Biofilm formation and enzymatic virulence attributes of pathogenic Candida isolates from clinical cases

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ABSTRACT: Clinical infections due to Candida species is increasing day by day. Virulence factors attributed are production of biofilm, enzymes etc. Biofilm formation help Candida species overcome host defence mechanisms and also antifungal treatment. Detection of biofilm production was done by 3 methods – micro titre plate method, tube method and Congo red agar method. Enzyme production also plays a vital role in pathogenesis of Candida infections. Enzyme demonstrated in this study are phospholipase, esterase, hemolysins. Early detection of these can direct prompt and correct clinical management.

Index Terms: Virulence factors, biofilm, enzymes, *Candida* species

I. INTRODUCTION

The incidence of fungal infections has significantly increased worldwide during the last few decades, with *Candida* species emerging as major global contributors. [1] *Candida* species are widespread opportunistic fungus in clinical samples, having taken over as the fourth most common cause of hospital-acquired systemic infections. [2,3]

Although *Candida albicans* has historically been the main pathogen, non-albicans species such as *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, and *Candida krusei* have become more common in the past ten years. [4,5] Numerous reasons, including chronic antibiotic usage, the use of indwelling intravascular catheters, immunosuppressive medications, cytotoxic therapy, immunological inadequacies, and the development of AIDS, are responsible for this change in the landscape of *Candida* infections. [6] As the most prevalent and deadly form of invasive candidiasis, candidemia—a bloodstream infection brought on by the species *Candida*—has a major negative influence on hospitalized patients' morbidity and mortality. [7]

Candida virulence traits include surface recognition, hyphal switching, germination, and extracellular hydrolytic enzyme synthesis. [8] Extracellular hydrolytic enzymes are one of these, and they are crucial to *Candida* adhesion and infection. [9] Enzymes have a crucial role in pathogenesis of candida as they facilitate hyphal invasion. Various hydrolytic enzymes, including proteases, lipases, phospholipases, esterases, and hemolysins, can be produced by Candida species.

A matrix of exopolymeric material encases structured microbial colonies called biofilms, which are crucial for the development of clinical infection. [10] Production of biofilm leads to intrinsic resistance against various antifungal agents. The pathophysiology of candidiasis is largely dependent on the outermost coats of *Candida* cells, which are necessary for their adhesion to the host surface. [11] Amphotericin B (AMB) and fluconazole (FLU) are two antifungal medicines to which biofilms are genetically resistant. Biofilm formation varies depending on the *Candida* species. [12] Currently there are various methods for detection of biofilm production like microtiter plate method, tube method, Congo red agar method, bioluminescent assay, piezoelectric sensors etc. [13]

The present study demonstrates the in vitro production of virulence factors i.e. enzymes namely phospholipase, esterase, hemolysins and biofilm production by above 3 methods – microtiter plate method, tube method and Congo red agar method.

II. OBJECTIVE

To demonstrate virulence factors of the *Candida* isolates – biofilm formation and enzymes

III. MATERIALS AND METHODS

Virulence factors – Enzymes [14]

PHOSPHOLIPASE ACTIVITY

Polak used Price et al.'s egg yolk agar plate method to measure the isolates' phospholipase production. *Candida* species were selected based on their ability to produce a zone of precipitation after growing on egg yolk agar in order to screen for extracellular phospholipase activity. SDA 13.0g, NaCl 11.7g, CaCl2 0.11g, and 10% sterile egg yolk made up the egg yolk agar media.

1] Components were combined and sterilized without the use of egg yolk.

2] A 500g centrifuge was run for 10 minutes at room temperature on the egg yolk.

3] The sterilized medium was mixed with 20 milliliters of supernatant.

4] To achieve visible turbidity, yeasts were extracted and suspended in sterile phosphate buffer saline (PBS) after a 24-hour incubation period of *Candida* species cultured on fresh SDA.

5] After plating a 10µl suspension of PBS on top of the egg yolk medium, it is incubated for 72 hours at 370C.

