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The Resistance Blueprint: Virulence Factors Steering Azole Failure In *Candida Tropicalis*

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ABSTRACT
Among the other forms of Candida (NAC) species that cause candidemia, Candida tropicalis has the highest mortality rate and is the fourth most common infectious agent among Candida species in many developing countries. Seventy clinically known Candida isolates are recovered in affirmatively flagged blood samples from BacT/ALERT 3D from various wards. Virulence parameters like hemolysin production, coagulase activity, phospholipase activity, and biofilm development were explored, and they were associated with antifungal susceptibility tests and interpreted as Minimum Inhibitory Concentration (MIC) values. The most common organism discovered among the 70 clinical Candida isolates was Candida tropicalis (27, 38.57%), followed by Candida albicans (19, 27.14%), Candida parapsilosis (13, 18.57%), Candida glabrata (6, 8.57%), and Candida krusei (5, 7.14%). By using the crystal violet assay, 49 (70%) of the 70 Candida isolates produced hemolysin, 43 (61.42%) exhibited phospholipase activity, 34 (48.57%) showed coagulase activity, and 55 (78.57%) indicated biofilm development. Virulence factors such hemolysin production, coagulase activity, phospholipase activity, and biofilm growth were studied, and they were related with antifungal susceptibility tests and interpreted as Minimum Inhibitory Concentration (MIC) values. Among the 70 clinical isolates of Candida, Candida tropicalis was found most frequently (27, 38.57%), followed by Candida albicans (19, 27.14%), Candida parapsilosis (13, 18.57%), Candida glabrata (6, 8.57%), and Candida krusei (5, 7.14%). 49 (70%) of the 70 Candida isolates produced hemolysin, 43 (61.42%) had phospholipase activity, 34 (48.57%) had coagulase activity, and 55 (78.57%) had biofilm formation, according to the crystal violet assay.

INTRODUCTION:
Candidemia, a common form of invasive candidiasis, is another name for Candida species that cause bloodstream infections (BSIs) [1]. Candidaemia is one of the most prominent reasons for BSIs among hospitalised patients in the United States, and it usually leads to prolonged hospital stays as well as mortality [2]. Candida tropicalis is the fourth main infective agent of Candida species in various impoverished nations and contributes to the greatest fatality rate among the NAC species that cause candidemia. The extensive usage of antifungal drugs develops C. tropicalis antifungal resistance, notably to azoles, which might finally result in the treatment failure of candidemia [3, 4].

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Due to the exorbitant cost of echinocandins, fluconazole is used to treat a large number of cases of Candida tropicalis, which is thought to be the most common species causing candidemia in developing countries [5]. In comparison to other continents, tropical Asia and Latin America have the proportionally biggest proportion of Candida tropicalis candidaemia [6]. Approximately 25,000 instances of candidemia are recorded nationwide each year, according to the Centers for Disease Control and Prevention (CDC). C. tropicalis (38%) is the most common bacterium in India among candidemia cases [7]. Due to the excessive cost of echinocandins, fluconazole is used to treat a large number of cases of Candida tropicalis, which is regarded to be the most frequent species causing candidemia in developing countries [5]. In comparison to other continents, tropical Asia and Latin America have the proportionally greatest proportion of Candida tropicalis candidaemia [6]. Approximately 25,000 incidences of candidemia are registered nationwide each year, according to the Centers for Disease Control and Prevention (CDC). The C. tropicalis (38%) is the most frequent bacterium in India among candidemia cases [7]. On the other hand, isolates of C. tropicalis have expanded globally, and since 2010, this situation has been particularly contentious in the Asia-Pacific region. The majority of candidemia studies [8, 9, 10, 11] indicated a considerable increase in pan azole resistance and Amphotericin B resistance in C. tropicalis.

Several virulence factors of Candida species induce BSIs, including hemolytic activity, coagulase, proteinase, phospholipase, esterase, lipase, and biofilm development. All of these various virulence factors could boost BSIs of Candida species through the escape mechanism, which is part of the defence system, in order to harm the host tissue [12]. The appearance of virulence factors in Candida species can be differentiated based on species, type of infection, geographical region, host reaction and stage, and the site of infection. In addition to helping researchers find new antifungal targets for more successful treatment regimens, an understanding of these virulence factors is essential for comprehending the pathophysiology [13].

