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Determination of Biofilm Production Of Candida Albican From Various Clinical Samples.

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Abstract:

Recent advances in technology for research have enabled studying bacteria and fungi in their actual natural environments, and there are over 95% of bacteria occurring naturally as biofilms. Hence the present study is undertaken to isolate Candida species from various clinical specimens, detect biofilm formation, and to study their susceptibility pattern. This study was carried out at the Department of Microbiology at Tertiary Care Centre of Central India from January 2023 to Nov 2023. The study consisted of total 380 samples during the study period at tertiary care hospital. Among 164 total samples a total of 75 Candida species were isolated from different clinical specimens. And study was carried out as per standard operating procedures. In the current study, the distribution of patients according to sex and age group revealed that females were more frequently affected than males. Specifically, out of the total patients, 98 were female, accounting for 59.8% of the cases, while 66 were male, making up 40.2% of the cases. In the present study, sample-wise distribution of Candida isolates revealed that C. albicans was most frequently isolated from sputum samples, accounting for 35.7% of the total isolates. Non-albicans Candida species were also prevalent in sputum samples, representing 25.0%. Urine samples contained 17.9% C. albicans and 10.7% non-albicans Candida. HVS samples had 7.1% C. albicans and 5.4% non-albicans Candida. In the current study, the analysis of virulence factors among Candida albicans isolates revealed that 49.3% of the isolates exhibited biofilm activity, while 50.7% showed no activity. Phospholipase activity was present in 57.3% of the isolates, with 42.7% showing no activity. Proteinase activity was observed in 45.3% of the isolates, whereas 54.7% did not exhibit this activity. Hence we conclude that Speciation of Candida isolates, detection of ability to form the biofilm, and monitoring of antifungal susceptibility testing are necessary for appropriate treatment.

Keywords: Candida albicans, SDP, biofilm, non-albicans Candida

Introduction:

Recent advances in technology for research have enabled studying bacteria and fungi in their actual natural environments, and there are over 95% of bacteria occurring naturally as biofilms [1]. Candida species occur as normal flora in people with normal immune systems; however,

they are able to cause opportunistic infections with high morbidity and mortality rates, predominantly in immunocompromised individuals. [2]. Candida spp. cause systemic diseases, which are the fourth leading cause of nosocomial bloodstream infections in modern hospitals. The most challenging clinical problem is the increased rate of non-Candida albicans isolation and the rapidly growing resistance of Candida species [3].

The most common is Candida albicans, among the Candida spp., and can be associated with superficial and systemic infections. The remaining species include C. tropicalis, C. glabrata, C. parapsilosis, and C. krusei contributing to 25%, 8%, 7%, and 4% candidiasis cases, respectively [4]. Pathogenesis of candidiasis would depend upon the expression of virulence factors, which includes germ tube formation, adhesions, phenotypic switching, biofilm formation, and production of hydrolytic enzymes [5].

Most of the diseases caused by Candida spp. are a result of biofilm formation. Biofilms are the group of microorganisms that are embedded in an extracellular matrix (ECM), forming a complex three-dimensional architecture on biotic and abiotic surfaces [6]. Biofilm formation can occur on mucosal surfaces and plastic surfaces of indwelling devices. Biofilms are genetically resistant to antifungal agents including amphotericin B (AMB) and fluconazole (FLU). Biofilm formation is species-specific and varies with Candida spp. [7]. Most frequently, pathogenic effects are caused by Candida albicans and to a lesser extent by other Candida spp. [8].

Candida shows resistance to azole due to general and long-term use of it [9]. Candidiasis tends to recur multiple times. Some health-care providers place patients on long-term therapy with antifungal drugs, but this may be associated with drug-resistant candidiasis that is difficult to treat. Early diagnosis of Candida spp. and monitoring their susceptibility to antifungals enables treatment. Since there are very few studies on biofilm formation and drug resistance reported from India, the present study is undertaken to isolate Candida species from various clinical specimens, detect biofilm formation, and to study their susceptibility pattern.

