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Antifungal susceptibility testing of *Candida* isolates from PLHIV patients

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Abstract

Introduction- There is a growing prevalence of fungal infections among immunocompromised patients, and there is also a concerning rise in resistance to commonly used antifungal medications. As a result, antifungal drug susceptibility testing has become an invaluable tool for clinicians, specifically in the treatment of invasive fungal infections and cases where initial treatments have proven ineffective.

Methodology- We conducted an analysis of the antifungal drug susceptibility patterns of 62 *Candida* isolates derived from various infection sites in a cohort of 165 PLHIV patients using the broth macrodilution method.

Results- A high resistance was observed to azole group with highest being Fluconazole (27%) followed by Voriconazole (14.5%) and Itraconazole (12.9%). No resistance was recorded for Amphotericin B and Caspofungin.

Discussion- Our findings indicate a higher resistance to Fluconazole, specifically in *non-albicans Candida* (NAC) species compared to *Candida albicans*. The presence of cross-resistance between Fluconazole and voriconazole is a matter of concern. However, it is important to note that no instances of resistance were observed for both Amphotericin B and Caspofungin. Nevertheless, elevated minimum inhibitory concentrations (MICs) were noted for these antifungal agents.

Conclusion- Routinely performing antifungal susceptibility testing in laboratories is crucial due to the growing resistance among antifungals, especially in the azole group. This resistance has led to the emergence of highly resistant and more harmful non-albicans *Candida* species. Thanks to the availability of standardised automated and manual methods, conducting such tests has become more accessible. Therefore, we strongly recommend performing routine susceptibility testing in laboratories to ensure appropriate treatment decisions for patients.

Keywords: Antifungal susceptibility; *Candida*; Non-albicans; CLSI; Fungi

1. Introduction

Candida species are the most prevalent among fungi causing infections. These species are responsible for a wide range of infections, ranging from superficial to life-threatening. Infections with drug-resistant *Candida* species, especially in patients with underlying diseases or immunosuppression, pose a higher risk of serious complications [1].

The development of resistance makes treatment less effective, which can jeopardize patient care and lead to prolonged hospitalization and associated complications.

The management of *Candida auris*, an emerging fungal pathogen, necessitates the use of antifungal susceptibility testing (AFST). This pathogen, known for its multidrug resistance and its presence in crucial areas of hospitals, has significantly impacted the approach to *Candida* infections for clinicians and infection control personnel.

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C. auris displays intrinsic resistance to fluconazole and has the potential to develop resistance to other antifungal agents such as amphotericin B, echinocandins, and other azoles.

Moreover, the diagnostic process for *C. auris* is challenging, necessitating the utilization of molecular methods to ensure accurate diagnosis. Therefore, in resource-limited settings where access to alternative antifungal agents and molecular diagnostic techniques may be limited, *C. auris* represents a significant threat.

Hence, there is a pressing demand for dependable, rapid, and user-friendly diagnostic as well as AFST methods [2].

To ensure accurate MIC values and sensitivity predictions, the primary function of AFST is to support clinicians in making informed decisions regarding patient therapy. Additionally, these results hold significance in terms of epidemiological comprehension and aiding in the estimation of antifungal drug resistance rates in the given population.

However, it is crucial to exercise caution when selecting an AFST method, as many existing methods lack adequate in vitro data and thus require careful interpretation of their outcomes [3].

This local resistance data must be considered by hospital clinicians while formulating empirical antifungal policy. Finally, standardization of antifungal susceptibility testing is also very important for drug researchers to determine therapeutic potential of newly developed antifungal agents [4,5].

The limited treatment options available against fungi include three classes of antifungals, azoles, polyenes and echinocandins. Echinocandins is new class of drug with unique mechanism of action which prevents cross resistance with azoles and polyenes. This makes them good candidate for refractory cases [6]. However they are more expensive and unavailable for oral use. Therefore Fluconazole remains most commonly used antifungal drug in India [7].

