Emerging Candida Species Isolated from Tertiary Care Hospital, Lahore: Species Distribution and Susceptibility Profile

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ABSTRACT

Objective: In the last five years, invasive Candida infections have become more prevalent. Along with Candida albicans, non-albicans candida (NAC), infections are also increasing. The study aimed to identify the emerging candida species from clinical samples and to estimate their susceptibility profile.

Materials and Methods: All Candida suspected samples were collected from Tertiary Care Hospital, Lahore. Samples were processed on sabouraud and blood agar. Wet prep was done for confirmation of Candida. Germ tube testing was done to differentiate C. albicans from NAC. Specie identification and antifungal susceptibility were performed by VITEK 2.0 compact system. 18sr RNA Sequencing was performed for unidentified Candida species.

Results: Out of 86 confirmed Candida samples, 41 were identified as C. albicans by germ tube testing, and 45 were NAC. VITEK 2.0 results indicated that C. tropicalis was isolated from a maximum of 33.3% of samples among NAC. There were 5 cases of candida co-existing with bacterial pathogens, while more than half (58%) were obtained from the Intensive Care Unit (ICU) patients. The antifungal susceptibility pattern of NAC species indicated that most NAC was susceptible. Out of 15 isolates of Candida tropicalis, 11(73.0%) were susceptible to fluconazole, and susceptibility against the other antifungals was 100%. All of the isolates of C. glabrata were 100 % susceptible to micafungin, amphotericin B, and flucytosine. All C. krusei, and C. guilliermondii isolates were susceptible to all the tested antifungals except fluconazole, and amphotericin B, respectively. C. ciferrii showed 100% susceptibility to all antifungals.

Conclusion: All ten unidentified isolates showed 100% susceptibility to all the drugs except fluconazole. This study has shown increased NAC and high susceptibility to antifungals.

Keywords: Candida albicans, non albicans Candida, susceptibility testing, VITEK

Original Research Article

Introduction:

Candida species are well-known opportunistic fungi that can cause fungal infections in immunocompromised individuals [(1)]. Candida species rank seventh in the United States as the most common cause of bloodstream infections. These infections contribute to a significant number of morbidities and mortalities worldwide. Being an opportunistic pathogen, it affects the subjects with predisposing risk factors. Among these risk factors are immunosuppression, exposure to broad-spectrum antimicrobials, cytotoxic drugs, catheterization, malignancies, diabetes myelitis, chronic kidney disease, post-surgery, hemodialysis, neutropenia, prolonged hospitalization, organ transplantation, parenteral feeding, intensive care setting, and elderly age [(2)].

Candida species are typically found as part of the normal flora in healthy individuals, residing in various body sites such as the skin and mucosal linings of the oral cavity, vagina, and gastrointestinal tract [(3)]. While numerous species are within the Candida genus, only a few are known to cause infections in humans. The most prevalent species responsible for most cases is C. albicans, accounting for approximately 80% of candidiasis cases. However, there is also a growing trend in cases of non-albicans Candida (NAC) infections. [(4)]. The worldwide studies showed a grave danger of increasing infections from the NACs. Multiple reasons for this sudden rise of NACs have been proposed, including excessive use of azoles and polyenes, improved diagnostic technologies, geographical factors, increased life expectancy and malignancies [(5)]. Among these NACs, C. glabrata, C. tropicalis, C. parapsilosis, C. krusei, C. dubliniensis, C. kefyr and C. guilliermondii are said to be associated with most of the infections (10). Among these, C. glabrata, C. krusei, C. parapsilosis and C. tropicalis are more common [(6)].

The incidence of different NAC species varies geographically and depends upon multiple factors. Geographically, C. tropicalis is more prevalent in the Middle East and Africa while

C. glabrata is common in the European Union (EU) and Asia-Pacific. Most cases of C. parapsilosis are reported in Latin and North America. As far as the physiological factors are concerned, most Candida spp. especially C. glabrata infect older adults (>60 years). However, C. parapsilosis affects infants less than one year of age [7]. These Candida species have been documented as emerging human pathogens in global surveillance programs such as SENTRY and ARTEMIS. NACs accounted for 10-40% of all candidiasis cases during 1970-1990, but Krcmery et al reported 2002 that this percentage has increased to 35%-65% [8]. Therefore, these NACs are emerging as serious public health risks and must be given equal attention to C. albicans to minimize the incidence rate.