Materials and Methods:

Material & Method

This study was carried out at the Department of Microbiology at Tertiary Care Centre of Central India from January 2023 to Nov 2023. The study consisted of total 380 samples during the study period at tertiary care hospital. Among 164 total samples a total of 75 Candida species were isolated from different clinical specimens. Different samples taken were urine, blood, sputum, stool, body fluid, vaginal swabs, pus etc. Details of the patients were recorded. A detail history was taken with demographic details [name, age, sex, IPD no, presenting complaints, sign and symptoms, presence of predisposing factors and treatment]. Patient's hospital records were used to know the use of any antifungal agents and past medical conditions.

Inclusion Criteria

All types of samples were included in this study.

Strains criteria- only Candida species was included.

Both male and female were included in this study.

Exclusion Criteria

Second repeat isolate from same patient.

Then biofilm preparation was done to identify the growth of the Candida as follows

Biofilm Detection-

A. Microtiter Plate Method-

It is used for Candida albicans and non albicans by using spectrophotometer.

Principle-

Polystyrene is a commonly used material in vitro biofilm studies. It is used as a test surface to take the biofilm forming ability of each Candida species. The species of Candida would be inoculated in proper media on the microtiter plate wells and the produced biofilm on the surface will be qualified by the percent transmission of light.

Method-

-Sterile microtiter plate will be taken.

-Each well will be inoculated with20 microliter yeast cell suspension.

-Two separate wells will be inoculated with 20 microliters each of the two-control strain.

-To each of the wells 180 microliter of sabauraud's dextrose broth with8% glucose will be added.

-The microtiter plate will be incubated at 37^0c for 24 hrs.

-The wells will be washed twice with 0.15M of phosphate buffer saline.

-Finally wells will be washed twice with200 microlitre of phosphate buffer saline and air dried the plate.

-Biofilm production measured by spectrophotometer at 630 nm [ELISA READER].

B. Tube Method-

-It is qualitative method.

-A loopful of test organism will be inoculated in 10 ml of tryypticase soya broth with1% glucose in test tube.

-The tube will be incubated at 37 ^{0}c for 24 hrs.

-After incubation, tube will be decanted and wash with phosphate buffer saline [Ph7.3] and dried

The test tube stain with crystal violet [0.1%]

-Excess stain wash out

-Tube will be in inverted position

-The scoring for tube method will be done according to the result control strain.

-Biofilm production will be considered positive when a visible film lined the wall and bottom of tube.

-Amount of biofilm will be scored as

1-well/None

2-Moderate

3-High/Strong

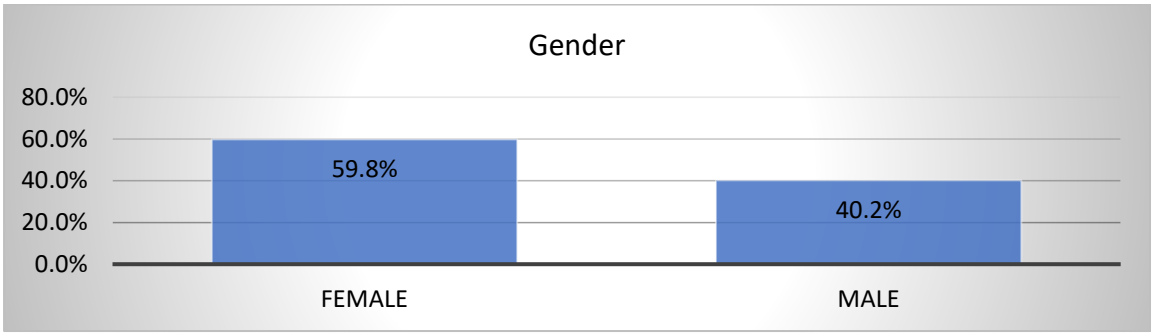
Results:

A Prospective analytical clinical study to study the virulence factors, and molecular characterization of Candida albicans from various clinical samples in tertiary care hospital [AVBRH].