The value of phospholipase production (Pz) is determined by:

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

Four classes: Very high (+) 0.90-0.99
High (++) 0.80-0.89
Low (+++) 0.70-0.79
Very low (++++) 0.69 or lower

HEMOLYTIC ACTIVITY

Determination of hemolysin activity was determined by Manns et al.

1] From each culture, a 10µl aliquot was obtained, and it was inoculated onto SDA enriched with 3% glucose and 7% fresh blood. It was then incubated for 48 hours at 37^0C with 5% CO2.

2] When observed under transmitted light, the existence of a conspicuous translucent halo and/or a greenish-black ring surrounding the inoculum site was interpreted as positive hemolytic activity.

Three hemolysis tests were performed, and the results show mean values±SD.

4] To assess the degree of hemolysin production displayed by several *Candida* strains, the widths of the zones of hemolysis and colony were measured.

5] The colony size diameters were divided by the diameter of the translucent radial zone of hemolysis, and the resulting ratio (equal to or larger than 1) was used as hemolytic index:

$$HI = \frac{\text{colony diameter}}{\text{Colony diameter + zone of precipitation}}$$

Hemolytic index grouped into 4 classes: Very high (++++) 0.90-1
High (+++) 0.80-1
Low (++) 0.70-1
Very low (+) 0.69 or lower

ESTERASE ACTIVITY

Lipolytic activities in *Candida* species was done by Walter Rudek.

1] 1L of distilled water, 10g of peptone, 5g of NaCl, 0.1 CaCl2, and 15g of agar made up the Tween 80 opacity medium.

2] After 15 minutes of autoclaving at 121^0C, the medium was allowed to cool to roughly 50^0C.

3] Blended with 5ml of Tween 80 that had been pre-autoclaved and cooled, and then transferred into sterile petri dishes with a 90mm diameter (25ml of agar per plate).

4] After being cultured overnight in SDA, each Candida isolate was moved to the Tween 80 opacity medium and dispersed throughout a roughly 10-mm-diameter circular inoculation site.

5] For ten days, inoculation plates were incubated aerobically at 35^0C and checked every day.

6] Esterase activity was identified by looking for precipitation haloes surrounding the inoculum under transmitted light.

DETECTION OF BIOFILM PRODUCTION [15]

A] Micro titer plate method

1] BHI broth containing 2% sucrose was used to inoculate isolates from agar plates.

2] Incubated for 18 to 24 hours at 37^0C.

3] Using fresh medium, the broth was diluted at a ratio of 1 in 100.

Each well was filled with 0.2 milliliters of diluted culture.

For the purpose of sterility testing and non-specific medium binding, only one broth was used as a control.

4] For 24 hours, plates were incubated at 37^0C.

5] To get rid of free-floating "planktonic" bacteria, the well's contents were taken out and cleaned four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2).

6] Biofilms created by adhering "sessile" organisms on a plate were fixed for 30 minutes using 2% sodium acetate and then stained for an additional 30 minutes using 0.1% w/v crystal violet.

7] After washing with deionized water to remove any remaining stain, the plates were let to dry.

8] Adherent bacterial cells were consistently stained with crystal violet and typically developed biofilm on the well walls.

9] An Enzyme-Linked Immunosorbent Assay auto reader was used to measure the optical densities (OD) of stained adherent bacteria at a wavelength of 570 nm (OD 570 nm), and the bacteria were graded in accordance with Christensen et al.

MEAN OPTICAL DENSITIES VALUE	BIOFILM FORMATION
< 0.120	NONE/WEAK
0.120-0.240	MODERATE
≥0.240	HIGH

B] Tube method

1] The isolates of Candida were infused into 10 milliliters of Brain Heart Infusion Broth (BHIB) containing 2% sucrose.

2] Incubate for 18 to 24 hours at 37^0C.

3] After decanting, the tubes were cleaned with phosphate buffer saline (PBS, pH 7.2).

4] After drying, tubes were dyed for 30 minutes using crystal violet (0.1% w/v).

5] The tubes were dried after extra stain was removed.

6] When a discernible film covered the tube's wall and bottom, biofilm production was deemed to be positive.
7] After tubes were inspected, the level of biofilm formation was rated as strong, moderate, or missing.