It was not until 2004, Clinical Laboratory Standard Institute (CLSI) established standardized method and clinical breakpoints (CBP) to determine *Candida* susceptibility for antifungals based on data from several clinical studies, pharmacodynamics and pharmacokinetics of drugs thus, providing accurate, reproducible results with good clinical correlation. In 2012, CLSI published revised version of clinical breakpoints (CBP) for MIC to determine *Candida* susceptibility for antifungals- fluconazole, voriconazole, amphotericin B and echinocandins group [8].

The advancement of automated methods for drug susceptibility testing in *Candida* species has greatly simplified processes and significantly reduced turnaround time. This improvement is particularly beneficial for clinicians as it allows them to make faster clinical decisions [9]. Nevertheless, in resource-constrained settings, reliance on more laborious conventional methods is still necessary.

Herewith we undertook a study to analyze antifungal drug susceptibility pattern of different *Candida* isolates recovered from various infection sites of PLHIV (people living with HIV infection) patients admitted in wards, ICU or attending OPD of tertiary care hospital in western part of India. The antifungal drug susceptibility testing (AFST) was performed by broth Macro dilution method using revised antifungal break points (CLSI M27- S4) [10].

2. Material and methods

The prospective study was undertaken with approval of ethical committee of institute. Written informed consent was obtained from all patient's included in the study.

A total of 62 *Candida* isolates were recovered from January 2012 to May 2014 from 300 different specimens obtained from 165 PLHIV patients attending OPD or hospitalized at tertiary care hospital with signs and symptoms of *Candida* Infection. Specimens were collected based on body site affected and includes oral swab, blood, body fluid, BAL, tissue and biopsy, pus etc. Clinical significance of each *Candida* isolates was established by appropriate standard guidelines.

2.1. Identification

Identification was carried out by conventional methods including germ tube test, growth on chrome agar, sporulation on corn meal agar (Hi Media, India) and carbohydrate assimilation test using yeast nitrogen basal agar (Difco, BD, India) (Figure 2,3).

Antifungal drug susceptibility for *Candida* isolates were performed for six antifungals- Amphotericin B (AMB), Fluconazole (FLZ), Voriconazole (VRZ), Ketoconazole (KTZ), Miconazole (MIZ) and Caspofungin (CFG) (all from

Himedia, India). Macrobroth dilution method was followed as per CLSI protocol. RPMI 1640 with MOPS buffer and 0.2% dextrose was used as a basal growth medium. Isolates were grown on potato dextrose agar twice prior to inoculum preparation to ensure purity and viability. Final inoculum (5 X 10^4 cells/ml) prepared by adjusting turbidity to 0.5 McFarland standard. Doubling drug dilutions were made ranging from 0.03 to 64 ug/ml for Fluconazole, Ketoconazole, Miconazole, Voriconazole and Amphotericin B and 0.016 to 8 ug/ml for Caspofungin. Tubes were incubated at 35 C for 72 hours and visually assessed (Figure 4).

MIC (Minimum inhibitory concentration) is defined as the highest tube dilution that shows decrease in turbidity when compared to growth control. Further interpretation of MIC values done based on clinical breakpoints recommended by CLSI. MEC (Minimum effective concentration) is the highest drug dilution where trailing of growth begins.

Quality control was performed using strains of *Candida parasilosis* (ATCC22019) and *Candida albicans* (ATCC90028)

3. Results

Study was comprised of 76% male population and 24% female population. More than half of the study group belonged to the age group of 31 to 50 years (Figure 1).

A 33 out of 62 *Candida* isolates were identified as *C. albicans* while remaining 29 were *non-albicans Candida* (NAC) which includes 18 of *C. tropicalis*, 5 of *C. parasilosis*, 3 *C. guillermondii* and 3 of *C. glabrata.*

A overall high resistance was observed to azole group with highest resistance to Fluconazole (27%) followed by Voriconazole (14.5%), Itraconazole (12.9%).

All the *Candida* isolates were uniformly susceptible to amphotericin B and caspofungin. MIC ranges of these antifungal drugs showed that Caspofungin (MIC 50- 0.06, MIC 90- 0.5) had highest susceptibility, followed by amphotericin B (MIC 50- 0.5, MIC 90- 2).

The NAC showed higher level of resistance particularly to fluconazole (38%) when compared to *C. albicans* (19%). Cross resistance was observed between fluconazole and voriconazole in 8 (13%) *Candida* isolates. Out of 6 *C. albicans* isolates resistant to fluconazole (19%), concurrent resistance was observed in 3 isolates for voriconazole and two isolates for itraconazole.