Table 1. Distribution of patients according to sex

	Number	Percentage
Male	66	40.2%
Female	98	59.8%

Figure 1. Gender



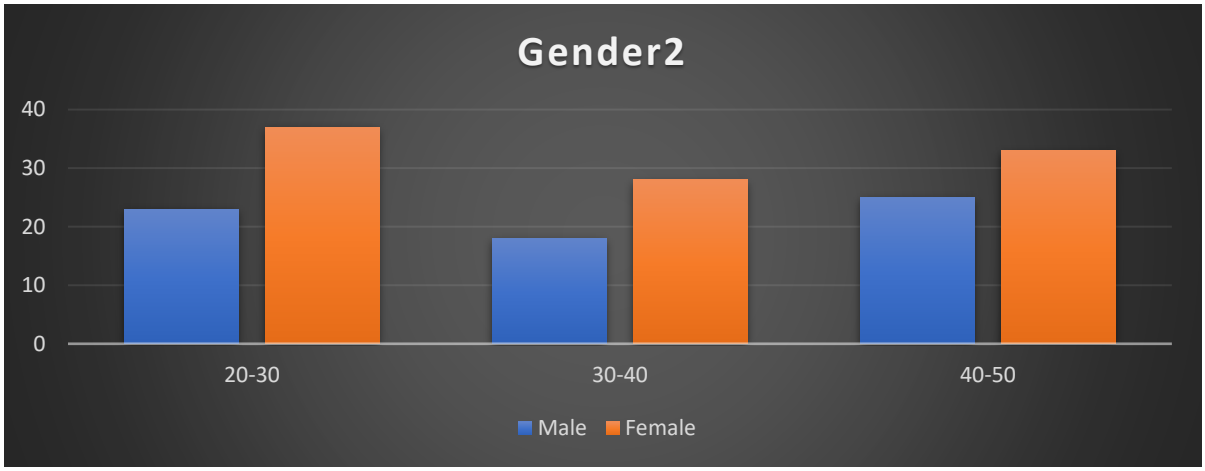
In the current study, the distribution of patients according to sex revealed that females were more frequently affected than males. Specifically, out of the total patients, 98 were female, accounting for 59.8% of the cases, while 66 were male, making up 40.2% of the cases. This

indicates a higher prevalence of the condition among female patients compared to male patients. Shown in Table 1 and Figure 1.

Table 2. Distribution of patients according to sex

	20-30	30-40	40-50	Total
Male	23	18	25	66
Female	37	28	33	98
Total	60	46	58	164

Figure 2. Distribution of patients according to sex



In the current study, the distribution of patients according to sex and age group revealed that females were more frequently affected than males. Specifically, out of the total patients, 98 were female, accounting for 59.8% of the cases, while 66 were male, making up 40.2% of the cases. The age group distribution showed that the highest number of patients fell in the 40-50 age group, with 33 females and 25 males. Shown in Table 2 and Figure 2.

Table 3. Candida Species

Candida Species	Number	Percentage
Candida albicans	75	45.7%
Non-albicans Candida	89	54.3%
Total	164	100.0%