Exceptionally high antifungal resistance among the NACs mentioned above is a severe concern. Commonly used antifungals belong to four classes: azoles, echinocandins, polyenes and pyrimidine analogues. Antifungal resistance can be intrinsic or acquired [9]. Compared to C. albicans, NACs exhibit varying degrees of acquired resistance to most antifungals. Intrinsic resistance is also quite common among them. This study aimed to identify emerging Candida species from clinical samples and estimate antifungal susceptibilities.

Methodology

This cross-sectional study was conducted in the Microbiology laboratory of a tertiary care hospital in Lahore in collaboration with the Institute of Microbiology and Molecular Genetics, University of the Punjab Pakistan, from September 2020 to April 2021. All Candida-suspected samples were received in a microbiology laboratory and passed through the inclusion and exclusion criteria. All of the suspected Candida species isolated from these clinical samples were included in the study. However, duplicate samples from the same patient during the same episode of illness were excluded.

Identification of Candida spp.

All the Candida suspected clinical samples were processed as the standard operating procedure of the lab. All the samples were inoculated on Sabouraud agar and blood agar to confirm Candida. They were incubated for 18 hours at 37℃ aerobically. Colonial growth was subjected to wet prep and gram staining. Candida spp. was preliminary identified based on cultural and microscopic morphologies. Germ tube testing and Sabouraud agar Candida were used to differentiate C. albicans from non-albicans Candida (NAC) species.

Germ tube testing

Germ tube testing were performed for C. albicans. The germ tube was the filamentous extension of the yeast cells. In Candida cells, some proteins and ribonucleic acid production are enhanced upon incubation at 37℃ in human or sheep serum for 3-4 hours. For this, colonies are incubated at 37℃ for 3 hours in sheep or human serum. In a test tube, 2 ml of pooled serum was added and inoculated with 1-2 colonies of suspected Candida species. Tubes were incubated at 37℃ for 3-4 hours. After incubation, one drop of suspension on a glass slide was placed with a Pasteur pipette and covered through a cover slip. The slide was examined microscopically for yeast cells with filamentous extension. C. albicans ATCC 10231 was used as the positive, and C. krusei ATCC 6258 was used as negative control.

Specie identification via VITEK 2.0. compact and Antifungal susceptibility testing of non-albicans Candida species

All isolates identified as NACs in the previous step were simultaneously subjected to species identification and antifungal susceptibility testing through VITEK 2.0. compact. In this system, the VITEK YST card was employed for specie identification, and the AST-YS08 card was used to measure MICs against fluconazole, voriconazole, flucytosine, amphotericin B and caspofungin. So, 3ml saline solution was taken in a tube and mixed with a pure bacterial culture. Mcfarland standard (0.50-0.63) was made. For gram-negative antibiotic susceptibility (GN-AST), a 145(l from GN-ID Mcfarland solution was mixed in another tube containing 3ml saline solution. For gram-positive antibiotic susceptibility (GP-AST), a 280 (l from GP-ID Mcfarland solution was mixed in another tube containing 3ml saline solution and different VITEK cards, i.e., GN-ID, GN-AST, GP-ID, and GP-AST, were placed in the tubes, respectively. The racks containing tubes in biomerieux VITEK 2 compact for loading samples were placed. When the samples were loaded, the racks were transferred to the other side to remove the cards and press start. Took out the racks after 5-7 minutes. Logged in to VITEK 2 software on the system. Clicked on cassette view, then clicked on samples showing incomplete status and entered the accession numbers of the samples and linked the AST of samples with their IDs. The results were noted after 18 hours of incubation.

Ribotyping

Two Isolated colonies of unidentified Candida spp. were sent for 18S rRNA sequencing. The sequencing results were subjected to nucleotide BLAST (Basic Local alignment search tool). The isolates were identified as species with the maximum similarity percentage in the database. The evolutionary history was inferred using the Neighbor-Joining method [10]. The optimal tree is drawn to show the rate of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) next to the branches [11]. The evolutionary distances were computed using the Maximum Composite Likelihood method [12] and are in the units of the number of base substitutions per site. The evolutionary analyses were conducted in MEGA11 [13].

