material and Methods: This study was carried out in a period of 1 year from October 2018-October 2019 in the Department of Microbiology, MGM Medical College and Hospital, Kamothe, Navi Mumbai, India. A total of 50 *Candida* were isolated from 167 clinical specimen suspected of fungal aetiology. All the samples were processed as under:

1. Microscopy
    a. KOH wet mount- For rapid detection of Pseudo-hyphae and budding yeast cell and to dissolve the organic matter such as Hair and Nail.
    b. Grams staining- for identification of budding yeast cells with or without Pseudo-hyphae.
2. Culture on SDA- The specimens were then inoculated onto Sabouraud Dextrose Agar (SDA- Hi-media) and incubated at 37ºC for 24-48 hours and observed for typical colonies of *Candida species*.
3. Species identification
    a. Urea hydrolysis- To rule out the possibility of Cryptococcus
    b. Germ tube (GT) test- To differentiate between *albicans* and *non-albicans* group.
    c. Sugar assimilation[12]- Yeast Nitrogen plates (Hi media laboratories) were made and flooded with yeast suspension, air dried and then sugar impregnated discs (Hi-media laboratories) were put and incubated 37ºC for 24-48 hours and observed for the hazy zones around.
4. Speciation by Chrom Agar- Final step in the species identification by distinct colour formation on CHROM agar plates (HiCHROM by Hi media laboratories) were used.
5. Demonstration of extracellular Phospholipase activity- Extracellular Phospholipase activity was determined by a method described Price *et al.* [13] that is Egg yolk agar Plate method. All the isolates were then subjected for Phospholipase production, by creating wells (keeping the diameter same) onto the plates, as it gives better infusibility, 10µ of the yeast suspension was inoculated and incubated at 37ºC for 4 days and observed for precipitation zone around the colonies. Egg yolk agar was prepared by 6.5gms of SDA, 5.84gms of NaCl and 0.55gms of $CaCl_2$ dissolved in 98ml of distilled water and sterilised by autoclaving at 121ºC for 15 minutes. Egg yolk was separated from Egg white and centrifuged at 4500 rpm for 45 minutes, 2ml of supernatant was then added to cooled medium (45-50ºC).



Phospholipase Activity

Candida spps. on Chrome Agar

Results and Observations: Microbiological analysis of all the specimens received for fungal cultures were processed and 54 isolates of yeasts were identified for further study. The isolation was done from various specimens including Sputum, Urine, Blood, High Vaginal Swab (HVS), Pus, Nail clipping and Foley's tip. It was also corelated with duration of infection as well as the Phospholipase activity of each isolate.

Fig. 1: Rateof Isolation of various Candida species in the Tertiary Care Hospital