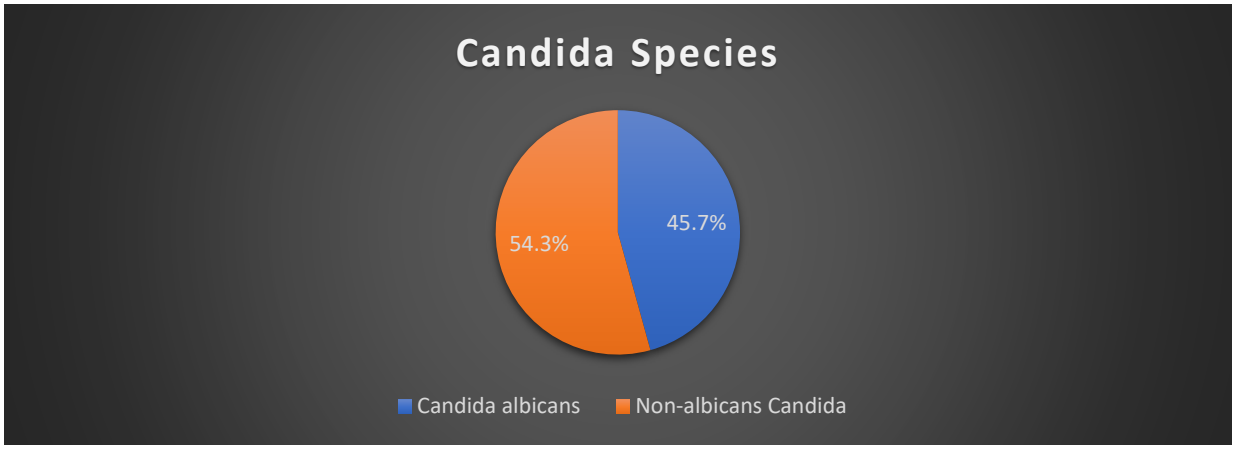


Figure 3. Candida Species

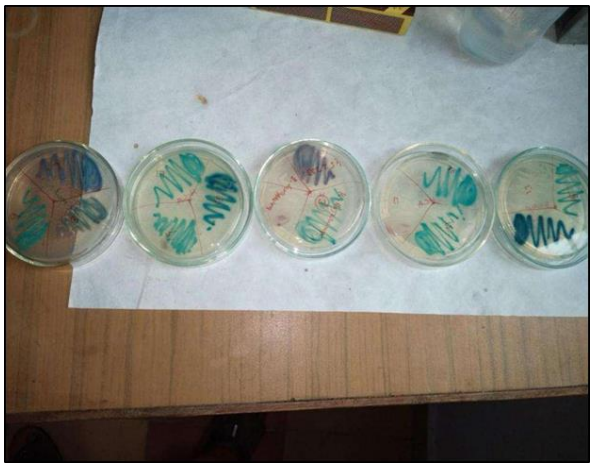


Figure 4. Growth of Candida on SDA plate

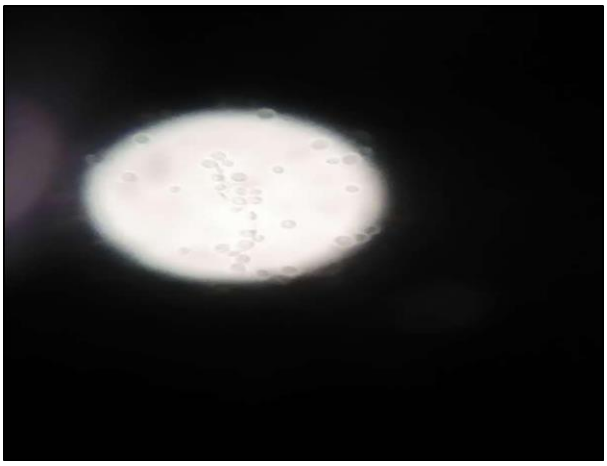


Figure 5. Candida under Microscope

In the present study, a total of 164 clinically suspected cases according to the sample size calculated in the present study in which the total 75 isolates were found to be culture positive for candida albicans and 89 for Non albicans. As shown in Table 3 and Figure 3. The growth of Candida on SDA plate depicted in Figure 4 &5.

Table 4. Sample-wise distribution of Candida isolates

Sample	C. albicans [%]	Non-albicans Candida [%]

Sputum	20 [35.7]	14 [25.0]
BAL	6 [10.7]	2 [3.6]
Pus	5 [8.9]	10 [17.9]
Urine	10 [17.9]	6 [10.7]
HVS	4 [7.1]	3 [5.4]
Body Fluids	3 [5.4]	2 [3.6]
Nail Clipping	2 [3.6]	1 [1.8]
Blood	0 [0.0]	2 [3.6]
Others	0 [0.0]	1 [1.8]