Among the NAC, *C. tropicalis* showed high resistance to fluconazole (33%, 6/18), while that to voriconazole and itraconazole were 11% and 9% respectively. All the isolates of *C. parasilosis* and *C. guillermondii* were susceptible to all three azoles (100%). Two out of three isolates of *C. grabrata* (66%) were resistant to fluconazole.

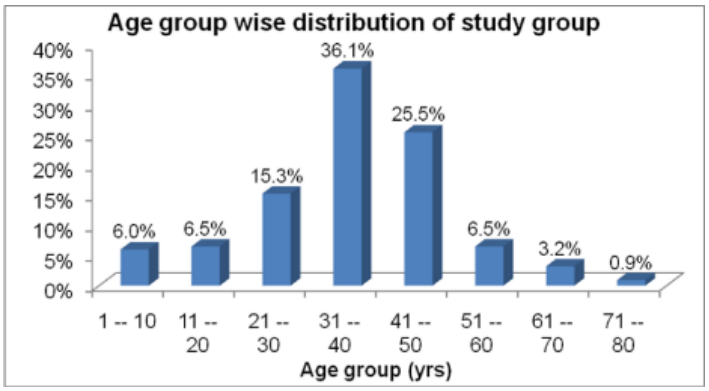

Figure 1 Age group wise distribution of the study group

Figure 2 Sabourauds dextrose agar showing colonies of *Candida albicans*

Figure 3 Green color colonies of *C albicans* and pink color colonies of *C krusei* on Hichrome agar

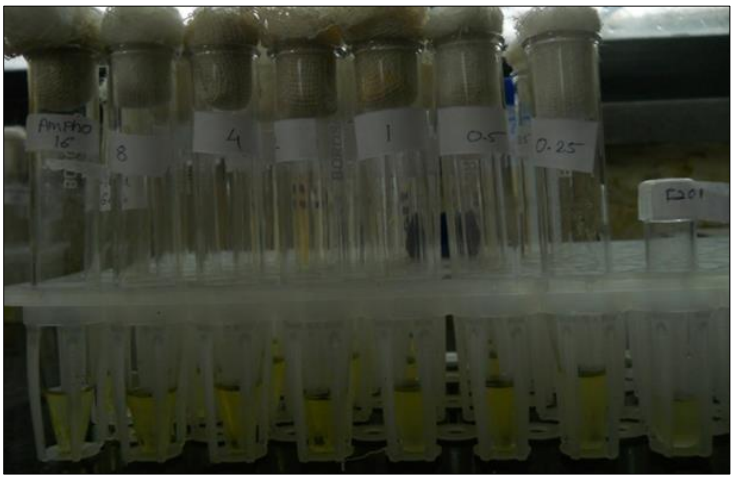

Figure 4 Antifungal susceptibility testing by Macro-broth dilution method

4. Discussion

For immunocompromised patients, early detection of resistant strain of *Candida* species is very important for successful management.

The prevalence of azole resistance varies significantly across regions and institutions, depending on the implementation of prophylactic and therapeutic antifungal practices [11]. Furthermore, several factors need to be considered before initiating an antifungal regimen. These factors include the drug's pharmacokinetics and pharmacodynamics, epidemiological data, hepatic or liver failure, potential interactions with other medications, immunosuppression, adverse events associated with the drug, and other risk factors [12].

It is important to note that different *Candida* species exhibit varying levels of virulence. Recent studies have indicated a higher incidence of complications and mortality associated with non-albicans *Candida* (NAC) compared to *C. albicans*. This is likely due to their frequent display of multidrug resistance [13].

The surveillance study conducted in US over 20 years The steady shift in paradigm of fungal infections from *C. albicans* to *non-albicans Candida* (NAC) species such as *C. glabrata*, *C. guillermondii* and *C. krusei* observed in this study is extremely concerning [14,15]. Limited treatment options available for these fungal species poses major threat particularly for immunocompromised patients [16]. A Multi-drug Resistant *non-albicans Candida* species – *C. auris* which was first detected in 2009, has already been reported from most of the countries and caused several outbreaks associated with health care settings. An emergence and rapid spread of this pathogen among different countries has created a global threat to public health [17,18].