The percentage of fluconazole-resistant Candida isolates has stayed largely constant over the past 20 years, according to CDC monitoring figures [14]. A statewide candidemia investigation in India found that the origin of multidrug-resistant (MDR) Candida tropicalis was in an equivalent percentage to that of MDR Candida auris [15].

The progression in drug tolerance permits the organism to procure lasting alterations of genes that result in the development of antifungal resistance, therapeutic failure, and mortality, which is another topic of concern [16]. In fact, a number of studies show that C. tropicalis is azole-tolerant because Fluconazole's effectiveness against C. tropicalis in blood samples declined, leading to Fluconazole therapeutic failure [17]. Despite the advancement of resistance, one or more antimicrobial agents should be a real worry in C. tropicalis candidemia cases because there are so few antifungals accessible to treat, particularly in developing countries where Fluconazole is one of the most often used antifungals [18].

Molecular researchers think that mutations in the ergosterol synthesis gene (ERG11) and the Cdr1 and Mdr1 efflux pumps are the main causes of azole resistance in C. tropicalis, even if we do not know how it developed [19] Alternative azole resistance routes have also been connected to biofilm development, mitochondrial defects, and other virulence factors [20]. In order to identify efficient methods of treating candidemia based on the novel regimen, the current study looks at the full clinico-mycological profile of Candida species, emphasizing virulence characteristics as well as the antibiotic sensitivity pattern of C. tropicalis isolates.

MATERIALS AND METHODS:

The Institute Ethical Committee (Human Studies) granted ethical permission(1213/IEC/2024) for a cross-sectional study conducted at a tertiary care facility between August 2024 and March 2025. Seventy clinically known Candida isolates were isolated from positively flagged blood samples incubated in BacT/ALERT 3D obtained from various wards and gramme stained directly to assess the existence of gram-positive budding yeast-like cells. The plates were incubated at 25 C and 37 C for 24 to 48 hours using normal culture techniques on Sabouraud Dextrose Agar (SDA) with antibiotics, MacConkey agar, and Blood agar. After that, every plate was investigated macroscopically and the colony characteristics were assessed. Colonies exhibited on SDA as smooth, creamy, convex, and pasty colonies (Figure 3). The colonies were then inspected under a microscope after being gram-stained. (Fig. 1).

Following the culture characterization, species identification was performed by inoculating the colonies onto Candida CHROM agar plates, which were incubated at 37 C for 48 hours, and they were identified and speciated based on the colour produced by the organisms on the chromogenic medium, i.e., C. albicans—apple green; C.

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tropicalis—metallic blue;

1. C. glabrata is purple, C. krusei is pink, and C. parapsilosis is cream to pink. In order to identify the Candida species that could not be identified by colour on Candida CHROM agar medium, the germ tube formation test and chlamydospore development on cornmeal agar were also conducted [26].

2. C. glabrata is purple, C. krusei is pink, and C. parapsilosis is cream to pink. In order to identify the Candida species that could not be identified by colour on Candida CHROM agar medium, the germ tube formation test and chlamydospore development on cornmeal agar were also undertaken [26].

Germ tube formation method:
In a sterile Eppendorf tube, a colony of yeast was suspended with 0.5 ml of human, sheep, or fetal bovine serum and incubated for two to four hours at 37 oC. Subsequently, a single drop of serum was placed on a glass slide, and a cover slip was retained over it. After that, a low-power objective was used to inspect it under a microscope. The emergence of long, tube-like projections linked to the yeast cells was named germ tube formation, which is frequently observed in Candida albicans or Candida dubliniensis.

Haemolysin Production:
According to Manns et al. [21], hemolysin production was shown on SDA containing sheep blood in addition to gentamicin. Ten LL of inoculum made with the isolates were aseptically injected into the medium. ATCC 90028 Candida albicans was utilized as a control, and Petri plates containing the aforementioned media were cultivated for 48 hours at 37 °C. Haemolysin activity was demonstrated by the existence of a halo zone of hemolysis encircling a colony. Hemolytic activity (Hz) is determined by dividing the colony diameter by the visible hemolysis zone (in millimeters).

Coagulase Activity.
Using the approach proposed by Yigit et al. [22], coagulase production was found. A tube containing 500 LL of rabbit plasma was aseptically inoculated with 0.1 mLL of an inoculum that had been stored aseptically for the whole night. The tubes were examined following incubation at 35 °C for 2, 4, 6, and 24 hours. A positive coagulase test demonstrates the clot formation, which cannot be revived by mild shaking. ATCC 25923 Staphylococcus aureus is the positive control, whereas ATCC 14990 Staphylococcus epidermidis is the negative control.