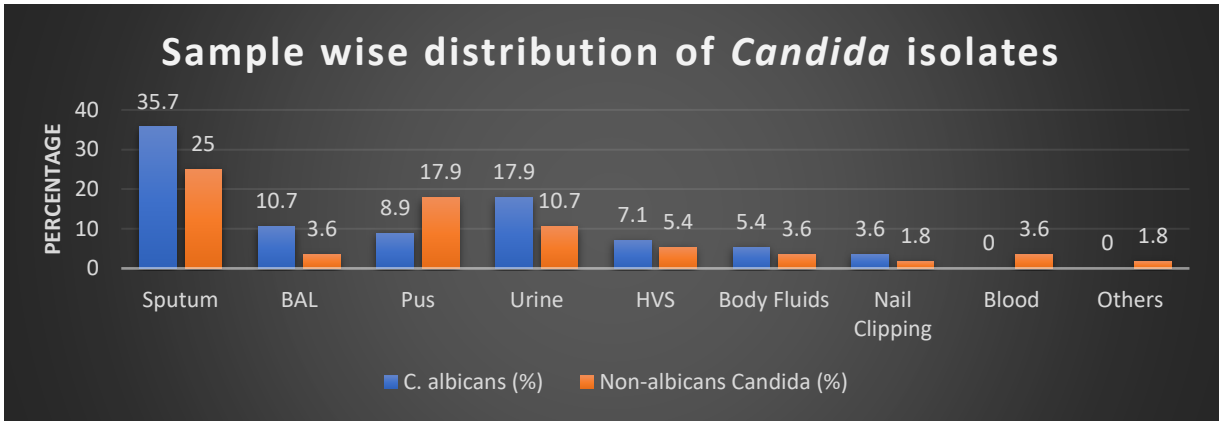


Figure 6. Sample wise distribution of Candida isolates

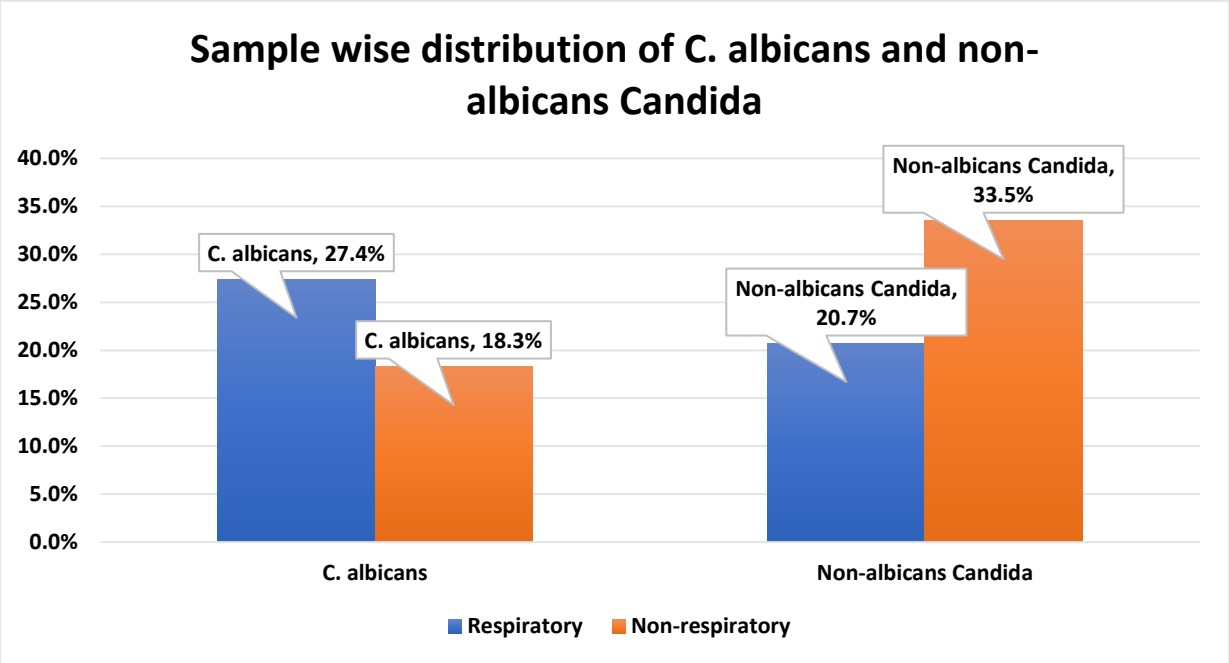
In the present study, sample-wise distribution of Candida isolates revealed that C. albicans was most frequently isolated from sputum samples, accounting for 35.7% of the total isolates. Non-albicans Candida species were also prevalent in sputum samples, representing 25.0%. Urine samples contained 17.9% C. albicans and 10.7% non-albicans Candida. HVS samples had 7.1% C. albicans and 5.4% non-albicans Candida. Blood samples had no C. albicans isolates but 3.6% non-albicans Candida. Other samples had no C. albicans isolates but 1.8% non-albicans Candida. Overall, C. albicans accounted for 45.7% of the total isolates, while non-albicans Candida species made up 54.3%. The result and graph are depicted in Table 4 and Figure 6.