Results

Among all the suspected clinical samples that were received within the study duration, 86 were identified as positive for Candida. Out of 86, 41(47.8%) isolates were identified as C. albicans by germ tube development, and the rest of the 45(52.3%) isolates were of non-albicans-Candida (NAC). These 45 NACs were considered emerging Candida spp. and further processing through VITEK 2.0. compact yielded the results of NAC. Candida glabrata was isolated from 8(17.8%) samples, C. tropicalis was also yielded from 15(33.3%) samples, C. krusei from 4(8.9%) and frequencies of C. ciferrii, C. famata, C. guilliermondii, Trichosporon asahii and unidentified Candida species were 3(6.7%), 1(2.2%), 1(2.2%), 3(6.7%) and 10(22.2%) respectively.

In this study, 27(60%) NAC species were male, and 18(40%) belonged to female samples. Out of 45, 39 (87.0%) NACs were isolated from urine samples, 4(9.0%) from blood, and in the rest of the cases, 2(4.4%) were isolated from pus and tracheal secretion. There were 5(11.1%) cases of co-existing bacterial pathogens. A single Enterococcus feacalis and 2, 2 (4.4%) cases of E. coli and Klebsiella pneumoniae were reported.

Out of 45, 2(4.4%) cases of Candida infection were reported in <1 year old and 4(9.0%), 0(0.0%), 8(17.8%), 11(24.4%) and 20(44.4%) was reported from 1-15, 16-30, 31-45, 46-60, >60 years old respectively (Fig. 1).

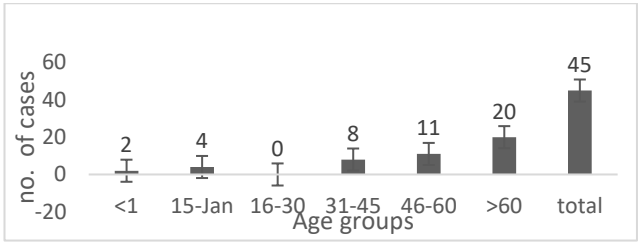

Fig-I Distribution of different NACs in different age groups

Out of 45 isolates, more than half 26(58%) isolates were from patients in the Medical ICU. And 9(20%) from Outpatient, 6(13%) from medical unit and 2(4%), 2(4%) from ICU-N and paediatrics department respectively (Fig. 2).

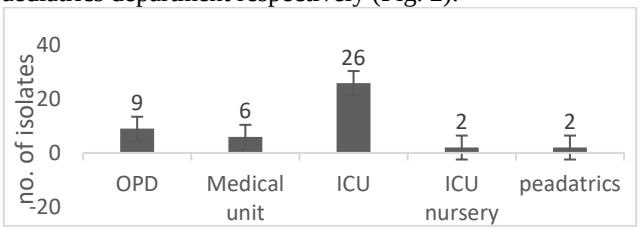

Fig-II Ward-wise distribution of NACs

The antifungal susceptibility pattern of NAC species indicated that out of 15 isolates of *Candida tropicalis*, 11(73.0%) isolates were susceptible to fluconazole and susceptibilities against the other antifungals were 100%. Out of 8 *C. glabrata* isolates, no one was susceptible to fluconazole, 6(75.0%) were susceptible to voriconazole, and 7(87.5%) were susceptible to caspofungin. The susceptibility of the isolates against micafungin, amphotericin B and flucytosine was 100%. All the isolates of *C. krusei* (4 out of 4) were susceptible to all the tested antifungal agents except fluconazole (because of intrinsic resistance pattern) and flucytosine (0.0% susceptible). A single isolate of *C. guilliermondii* was susceptible to all antifungals except amphotericin B. Out of 3 isolates of *Trichosporon asahii*, 2(66.7%) were susceptible to fluconazole, 3(100.0%) were susceptible to voriconazole none was susceptible to caspofungin and micafungin and 1(33%) isolate was susceptible to amphotericin B and flucytosine. All ten isolates of other *Candida species* (unidentified) showed 100% susceptibilities to all drugs, except susceptibilities to fluconazole were 0%. *C. ciferrii* showed 100% susceptibility to all antifungals but susceptibility to flucytosine was reduced to 66.7%. A single isolate of *C. famata* was susceptible to all tested antifungals (Fig. 3).