C] Congo red method [16]

1] The medium contained 37 g/l of brain heart infusion broth, 50 g/l of sucrose, 10 g/l of agar, and 0.8 g/l of Congo red.
2] After the agar had cooled to 55^0C, the concentrated aqueous solution containing Congo red stain was added. It was then autoclaved at 120^0C for 15 minutes.
3] After inoculating the medium plates, they were incubated aerobically at 37^0C for a full day.
4] Black colonies with a dry crystalline consistency signaled a successful outcome.
5] Red-colored colonies are thought to be unfavorable for the formation of biofilms.

IV. RESULTS

TABLE 1: Biofilm formation of *Candida* isolates by Micro titer plate method (MTP), Tube method (TM) and Congo red agar method (CRA)

Biofilm formation	Micro titer plate method (n=100)	Tube method (n=95)	Congo red agar (n=59)
	N (%)	N (%)	N (%)
High	42 (24.3)	29 (16.8)	15 (8.7)
Moderate	58 (33.5)	66 (38.2)	45 (26)
Weak/ Negative	73 (42.2)	78 (45.1)	113 (65.3)

A total of 173 isolates of *Candida* were studied for biofilm formation. Biofilm was detected in 100 (57.8%) isolates by micro titer plate method, 95 (55%) by tube method and 60 (34.7%) by Congo red agar method.

TABLE 2: Species level screening of the isolates for biofilm formation by Micro titer plate method (MTP), Tube method (TM) and Congo red agar method (CRA)

Candida species	Strong			Moderate			Weak/negative		
	MTP	TM	CRA	MTP	TM	CRA	MTP	TM	CRA
C. tropicalis (N=65)	21	08	08	29	32	26	15	25	31
C. albicans (N=49)	11	10	04	12	13	12	26	26	33
C. glabrata (N=32)	04	06	00	10	13	03	18	13	19
C. krusei (N=12)	02	02	01	05	04	02	05	06	09
C. guillermondii (N=9)	02	02	01	01	03	01	06	04	07
C. parapsilosis (N=4)	01	00	00	01	01	01	02	03	03
C. keyfr (N=1)	01	01	01	00	00	00	00	00	00
C. viswanathii (N=1)	00	00	00	00	00	00	01	01	01
Total	42	29	15	58	66	45	73	78	113

This table shows the distribution of biofilm formation among various species of *Candida* isolated from various clinical samples by 3 methods. Highest number of strong biofilm producers were *C. tropicalis* by Micro titer plate method and Congo red agar method and *C. albicans* by tube method. For moderate strength of biofilm producers, *C. tropicalis* showed highest number by all the three methods.