Table 5. Sample wise distribution of C. albicans and non- albicans Candida

Sample	C. albicans	Non-albicans Candida	Total
Respiratory	45 [27.4%]	34 [20.7%]	79 [48.2%]

Non-respiratory	30 [18.3%]	55 [33.5%]	85 [51.8%]
Total	75 [45.7%]	89 [54.3%]	164 [100%]

Figure 7. Sample wise distribution of C. albicans and non- albicans Candida



The present study shows the distribution of Candida albicans and non-albicans Candida across respiratory and non-respiratory samples. In respiratory samples, C. albicans was found in 45 cases [27.4%], while non-albicans Candida was present in 34 cases [20.7%]. In non-respiratory samples, C. albicans was found in 30 cases [18.3%], and non-albicans Candida was present in 55 cases [33.5%], making a total of 85 cases [51.8%]. Overall, C. albicans accounted for 75 cases [45.7%], and non-albicans Candida accounted for 89 cases [54.3%] out of the total 164 samples. The result and graph are depicted in Table 5 and Figure 7.

Table 6. Virulence factors amongst C. albicans isolates

	Biofilms	**Phospho-lipase**	**Proteinase**	**Haemolysin**	**Esterase**	**Coagulase**	**ALS**	**HWP**
Activity /Positive	37 [49.3]	43 [57.3]	34 [45.3]	38 [50.7]	36 [48]	32 [42.7]	31 [41.3]	40 [53.3]

No activity/ Negative	38 [50.7]	32 [42.7]	41 [54.7]	37 [49.3]	39 [52]	43 [57.3]	44 [58.7]	35 [46.7]
Grand Total	**75 [100]**	**75 [100]**	**75 [100]**	**75 [100]**	**75 [100]**	**75 [100]**	**75 [100]**	**75 [100]**

In the current study, the analysis of virulence factors among Candida albicans isolates revealed that 49.3% of the isolates exhibited biofilm activity, while 50.7% showed no activity. Phospholipase activity was present in 57.3% of the isolates, with 42.7% showing no activity. Proteinase activity was observed in 45.3% of the isolates, whereas 54.7% did not exhibit this activity. Haemolysin activity was found in 50.7% of the isolates, with 49.3% showing no activity. Esterase activity was present in 48% of the isolates, while 52% showed no activity. Lastly, coagulase activity was observed in 42.7% of the isolates, with 57.3% showing no activity. ALS gene was detected in 41.3% of the isolates, while the HWP gene was detected in 53.3% of the isolates. Conversely, the ALS gene was not detected in 58.7% of the isolates, and the HWP gene was not detected in 46.7% of the isolates. The result and graph are depicted in Table 6.

Table 7. Distribution of Virulence factors of C. albicans isolates in respect to site

	Biofilm	**Phospholipase**	**Proteinase**	**Haemolysin**	**Esterase**	**Coagulase**
BAL	12 [16]	12 [16]	10 [13.3]	8 [10.7]	9 [12]	19 [25.3]
Blood	18 [24]	11 [14.7]	10 [13.3]	13 [17.3]	10 [13.3]	8 [10.7]
Body Fluids	8 [10.7]	10 [13.3]	11 [14.7]	10 [13.3]	18 [24]	10 [13.3]
Pus	14 [18.7]	13 [17.3]	16 [21.3]	13 [17.3]	10 [13.3]	11 [14.7]
Sputum	11 [14.7]	14 [18.7]	9 [12]	17 [22.7]	14 [18.7]	10 [13.3]
Urine	12 [16]	15 [20]	19 [25.3]	14 [18.7]	14 [18.7]	17 [22.7]
Grand Total	**75 [100]**	**75 [100]**	**75 [100]**	**75 [100]**	**75 [100]**	**75 [100]**

In the current study, the distribution of virulence factors among Candida albicans isolates from different sample sites shows diverse pathogenic capabilities. BAL samples had 16% biofilm activity, 16% phospholipase, 13.3% proteinase, 10.7% haemolysin, 12% esterase, and 25.3% coagulase. Blood samples showed 24% biofilm, 14.7% phospholipase, 13.3% proteinase, 17.3% haemolysin, 13.3% esterase, and 10.7% coagulase. Body fluids had 10.7% biofilm,