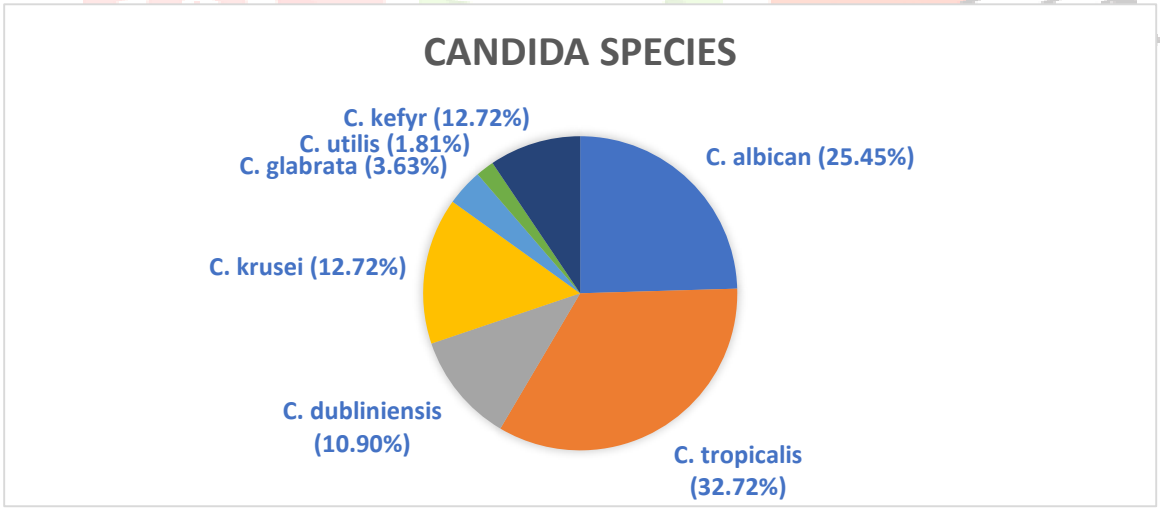
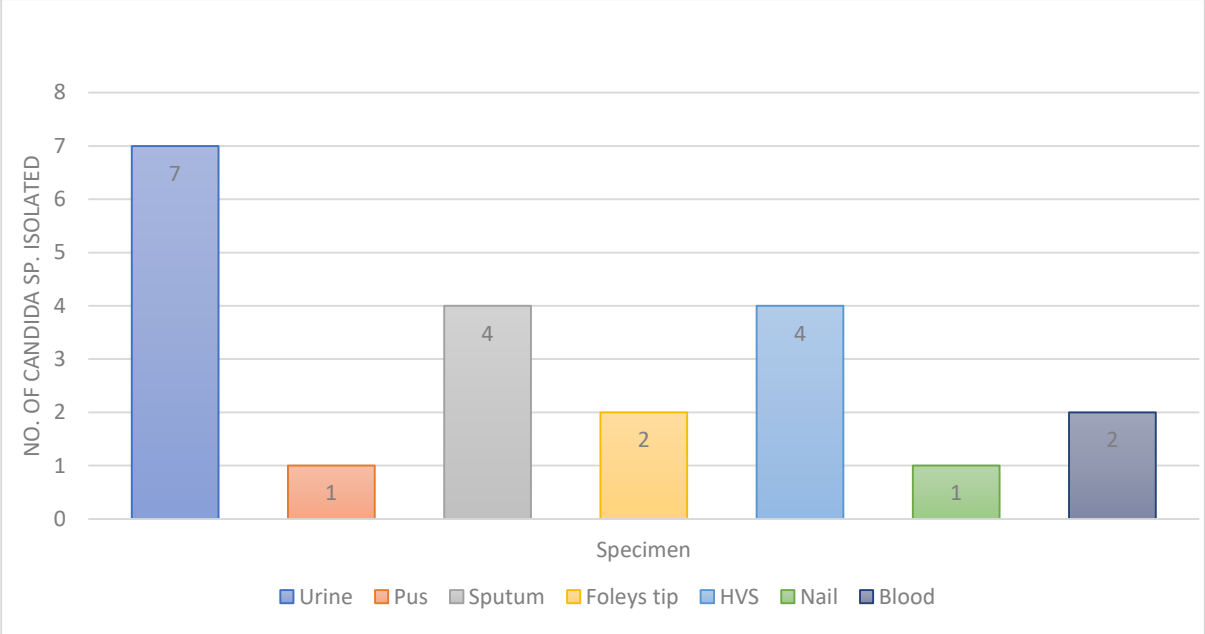


Figure 1 depicts the no. of species isolated and its percentage with respect to the total no. of *Candida* isolated. *Candida tropicalis* (32.72%) was found to be the predominant species whereas as *Candida utilis* (1.81%) was found to be the least common species in this study.

Graph 1: Variety of *Candida species* isolated in Various Specimens-



The above Graph depicts rate of isolation of various species of *Candida* in various clinical specimens. A total of 7 varieties of *Candida* were isolated from Urine whereas only 1 type of species were isolated from Pus and Nail Clipping.

All the isolates were also subjected to check Phospholipase activity. The Phospholipase activity was calculated in terms of Pz Value which was determined by the following formula[13]:

$$\text{Pz} = \frac{\text{Colony diameter}}{\text{Colony diameter} + \text{Zone of precipitation}}$$

The Pz value was classified in following based on intensity:

Pz value	Intensity
1 (Negative)	0
<0.90-0.99	+
0.80-0.89	++
0.70-0.79	+++
<0.70	++++

It was found that 29 (53.70%) isolates were positive for Phospholipase activity and all showed

Strong Phospholipase activity i.e., Pz value <0.70, whereas the rest 25 (46.29%) were negative for phospholipase activity.