Phospholipase Production.
Phospholipase synthesis was identified using the technique described by Samaranayake et al. [23]. A standard 5-liter test-strain inoculum was aseptically added to egg yolk-containing agar. Petri plates are dehydrated at ambient temperature; they were incubated for 48 hours at 37 °C. The presence of the phospholipase enzyme was indicated by the creation of a precipitation zone around the colony. Candia albicans ATCC 10231 served as the positive control. The phospholipase index (Pz) is computed as the colony diameter to the combined and precipitation zone ratio. Phospholipase production was missing when Pz was 1; positive activity was seen when Pz was 1. The test was carried out three times in duplicate for each isolate to limit experimental error.

Biofilm formation
A growth from SDA was suspended in a sterile brain-heart infusion (BHI) broth and cultivated overnight. The next step was a 1:100 dilution in BHI. Then, a commercially available polystyrene, pre-sterilized, round-bottomed, 96-well microtiter plate (HiMedia) was used to incubate 100 liters of diluted broth at 37 °C overnight for the growth of biofilm, and with distilled water, the microtiter plates were cleaned.

Crystal violet assay
The microtiter plate was washed with distilled water and then stained with a 0.1% aqueous solution of crystal violet (120 L) for 15 minutes at room temperature. The wells were cleansed four times with sterile distilled water. After de-staining with 125 L of 95% methanol, the wells were incubated for 15 minutes at room temperature. An ELISA reader was used to examine the de-stained wells spectrophotometrically at 570 nm. Each sample was ran 12 times in duplicate.

The biofilm formation was read as follows:
(ODC - OD cutoff value, Avg NC - Average OD value of negative control)
Avg NC=0.194, SD=0.013
ODC =Avg NC+3×SD, ODC =0.194+0.039, ODC=0.233
OD ≤ ODC= non-biofilm producers
ODC < OD ≤ 2 ODC=weak biofilm producers
2ODC < OD ≤ 4 ODC=moderate biofilm producers
4ODC < OD=strong biofilm producers

Antibiogram:
The antibiogram pattern for antifungals such Fluconazole (25 g), Amphotericin B (100 U), Itraconazole (10 g), and Voriconazole (1 g) (Hi-Media Laboratories) was done by the Kirby-Bauer disc diffusion method. Mueller-Hinton agar was used, along with 0.5 g of methylene blue per

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milliliter and 2% glucose, to enhance the measurement of growth margins. The Clinical and Laboratory Standards Institute (CLSI) criteria were followed in the interpretation of the zone sizes. Then, fluconazole-resistant isolates were exposed to broth microdilution M27-A3 in order to obtain MIC values, and the findings were interpreted according to the CLSI standards [24].

RESULT:

Blood samples were obtained from different wards over the course of the 16-month study period, and those marked with positive signals were examined under a microscope and cultured for Candida species using SDA, Blood Agar, and MacConkey Agar (Figure 2). A total of 70 consecutive and non-duplicate Candida species were included in our investigation. Organisms other than Candida isolates were eliminated.

Of the 70 isolates of Candida species, 13 (18.57%) showed a positive germ tube formation test result, while 57 (81.42%) did not. The most common organism among the 70 clinical Candida isolates was Candida tropicalis (27, 38.57%), followed by Candida albicans (19, 27.14%), Candida parapsilosis (13, 18.57%), Candida glabrata (8.57%), and Candida krusei (5, 7.14%) (Table 1) (Figure 3). However, all the isolates reported negative findings on the urea test.

In the interpretation of virulence factors of the 70 Candida isolates, phospholipase activity was demonstrated by 43 (61.43%) isolates (Table 2), out of which 25 (58.13%) were C. tropicalis, followed by 11 (25.58%) were C. albicans, 4 (9.30%) were C. glabrata, 2 (4.65%) isolates of C. parapsilosis, and 1 (2.32%) isolate of C. krusei (Table 1).

With respect to hemolysin, 49 (70% out of the 70 Candida isolates) indicated hemolysin production (Table 3) (Figure 4), of which 27 (55.10%) were C. tropicalis, 12 (24.48%) were C. albicans, C. parapsilosis 5 (10.20%), C. glabrata 3 (6.12%), and C. krusei 2 (4.08%). 34 (48.57%) Candida isolates showed coagulase activity; of these, 14 (38.24%) Candida albicans exhibited the highest coagulase activity, followed by 11 (32.35%) Candida tropicalis, 6 (17.64%) Candida parapsilosis, 3 (8.82%) Candida glabrata, and 1 (2.94%) Candida krusei (Table 1) (Figure 5).