13.3% phospholipase, 14.7% proteinase, 13.3% haemolysin, 24% esterase, and 13.3% coagulase. Pus samples exhibited 18.7% biofilm, 17.3% phospholipase, 21.3% proteinase, 17.3% haemolysin, 13.3% esterase, and 14.7% coagulase. Sputum samples showed 14.7% biofilm, 18.7% phospholipase, 12% proteinase, 22.7% haemolysin, 18.7% esterase, and 13.3% coagulase. Urine samples had 16% biofilm, 20% phospholipase, 25.3% proteinase, 18.7% haemolysin, 18.7% esterase, and 22.7% coagulase. The result and graph are depicted in Table 7.

Table 8. Distribution of the Pz value among the C. albicans isolates.

	Biofilm	**Phospholipase**	**Proteinase**	**Haemolysin**	**Esterase**	**Coagulase**
< 0.69	19 [25.3]	14 [18.7]	13 [17.3]	23 [30.7]	7 [9.3]	14 [18.7]
0.7 – 0.79	16 [21.3]	17 [22.7]	16 [21.3]	12 [16]	17 [22.7]	15 [20]
0.8 – 0.89	13 [17.3]	13 [17.3]	17 [22.7]	11 [14.7]	19 [25.3]	15 [20]
0.9 – 0.99	14 [18.7]	16 [21.3]	18 [24]	13 [17.3]	17 [22.7]	14 [18.7]
1	13 [17.3]	15 [20]	11 [14.7]	16 [21.3]	15 [20]	17 [22.7]
Grand Total	**75 [100]**	**75 [100]**	**75 [100]**	**75 [100]**	**75 [100]**	**75 [100]**

In the current study, the distribution of Pz values among C. albicans isolates for various enzymes shows a diverse range of activity. For biofilm formation, 25.3% of isolates had Pz values below 0.69, while 21.3% fell within the 0.7-0.79 range. Phospholipase activity was highest in the 0.7-0.79 range [22.7%], with 18.7% of isolates below 0.69. Proteinase activity was most common in the 0.9-0.99 range [24%], and haemolysin activity was highest below 0.69 [30.7%]. Esterase activity peaked in the 0.8-0.89 range [25.3%], and coagulase activity was most frequent in the 0.7-0.79 range [22.7%]. Each enzyme showed a unique distribution pattern, reflecting the variability in virulence factors among the isolates. The result and graph are depicted in Table 8.

Discussion:

Candida albicans, an opportunistic fungal pathogen, poses a significant global health burden due to its increasing multidrug resistance and ability to cause invasive infections. This ubiquitous organism, often colonizing healthy individuals, can transition into a virulent pathogen under favorable conditions. Early detection of C. albicans is crucial for effective treatment, yet resource constraints often limit the availability of advanced mycological techniques in many regions. The emergence of C. albicans strains with enhanced virulence factors contributes to their ability to displace commensal flora and establish infections ranging from superficial to systemic. These virulence factors facilitate adhesion, invasion, and tissue damage. [10] While phenotypic assays provide a simple and cost-effective method for detecting these traits, genetic analysis offers a more comprehensive understanding of the underlying mechanisms and can improve prognostic accuracy. However, data on both phenotypic and genotypic virulence factor analysis, particularly from developing countries, remains limited. Furthermore, studies directly correlating phenotypic activity with gene expression for specific virulence factors are relatively scarce. [11]

Hence, considering the aforementioned factors, a prospective, analytical study was conducted on 164 diverse clinical specimens from patients with suspected fungal infections at a tertiary care hospital [AVBRH].

Demographic data

A total of 164 clinical samples were collected from diverse patient populations. These samples underwent processing to determine the prevalence of Candida-associated infections, isolate and identify Candida species, characterize their distribution and antifungal susceptibility profiles, and detect the presence of virulence factors using both phenotypic and molecular PCR-based methods.

Distribution of patients according to age and sex

The current study population predominantly comprised female patients, with 59.8% [10] of the 164 participants identifying as female. The cohort aged 20-30 years exhibited the highest prevalence, with 37 female participants, while the 40–50-year age group also demonstrated the greatest impact among males, with 25 individuals affected. These findings corroborate previous research by Al-Karaawi et al and Fornari et al and may have implications for targeted clinical interventions [12, 13].