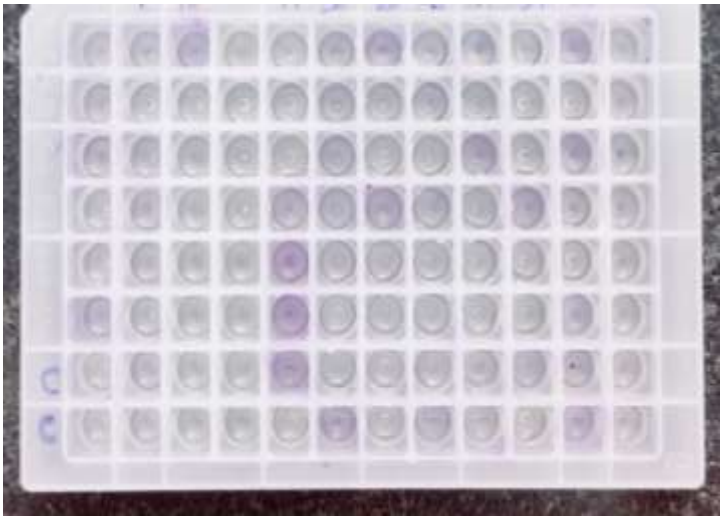

Plate 1: Biofilm production demonstrated by micro titer plate method

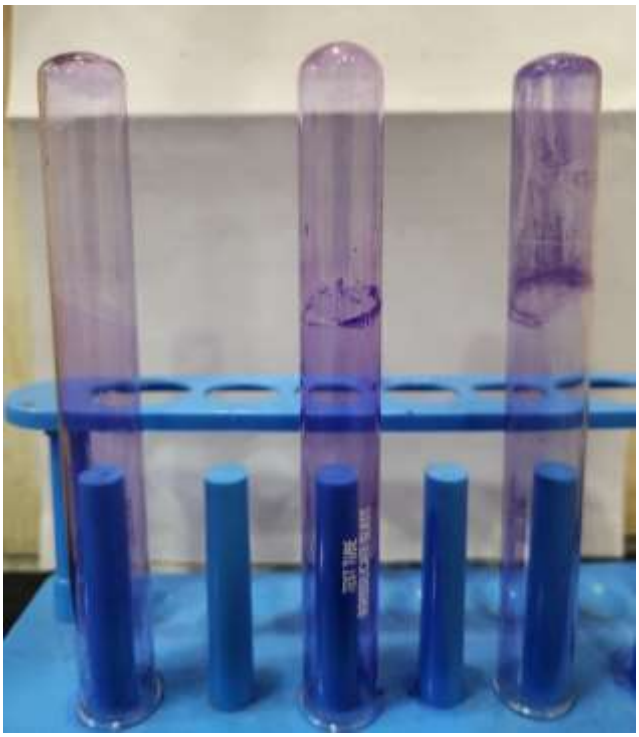

Plate 2: Biofilm production demonstrated by Tube method

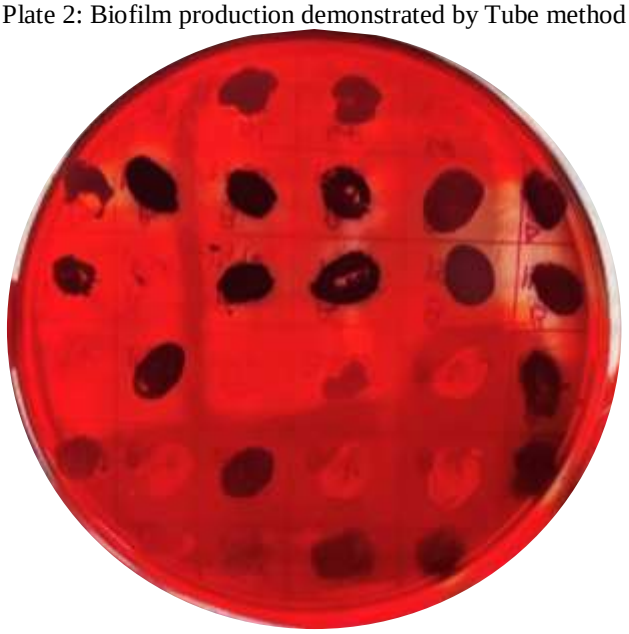

Plate 3: Biofilm production demonstrated by Congo red agar method

TABLE 3: Virulence factors (Enzymes) in various *Candida* species

Candida species	Phospholipase activity	Esterase activity	Hemolytic activity
C. tropicalis (N=65)	53 (81.54%)	60 (92.31%)	45 (69.23%)
C. albicans (N=49)	44 (89.80%)	42 (85.71%)	47 (95.92%)
C. glabrata (N=32)	09 (28.12%)	03 (9.37%)	06 (18.75%)
C. krusei (N=12)	02 (16.67%)	01 (8.33%)	01 (8.33%)
C. guilliermondii (N=9)	02 (22.22%)	-	-
C. parapsilosis (N=4)	01 (25%)	-	-
C. keyfr (N=1)	01 (100%)	-	-

This table shows the virulence factor i.e. enzymes demonstrated by different *Candida* species. Maximum phospholipase activity was showed by *C. kefyr* 100% followed by *C. albicans* 89.80%. Highest esterase activity was showed by *C. tropicalis* (92.31%) followed by *C. albicans* (85.71%), *C. glabrata* (9.37%), *C. krusei* (8.33%). Similarly, maximum hemolytic activity was showed by *C. albicans* (95.92%) followed by *C. tropicalis* (69.23%), *C. glabrata* (18.75%) and *C. krusei* (8.33%).