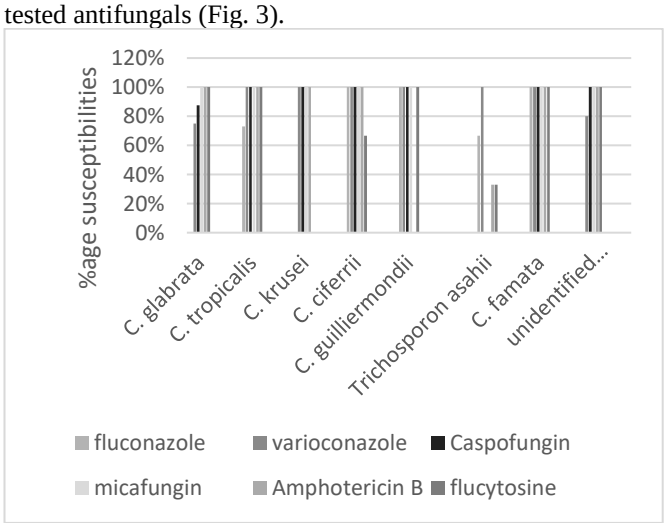

Fig-III Percentages of antifungal susceptibility patterns of NACs

Two isolates were randomly subjected to 18S rRNA sequencing for species identification among unidentified Candida species. Nucleotide BLAST (Basic local alignment search tool) of the 18S rRNA identified one isolate as Candida glabrata (with a percent similarity of 98%) with the accession number MZ562722.1. While the other was identified as *Saccharomyces boulardii* (similarity 99%) with accession number KT000033.1. The neighbour-joining phylogenetic tree was constructed based on the GenBank data of these two strains with similar strains, which was drawn to scale with the same branch length units as the evolutionary distances [(13)]. The maximum composite likelihood method was used to measure the evolutionary distances. The analysis involved ten nucleotide sequences, in which our strains showed close similarity (Fig. 4)

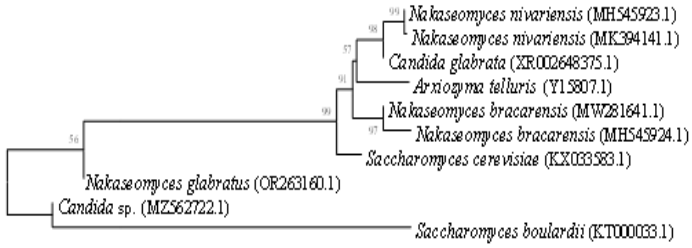

Fig-IV Molecular phylogenetic analysis by neighbour-joining method using MEGA 11

Discussion

In this study, the percentage of non- albicans *Candida* species (NACs) was higher than that of C. albicans and was isolated from more than half (52.3%) of the clinical samples. The frequency of *C. tropicalis* (33.3%) was the highest, followed by Unidentified *Candida* species (22.2%), *C. glabrata* (17.8%), *C. krusei* (8.9%), *Tricosporon asahii* (6.7%) and *C. ciferrii* (6.7%). Giacobbe *et al*. conducted a metanalysis of the data on NACs from 44 countries published between 1971 to 2018 to estimate the pooled prevalence of NAC [(14)]. This metanalysis revealed that in 49.5% ± 1.5 cases, NACs were responsible for candidiasis, which corresponds with our results. *C. tropicalis* was the most frequent NAC in Asian studies, with a pooled prevalence of 21.3%. We also report similar results. *C. tropicalis* remained to be the most frequent isolate. However, its prevalence is 33.3 % which is higher than that of the metanalysis conducted by Daniele.