Candida Species

The current study explores the prevalence and distribution of Candida species in clinical samples. Notably, non-albicans Candida species were found to be more prevalent than Candida albicans, constituting 89 [54.3%] and 75 [45.7%] of the total isolates, respectively. These findings corroborate previous research by Makled et al and may have implications for targeted clinical interventions [14,15].

Sample-wise distribution of Candida isolates

A comprehensive analysis of Candida isolates across various sample types was conducted in the current investigation. C. albicans was the most prevalent fungal species recovered from sputum samples, constituting 35.7% of all isolates. Furthermore, a substantial proportion [25.0%] of non-albicans Candida species were also identified in sputum samples. C. albicans was observed in 17.9% of urine samples, while non-albicans Candida constituted 10.7% of isolates from this source. In HVS samples, C. albicans accounted for 7.1% of isolates, and non-albicans Candida represented 5.4%. No C. albicans isolates were recovered from blood samples; however, 3.6% of isolates from this source were identified as non-albicans Candida. A similar trend was observed in other sample types, where no C. albicans isolates were detected, but 1.8% of isolates were classified as non-albicans Candida. In total, C. albicans comprised 56.62% of the total isolates, whereas non-albicans Candida species accounted for the remaining 43.38%. This result was in accordance with the previously reported by Alim et al and Gandham et al [16-17]

This study also investigated the distribution of Candida albicans and non-albicans Candida species across respiratory and non-respiratory samples. In respiratory samples, C. albicans was detected in 27.4% of cases, while non-albicans Candida was found in 20.7%. In non-respiratory samples, C. albicans was identified in 18.3% of cases, and non-albicans Candida was present in 33.5% of cases. Overall, C. albicans accounted for 45.7% of all Candida isolates, and non-albicans Candida accounted for 54.3% of the total 164 samples analyzed. These results are consistent with earlier findings by Alim et al and Neves-Junior et al [18 -20].

Virulence factors amongst C. albicans isolates

Biofilm

In the current study, the analysis of virulence factors among Candida albicans isolates revealed that 49.3% of the isolates exhibited biofilm activity, while 50.7% showed no activity. This result was align with previously reported study by Benmessaoud et al, Zarrin, & Kiasat [21,22].

The distribution of biofilm production among Candida albicans isolates from different sites reveals some notable patterns. The highest percentage of biofilm production was observed in blood samples, with 18 isolates [24%] exhibiting this virulence factor. This is particularly concerning as biofilm formation in blood can lead to systemic infections, which are more challenging to treat. In contrast, the lowest percentage of biofilm production was found in body fluids, with 8 isolates [10.7%]. Other sites showed a relatively even distribution, with BAL [Bronchoalveolar Lavage] and urine samples each having 12 isolates [16%], pus samples having 14 isolates [18.7%], and sputum samples having 11 isolates [14.7%]. Overall, the ability of Candida albicans to form biofilms across various sites underscores the importance of monitoring and managing this virulence factor to effectively prevent and control infections. Our findings are consistent with those of previous studies by Ferreiraet al, Makled et al and other [23,24].

The distribution of the Pz value among the C. albicans isolates for biofilm formation shows a varied range. The majority of isolates [25.3%] had a Pz value of less than 0.69, indicating a

significant presence of biofilm formation. This was followed by 21.3% of isolates with a Pz value between 0.7 and 0.79, and 17.3% of isolates with a Pz value between 0.8 and 0.89. Additionally, 18.7% of isolates had a Pz value between 0.9 and 0.99, and 17.3% of isolates had a Pz value of 1. Overall, the distribution indicates that biofilm formation is a common trait among the C. albicans isolates, with a notable proportion exhibiting strong biofilm-forming capabilities. These results are congruent with prior findings by Dabiri, Shams-Ghahfarokhi, & Razzaghi-Abyaneh, 2018 [25].

Hence we conclude that Speciation of Candida isolates, detection of ability to form the biofilm, and monitoring of antifungal susceptibility testing are necessary for appropriate treatment.

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