Table 1: Average Pz value with Mean Percentage of Various *Candida species* Isolated from Various Specimens-

Candida species	**Average Pz Value**	**Mean %**
C. albicans	0.0442	4.42
C. tropicalis	0.046	4.65
C. dubliniensis	0.045	4.5
C. krusei	0.067	6.73
C. kefyr	0.041	4.1
C. utilis	0.055	5.5
C. glabrata	0.00	0

It was found that 29 of 54 isolates were positive for Phospholipase activity and they all showed strong Phospholipase activity i.e., Phospholipase positive (Pz <0.70).

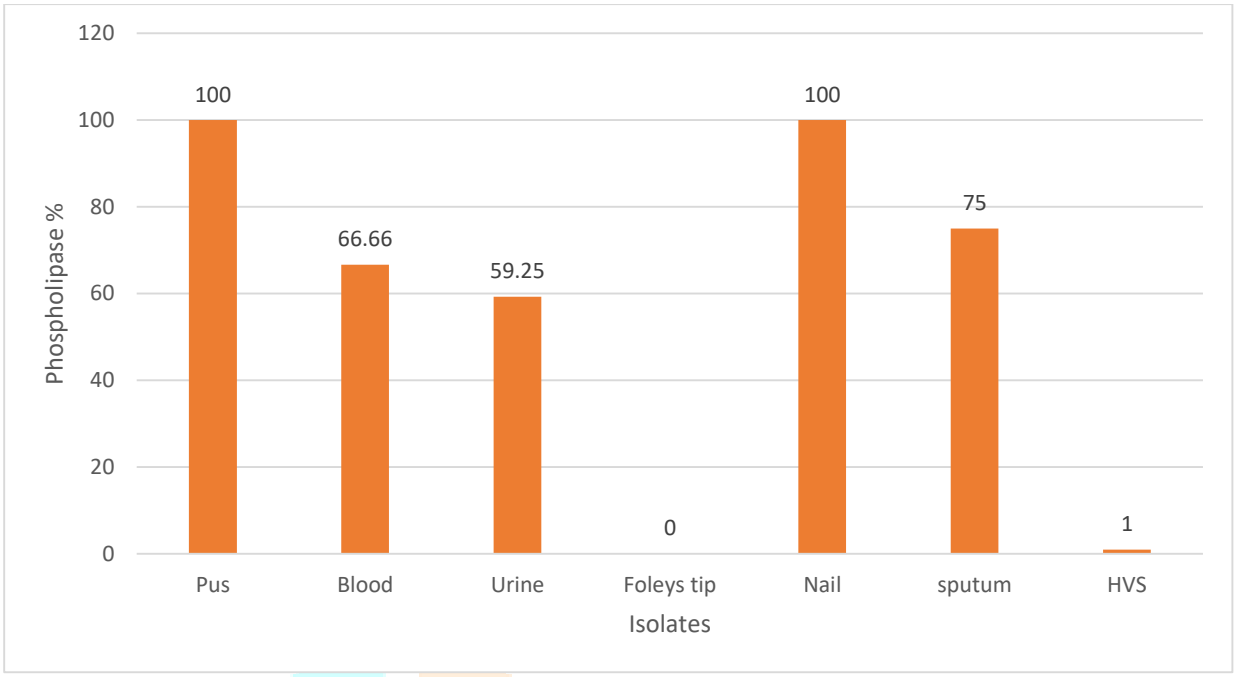
The above Table shows that Pz value of *Candida* isolates range between 0.041-0.067. Highest activity was recorded in the isolates of *C. krusei* and lowest in *C. kefyr*. The commonly isolated *Candida species* were *C. albicans and C. tropicalis* which showed Phospholipase activity in the range of 0.44-0.046.

Table 2: Phospholipase Activity of Various *Candida species*:

Candida species	**Total isolate**	**Phospholipase (+)**	**Percentage (%)**
Candida albicans	13	8	69.23
Candida tropicalis	18	12	66.66
Candida dubliniensis	6	1	16.66
Candida krusei	8	3	37.5
Candida kefyr	5	3	60
Candida utilis	1	1	100
Candida glabrata	2	0	NA

The Table 2 shows percentage of Phospholipase activity in different species of Candida. It was observed that both *Candida tropicalis* and *Candida albicans* were potential Phospholipase producers with more than 60% isolates showing Phospholipase production.