A study conducted by Jeon JS and Kim JK in South Korea from 2014 to 2018 examined the prevalence of Candida species [(15)]. Similar to our research, they utilized VITEK 2.0 for species identification. Their study reported that NAC species were the most frequently isolated from clinical samples. The percentage of NAC isolation was 59.5%, slightly higher than what we observed in our study. It is important to note that their research specifically focused on Candida species isolated from blood samples. However, *Candida* species isolated from all types of clinical samples were included. Out of all NACs, 87.0% were isolated from urine samples. Satyendu Saha concluded that maximum NACs were isolated from urine samples followed by vaginal swabs [(16)]. However, in the study, after urine samples, most of the isolation was from blood samples, and no NAC was isolated from vaginal swabs.

A study from Taiwan reported a higher incidence of NACs (54.2%) candidemia in children than that of *C. albicans* [(17)], which also agrees with the findings of our study. Pfaller *et al* reported in their research that NAC mostly affects people in the

age group >60, which corresponds to our research. Here the most affected age group was also >60 [18]. Kothalawala *et al* from Sri Lanka also associated 69% of cases of candidemia with NACs using VITEK 2.0 for identification [19]. A 6-year surveillance in Korea also concluded that *C. tropicalis* was the most prevalent NAC and constituted 36.4% of all NACs, approximating our results [20].

Chakrabarti *et al* conducted a prospective study and concluded that *C. tropicalis* (42.1%) was the most frequent NAC with azole resistance of 10.2%-13.6% [21]. While in our study, *C. tropicalis* showed 0% resistance against voriconazole and 27% resistance against fluconazole. In the present study, no NAC showed resistance against echinocandins such as caspofungin and micafungin except one *C. glabrata* isolate resistant to caspofungin but susceptible to micafungin. The same results were obtained for echinocandin susceptibility in a study by Yenisehirli *et al* [22]. Many other studies also concluded that echinocandin was active against NACs [23]. In our study, the gene sequencing and phylogenetic analysis by MEGA 11 suggested a close relation of *C. glabrata* with *S. boulardii*, which may be the reason this *Candida* spp resists echinocandin as described by Vermitsky *et al*., 2006 [24].

Resistance to amphotericin B was also rare, and only 4.4% of isolates showed resistance against it including *C. guilliermondii* and *Trichosporon asahii*. Hitkova *et al* also revealed that resistance against amphotericin B was uncommon [25]. In this study, only 5.97% of isolates resisted it, which is very close to our findings. El-Ganiny *et al* also discussed this uncommon amphotericin B resistance in NACs [26]. Shawky *et al* reported that out % of all *C. krusei* isolates, 84.6% were resistant to flucytosine [27]. Resistance to azoles is increasing among *Candida* species, especially in the NACs; 60% of NAC showed resistance to fluconazole, while only 8.9% were resistant to voriconazole in the present study. None of the *C. glabrata* and *C. krusei* were susceptible to fluconazole, while resistance to voriconazole was 25% and 0%, respectively.

The percentage of resistance against fluconazole showed by *C. tropicalis*, and *Trichosporon asahii* was 27% and 33.3%. While the *C. krusei* showed 100% resistance to fluconazole which may be due to its intrinsic resistance pattern and following the results obtained by Satyendu Saha [16]. In contrast to the finding of these studies, 60%-69% isolates of *C. glabrata* were susceptible to fluconazole. However, another study conducted by Mashaly and Shrief from Egypt also reported 0% susceptibility of *C. glabrata* to fluconazole [28]. A study from US reported that no isolate of *C. glabrata* showed susceptibility to fluconazole while using E-test. For *C. tropicalis*, Sadeghi *et al* also observed the emergence of antifungal resistance to fluconazole with the percentage of 14.3% which is slightly lower than our findings [29].

Interestingly, ten isolates remained unidentified in our study on VITEK 2.0; to our surprise, no one was susceptible to fluconazole. These were supposed to be the *C. auris* which cannot be identified on VITEK 2.0 and shows a high resistance to fluconazole [30]. Owing to our limited resources, we could not identify these isolates. Only two isolates were processed.

In conclusion, non-albicans *Candida* species are emerging as dominant pathogens, surpassing *C. albicans*, and showing significant resistance to antifungal treatments.

Author contribution

NS: experiment, data and strain collection; FR: strain collection and demographic data; ANS and SR: designing the experiment, data analysis, and manuscript writeup.

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Conflict of interest

None to declare

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