For each of the 70 isolates of Candida species, biofilm formation was measured using the crystal violet technique and the results were as follows: 55 (78.57%) isolates showed biofilm generation, and among them, 25 (45.46%) C. tropicalis isolates showed biofilm production, followed by 13 (23.63%) C. albicans, 5 (11.11%) C. krusei, 9 (16.36%) C. parapsilosis, and 3 (5.4%) C. glabrata. C. tropicalis stood out among them as the most frequent source of biofilm production. Of them, 18 isolates were discovered to create strong biofilm (3+), and 10 isolates were observed to display moderate biofilm development (2+). Also, 8 Candida species displayed weak biofilm formation (+1; Table 1).

The antibiogram profile of the 70 isolates of Candida was demonstrated for Voriconazole, Amphotericin B, Fluconazole, and Itraconazole. A high degree of fluconazole resistance has been found in 23 (32.8%) Candida species when compared to other antimicrobials used in this investigation. C. tropicalis exhibited the highest level of fluconazole resistance among them. Notably, all C. krusei isolates were resistant to fluconazole. Of the 70 Candida isolates, 56 (80%) were responsive to itraconazole, whereas 13 (20%) demonstrated resistance. 63 (90%) of the isolates were responsive to voriconazole, whereas 7 (10%) displayed resistance. While the antibiogram of Amphotericin B was evaluated, only 1 (1.42%) isolate of C. tropicalis was found as resistant and the remainder, 98.57%, were sensitive (Table 2) (Figure 6).

MICs for fluconazole for every C. tropicalis isolates were determined using the microdilution method, and the findings were interpreted based on CLSI standards, in which 1 (14.28%) C. tropicalis isolate had a MIC of 2 g/mL and 2 (28.57%) isolates showed intermediate resistance to Fluconazole, exhibiting a MIC of 4-32 g/mL. The increased fluconazole MIC value of 64 g/mL was demonstrated by 4 (57.14%) isolates of C. tropicalis (Table 3).

DISCUSSION:

Candidemia is a serious problem, particularly in patients with impaired immune systems. In recent years, NAC species have replaced Candida albicans as the most common cause of candidemia. In southern India, the most prevalent isolates of candidemia were C. tropicalis and C. parapsilosis [25, 26]. However, when compared to other Candida species, candidemia brought on by C. tropicalis isolates is a potentially lethal infection that increases patient mortality. There are risk factors that could improve a person's vulnerability to candidemia, including increasing usage of antibiotics and corticosteroids, frequent hospitalisations, neutropenia, malignancy, AIDS, intravascular catheterization, chemotherapy, and other conditions that affect immunity [27, 28].

The isolation of NAC species from candidemia patients has steadily increased during the past few

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years [28, 29–30]. Numerous investigations have isolated and noted similar trends among NAC species, although other study indicates that C. glabrata and C. albicans prevail [31, 32, 33]. The traditional approach was used in this work to identify 70 consecutive, non-repetitive Candida isolates. In this investigation, NAC species accounted for roughly 72.85% of the total, while C. albicans and comparable results were revealed by Sachin C et al. [34]. Among these, 27 (38.57%) Candida species were the most frequent isolates, and comparable results were also reported in the study findings of Tak et al. and Chakrabarti et al. [35, 36].

These virulence factors have been extensively studied since Candida albicans was identified as the most common pathogenic organism in candidemia. Nonetheless, the evolution of virulence in Candida non-albicans species is covered in numerous study publications [37, 38, 39]. The demonstration of virulence factors demonstrates that 43 (61.43%) isolates showed phospholipase activity, while 25 isolates of C. tropicalis showed maximal phospholipase activity. These outcomes were consistent with research by Sachin C. et al., which revealed that 60.9% of isolates had phospholipase activity, with 19 isolates of C. tropicalis producing the majority of this activity [34].

On the other hand, phospholipase activity was more common in Candida albicans (45.62%) than in Candida non-albicans species, according to Khater et al. [40]. However, on the contrary, Figueiredo-Carvalho et al. showed no phospholipase activity across NAC species [41].