Graph 2:Percentage of Phospholipase Activity in *Candida* Isolated from Various Clinical Samples.



Majority of *Candida* isolated from Nail, Pus and Sputum samples showed phospholipase activity.

Discussion: *Candida albicans* is the most common cause of Candidiasis, whereas Other *Candida species* are now replacing *C. albicans* in recent years. The extracellular Phospholipase has been known for its pathogenicity causing membrane disruption and colonisation, which was even reported in few cases **[5].**The state of host is of primary importance in determining *Candida* Pathogenicity [14]. This study was done on various clinical specimens isolated from suspected case of Candidiasis.

In this study maximum *Candida* were isolated from cases of Urinary tract infection (UTI) that is from Urine (56%) followed by Sputum (20%), High Vaginal Swab (8%), Blood (6%), Tip and Pus (8%) and Nail (2%).It shows that *Candida* is major cause of UTI followed by Respiratory Infections and vulvovaginal infections. Similar findings were reported by Das Mohandas*et al.* [4]

In majority of the studies done across the Globe showed *C. albicans* as the major pathogen.**(4,15)** However in the present study we found *C. tropicalis* as most frequently isolated species. In a study by Sudhason Ye [16]and Shivprakasha[17] other *Candida species* were more predominant. This shows that *non-albicans-candida* was more prevalent in our set up.

This study shows the rate of isolation of various species of *Candida*, in which *Candida albicans* was 14 (25.45%), whereas *Candida tropicalis* was 18 (32.72%). Amongst the *non-albicans species, C. tropicalis* 18 (32.72%) was the most predominent followed by *C. krusei* 7 (12.72%) and *C. kefyr* 7 (12.72%), *C. dubliniensis* 6 (10.90%), *C. glabrata* 2 (3.63%) and *C. utilis*1 (1.81%). The ratio of *C. albicans* to *C. non-albicans* group was found to be 2.8:11 in this study, whereas in the study done by SR Fule[14], the ratio was found to be 1.68:1, which is much lesser than what we have got in our study.

There are many reports describing the features of *C. albicans* with probable virulence factors that enables the organism to cause infection in a susceptible host.[14]A study has been done on Role of Phospholipase in virulence and fungal pathogenesis.[18] The highest phospholipase intensity was seen in *C. albicans*, whereas it was minimal in *C. krusei*. All the clinical isolates in this study showed either intense Phospholipase activity or no Phospholipase activity at all, which confers its virulence and pathogenicity as an opportunistic pathogen. Lower is the Pz value, more is the enzymatic activity, and more is the pathogenicity.

The average Pz value ranged from 0.041-0.067. *C. albicans, C.dubliniensis and C. tropicalis* were found nearly equal in terms of Phospholipase activity this could be due to varying no. of species isolated. More than 60% of *C.albicans* and *C. tropicalis* isolates were Phospholipase producers. In this study, 9 of 13 (69.23%) *C. albicans* showed the enzymatic activity whereas, *C.tropicalis* 12 of 18 (66.66%), 1 isolate of *C. utilis*, *C. dubliniensis* 1 of 6 (16.66%), *C. krusei* 3 of 8 (37.5%) and *C. kefyr* 3 of 5 (60%) and in *C. glabrata* none of the strains showed Phospholipase activity. *Albicans* and *non-albicans Candida* showed this activity, whereas as Fule SR [14]. reported that none of the *non-albicans* group showed the Phospholipase activity. Another study by Vinitha M [4] showed that Phospholipase activity was shown by both *albicans* (46.93%) and *non-albicans* (42%) group which correlates with our study.

One important observation noted in this study was 69.23 % *C. albicans* (n= 13) and 66.66% of *C.tropicalis* (n=18) were Phospholipase positive. This indicates that though non albicans Candida were isolated in higher number as compared to *Candida albicans*, phospholipase activity was the common factor contributing to their pathogenicity.

Conclusion: This study was carried out for a period of one year with 50 isolates of *Candida* due to time constrain. However, it clearly highlights the fact that Phospholipase activity could be one of the important factors for pathogenicity of *Candida*. The Authors would like to take this study further to study the effect of phospholipase activity on prognosis of infection.

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