WHO surveillance report (2014) covering several countries shows great variation in fluconazole drug resistance among Candidemia patients by geographic location ranging from highest 33% in Denmark and lowest in Korea (0.9%). It also reports higher fluconazole resistance among NAC than among *C. albicans* in most of the countries [19]. We also observed higher resistance with NAC particularly for fluconazole. Resistance among NAC for other antifungals were comparable or on higher side than among *C. albicans*.

No resistance was observed to azoles among *C. parasilosis* and *C. guillermondii*, Nevertheless, CDC surveillance of Candidemia patients conducted over the period of 4 years demonstrated increasing Fluconazole resistance among *C. parasilosis* isolates [20].

In present study, a 13% of isolates were resistant to both fluconazole and voriconazole, two important azole group drugs which is similar to 11.3% which was observed in Oberoi et al [21]. The reason for higher variconazole resistance is unclear, although it could be because of cross resistance owing to similar mechanism of action rendering voriconazole, a newer, less commonly used and more effective antifungal ineffective. Development of cross resistance may limit the use of voriconazole as a prophylactic therapy for *Candida* infections [22].

We didn't detect any resistance to Amphotericin B which is reassuring. Similar finding was observed in certain studies [23]. Although it's standard drug to treat *Candida* species, unfortunately it requires parenteral use, in addition to this it's toxic effects also limits its usefulness. Fluconazole is reported as equally effective and better tolerated drug than amphotericin B for treatment of systemic candidiasis [24]. Other Indian studies had also failed to report any resistance to amphotericin B [25,26]. A study from southwestern part of India showed resistance of 8% which was more evident with NAC [27].

A study conducted on *Candida* isolates obtained from immunocompromised patients implemented the disc diffusion method to assess sensitivity. The results indicated that amphotericin B and nystatin exhibited complete sensitivity, while ketoconazole displayed a sensitivity rate of 37.09%. clotrimazole showed a sensitivity rate of 20.9%, itraconazole at 19.35%, and fluconazole at 14.5%. The *Candida* isolates consisted of various species, with *C. tropicalis* representing 43.1% of the isolates, *C. albicans* at 32.25%, *C. glabrata* at 24.19%, and *C. parapsilosis* at 6.45% [28].

Ketoconazole and miconazole are antifungal agents for topical application. Though these agents had been used in past as a systemic therapy for serious fungal infections, they have been substituted because of limited efficacy and high toxicity. Our resistance pattern for ketoconazole and miconazole is supported by study from northern India which reported 7.1% resistance to both ketoconazole and miconazole [29]. However few studies from outside countries have shown relatively low resistance against these agents [30, 31].

In a study conducted in Manipur, it was observed that *non-albicans Candida* (NAC) species were more prevalent (56%) than *C. albicans* (44%). Among the NAC species, *C. tropicalis* accounted for 32% of the total. The AFST by disc diffusion method revealed that voriconazole exhibited the highest sensitivity (86%), while ketoconazole displayed the lowest sensitivity (56%). Additionally, a sensitivity rate of 81% was observed for amphotericin B [32].

Limitations of the study-

The underlying etiology of immunosuppression was solely HIV infection, and the sample size was small. Additionally, there was no available information regarding previous use of azoles.

5. Conclusion

Our study focuses on exploring the susceptibility of *Candida* species, including both *C. albicans* and non-albicans strains, among immunocompromised patients. We have observed a concerning trend of increasing resistance, particularly within the azole group of antifungal drugs. This pattern of resistance has led to the emergence of highly resistant *non-albicans Candida* species, which pose a significant threat to immunocompromised patients. Therefore, it is crucial to implement continuous surveillance studies to monitor and control the ongoing use of antifungal medications.

Fortunately, with the availability of standardized automated and manual methods, antifungal susceptibility testing has become clinically relevant. Therefore, we strongly recommend performing routine susceptibility testing in laboratories to ensure appropriate treatment decisions for patients.

Compliance with ethical standards

Disclosure of conflict of interest

Author states that there is no conflict of interest.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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