Plate 4: Demonstration of Phosphatase activity

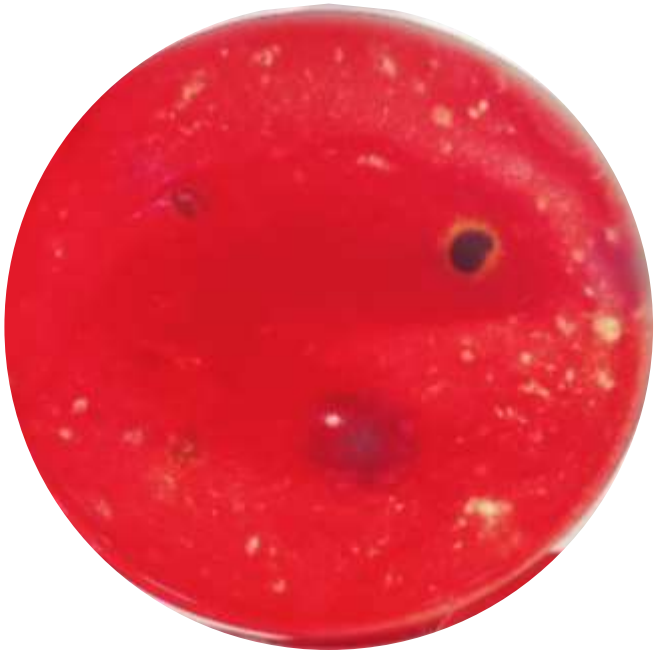

Plate 5: Demonstration of Hemolytic activity

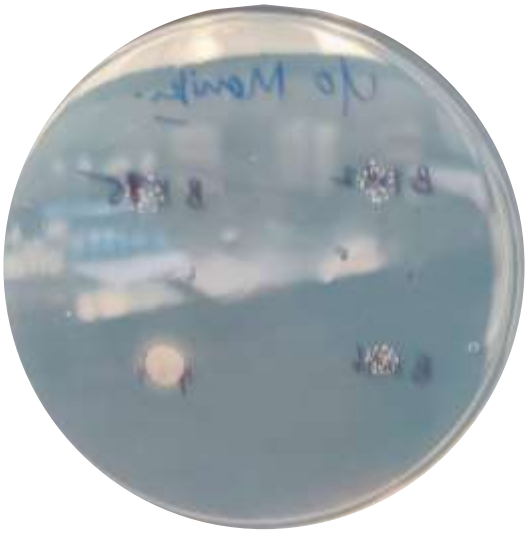

Plate 6: Demonstration of Esterase activity

V. DISCUSSION

A community of bacteria adhering to a surface by their extracellular polymers is called a biofilm. [17] Microorganisms encased in their secreted slime form biofilms. Since the formation of biofilms is linked to pathogenicity, it is appropriate to view this ability as a significant virulence factor in the context of candidiasis. Due to their ability to elude host immune systems, endure the effects of antifungal medication, and withstand pressure from competing species, biofilms may assist preserve the roles of fungi as both commensals and pathogens. As such, treating illnesses linked to biofilms is challenging. [18] The high level of antimicrobial resistance exhibited by the related organisms is also linked to the formation of biofilms. [19]

In this study, the production of biofilms was examined in 173 distinct *Candida* isolates. By using the micro titre plate method, biofilm was detected in 100 isolates (57.8%), by Tube method in 95 isolates (55%) and 60 isolates (34.7%) by using the Congo red agar method. This implies that the sensitivity of the microtiter plate method is higher than that of the other two methods.

Higher biofilm formation rates were seen in other studies. Renuka Devi et al. [20] found that 73.4% of the isolates in their study produced biofilms. Janakiram et al. (74%), Shilpa Khatri (61.25%), and Vinitha et al. (73%), all reported similar outcomes. [21,22,23]

Candida species have the ability to create a wide range of hydrolytic enzymes, such as hemolysins, lipases, phospholipases, proteinases, and esterases. Since these enzymes facilitate host invasion through tissue penetration and adhesion, extracellular hydrolytic enzymes seem to play a critical role in the establishment of *Candida* overgrowth. [24] The most important hydrolytic enzymes include hemolysin, esterase, proteinases, and phospholipases.