Among 70 Candida isolates, 49 (70%) showed hemolysin production, and the present study results were associated with the study findings of Luo et al. and M. A. Galán-Ladero et al., which indicated [42, 43]. Although investigations have indicated that C. tropicalis produces hemolysin, it is vital to determine if the hemolytic activity exhibited is genuinely occurring or whether it is the consequence of phospholipase synthesis [44]. Therefore, in order to clarify the hemolysin production that contributes to the pathogenesis of C. tropicalis, more advanced research based on the molecular analysis seems to be required [22]. Thirteen Candida albicans exhibited the greatest level of coagulase activity among the 34 (48.57%) Candida isolates. These findings were in line with those of Yigit N et al., who found coagulase activity in 50.6% of the Candida isolates. 14 C. albicans were discovered to have coagulase activity [22].

The development of a mature, highly organized biofilm commences when the yeast cell clings to a surface and starts to grow. Biofilm formation is thought to be a potent infectious characteristic that results in recurrent infections and therapeutic failures [46]. 55 (78.57%) of the 70 Candida isolates exhibited biofilm generation, which was consistent with the findings of Sanyuktha Tulasidas et al.'s investigation, which revealed biofilm activity in 74% of the isolates [47]. It was interpreted that 25 (45.46%) of the Candida tropicalis isolates demonstrated highest biofilm formation. This discovery is in accord with the results obtained in a study conducted by Sasani et al., who similarly reported C. tropicalis (47%) as the prevalent isolate that caused biofilm activity [48].

The development of a mature, highly organized biofilm commences when the yeast cell adheres to a surface and starts to proliferate. It is believed that biofilm development is a powerful infectious trait that causes recurring infections and treatment failures [46]. In line with the results of Sanyuktha Tulasidas et al.'s study, which found biofilm activity in 74% of the isolates, 55 (78.57%) of the 70 Candida isolates showed biofilm formation [47]. Twenty-five (45.46%) of the isolates of Candida tropicalis were found to have the maximum biofilm development. This finding is consistent with the findings of a research by Sasani et al., who similarly identified C. tropicalis (47%) as the most common isolate responsible for biofilm activity [48]. The creation of a mature, highly organized biofilm commences when the yeast cell clings to a surface and starts to proliferate. It is believed that biofilm formation is a potent infectious characteristic that causes recurring infections and treatment failures [46]. In line with the results of Sanyuktha Tulasidas et al.'s research, which found biofilm activity in 74% of the isolates, 55 (78.57%) of the 70 Candida isolates showed biofilm formation [47]. The maximum biofilm growth was reported in 25 (45.46%) of the isolates of Candida tropicalis. This result is consistent with a study conducted by Sasani et al. [48], which also found that the most prevalent isolate causing biofilm activity was C. tropicalis (47%).

Candida tropicalis, which is becoming one of the most important and concerning Candida isolates developing antifungal resistance, had the greatest resistance pattern [58]. So, the MIC of all fluconazole-resistant C. tropicalis isolates was demonstrated and interpreted by the microdilution method as per CLSI standards [59]. The current study results were similar to the study findings of Gandham et al. and revealed that AFST of fluconazole showed a higher MIC of 2 g/ml in 4 isolates of C. tropicalis and 1 of C. parapsilosis, while 2 of C. albicans were resistant [60]. The misuse of fluconazole, which allowed resistant species like

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NAC species to survive, seems to be the most likely source of this shift [61].

The significance of regularly monitoring these occurrences of candidemia is underlined by the shifting epidemiology and developing antifungal resistance. Therefore, a rigorous antifungal stewardship approach and correct identification of Candida species may help manage infections and minimize mortality rates [62, 63].

CONCLUSION:

In this investigation, the prevalence of candidemia among the patients from various wards occurred with a predominance of C. Tropical. Fluconazole has the least antifungal activity, which is reflected in the highest level of antifungal resistance observed, while amphotericin B has the greatest antifungal action. The interpretation of numerous virulence factors and antifungal drug resistance were seen predominantly among NAC species, therefore demonstrating its vital significance in immunocompromised persons' treatment. Timely detection of Candida BSI and understanding of its resistance profile are crucial for improving outcomes, especially for critically ill infants and immunocompromised individuals. In order to reduce the total impact of increasingly common Candida infections, treatment failures, and financial burden, this knowledge on virulence factors and antifungal susceptibility is necessary.

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DATA AVAILABILITY:

All the datasets created or analysed during this study are incorporated into the manuscript.

CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest.

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