Phospholipases may be associated with the active invasion of host tissue in candidiasis lesions by rupturing epithelial cell membranes and facilitating the entry of the hyphal tip into the cytoplasm. [25] The production of proteinases is thought to improve an organism's capacity to colonize and penetrate host tissues as well as elude the host's immune system by breaking down several proteins crucial to host defence, including complement, cytokines, and immunoglobulins. [26-28]

It has been reported that *Candida albicans* excretes a hemolytic factor that causes hemoglobin to be released and is subsequently used by the organism as an iron source. This ability of *Candida* to acquire elemental iron through the production of hemolysin is crucial to its survival and ability to establish infections in humans, particularly in disseminated candidiasis. A colonizing strain of *Candida* can enter the bloodstream and deeper structures when the host's defenses are weakened, leading to invasive infections. Disseminated infection risk is also probably influenced by the virulence characteristics of the colonizing organism.

The virulence factors of *Candida*, such as phospholipase, proteinase, hemolysin, and esterase production, are of great interest due to the rising incidence of invasive *Candida* infections. These factors can be used to develop novel therapeutic interventions or as a target for controlling and preventing candidiasis. [29,30]

The objective of the current investigation was to measure the in vitro esterase, hemolysin, and phospholipase activities of 173 clinical isolates belonging to different species of *Candida*.

All of the species in this study exhibited phospholipase activity, with the exception of *C. viswanathii*. *C. kefyr* displayed the highest activity at 100%, followed by *C. albicans* at 89.80%. *C. tropicalis* had the highest phospholipase activity (18.7%) in the study by Aparna T et al. [24], followed by *C. albicans* (16.3%) and *C. krusei* (16.6%).

Investigators like as Thangam et al. found that *C. tropicalis* isolates from non albicans *Candida* species had strong phospholipase activity. [31] However, some, like Samaranayake et al., did not report any action. [32] The biological variations among the isolates examined could be the cause of these inconsistent observations.

According to the current study, *Candida albicans* has the highest hemolytic activity (95.92%), followed by *Candida tropicalis* (69.23%) and *Candida glabrata* (18.75%). According to the study by Tsang et al., whose values varied from 0.60 to 0.79, the Hemolytic Index (HI) values vary from 0.6 to 0.91. [33]

Few research has been done on hemolysin activity in *Candida* species. The circumstances in which *Candida albicans* can exhibit hemolytic activity were established by Manns et al. However, they discovered that in the absence of glucose in the culture media, hemolysis does not occur. [34] However, Luo et al. investigated 80 isolates of *Candida* from clinical sources across various geographic locations, and in trials using glucose-free sheep blood agar, they found only alpha hemolysis and no beta hemolysis. [35] Elevated blood glucose levels can either directly or indirectly be linked to higher hemolysin activity in isolates of *Candida albicans.*

Of the 49 strains of *Candida albicans* that we tested for esterase activity, 42 (85.71%) gave positive results; of the 65 strains of *Candida tropicalis*, 60 (92.31%) produced halo, and of the 32 strains of *Candida glabrata*, 3 (9.37%) produced hallo. In contrast to Aktas et al.'s work, which used a larger sample size to achieve favorable results for 58 out of 59 *C. albicans* strains (98%) and 38 out of 59 *C. tropicalis* strains (100%). [36]

VI. CONCLUSION

The majority of medical devices including indwelling catheters include *Candida* biofilms. We contrasted the three approaches—Microtiter plate, Tube Method, and Congo Red Agar—that we used in the present study. After comparison, the MTP method was found to be the most specific, sensitive, repeatable, accurate, and efficient way to identify the creation of biofilm. As such, it can be suggested as a general screening technique for the identification of *Candida* that produces biofilm in laboratory settings.

Contrary to most studies that demonstrate *C. albicans*, *C. tropicalis* showed stronger phospholipase and esterase activity. However, *C. albicans* had greater hemolytic activity. Extracellular enzyme secretion can therefore be regarded as a potential virulence factor, however the degree of their synthesis and their effect on the host are mostly determined by the host's constitution.

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