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Screening of Transcriptomic Factors Associated with Multidrug-Resistant *Candida krusei*

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Abstract

Oral candidiasis is a common fungal infection caused by *Candida* species, often exhibiting resistance to antifungal agents. Among non-*albicans Candida* species, *Candida krusei* is of clinical concern due to the development of resistance mechanisms, including drug efflux pumps and gene overexpression. This study investigates drug susceptibility, minimum inhibitory concentration (MIC), efflux pump activity, and gene expression in *C. krusei* isolates. After identification using chromogenic media (HiCrome Candida Differential Agar), clinical isolates were subjected to antifungal susceptibility testing and MIC determination against fluconazole, voriconazole, miconazole, clotrimazole, and nystatin following CLSI guidelines. Efflux pump activity was evaluated using the Rhodamine 6G (R6G) efflux assay. Gene expression of *CDR1, CDR2, TAC1,* and *ERG11*- key regulators of azole resistance—was analyzed using RT-qPCR and the $2^{-\Delta\Delta CT}$ method. The antifungal susceptibility profiling revealed that the majority of the clinical isolates exhibited resistance to nystatin with an MIC of ≥ 8µg/mL, while the MIC ranges 0.25-2µg/mL for other drugs. Notably, higher R6G efflux was observed in nystatin exposure compared to other antifungals, indicating a contributory role of ATP-binding cassette (ABC) transporters to antifungal resistance- especially in reducing intracellular drug accumulation. Further support for this mechanism was provided by RT-qPCR analysis, which showed upregulation of resistance-associated genes- *CDR1, CDR2, TAC1*, and *ERG11*- pre-treated with nystatin. The increased efflux activity observed in the R6G assay and overexpression of these efflux genes, especially in response to nystatin, underscores the pivotal role of efflux pumps in mediating resistance. These findings suggest that targeting efflux-mediated pathways may be a promising strategy in overcoming resistance in *Candida* infections.

Key words: Antifungal resistance, *Candida krusei*, Efflux pumps, Gene expression, Oral candidiasis.

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Introduction

Oral candidiasis is a common opportunistic fungal infection caused by *Candida* species, particularly in immunocompromised individuals, such as those with HIV/AIDS, diabetes, or those undergoing chemotherapy and organ transplantation (Gow et al. 2011). *Candida* species are normally harmless in the human microbiome, but they can become harmful in some situations and cause infections in the mouth, esophagus, and even the whole body in severe cases (Calderone and Clancy 2011). The increasing prevalence of drug-resistant *Candida* species has become a significant concern in clinical settings. While *Candida albicans* remains the most commonly isolated species in oral candidiasis, non-*albicans Candida* (NAC) species such as *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* are emerging as significant pathogens (Arendrup and Patterson 2017). These species often exhibit intrinsic or acquired resistance to commonly used antifungal drugs, particularly azoles (Pristov and Ghannoum 2019). The rise in resistance necessitates a deeper understanding of drug susceptibility patterns and resistance mechanisms.

Azole antifungal agents, including fluconazole, voriconazole, miconazole, and clotrimazole, are commonly prescribed for treating oral candidiasis due to their broad-spectrum activity and favorable pharmacokinetics (Odds et al. 2003). In contrast to azoles, nystatin, a polyene antifungal, exerts its fungicidal action by binding to ergosterol in the fungal cell membrane, leading to increased membrane permeability and cell death (Laniado-Laborin and Cabrales-Vargas 2009). It is widely used as a topical agent for treating oral and mucocutaneous candidiasis and is considered effective, particularly against strains resistant to azoles (Pappas et al. 2016). However, resistance to

these agents has been increasingly reported, contributing to treatment failure and recurrent infections (Silva et al. 2017). The Clinical and Laboratory Standards Institute (CLSI) provides standardized guidelines for determining the minimum inhibitory concentration (MIC) of antifungal agents, facilitating the assessment of drug efficacy against *Candida* isolates (PA 2002). MIC testing is crucial for identifying resistant strains and guiding appropriate therapeutic strategies.

One of the major mechanisms contributing to azole resistance in *Candida* species is the overexpression of efflux pumps (Homes et al. 2016). These membrane transport proteins actively expel antifungal agents from fungal cells, reducing intracellular drug accumulation and diminishing drug efficacy. ATP-binding cassette (ABC) transporters such as CDR1 and CDR2, and major facilitator superfamily (MFS) transporters, play crucial roles in mediating azole resistance (Morschhauser 2016). Studies have shown that efflux pump activity significantly contributes to fluconazole resistance, making it imperative to evaluate the role of efflux mechanisms in clinical isolates (Sanglard et al. 2009). Drug resistance in *Candida* species is often associated with the upregulation of specific genes involved in ergosterol biosynthesis and drug efflux (Liu et al. 2015). *ERG11*, which encodes lanosterol 14α-demethylase, is a key target of azoles, and its overexpression or mutation leads to reduced drug susceptibility (Flowers et al. 2015). Additionally, *TAC1* (Transcriptional Activator of *CDR* genes) is a transcription factor that regulates the expression of efflux pump genes *CDR1* and *CDR2*, contributing to azole resistance (Coste et al. 2004). Gene expression studies using real-time quantitative PCR (RT-qPCR) provide valuable insights into the molecular basis of antifungal resistance, facilitating the development of targeted therapeutic approaches (Paul et al. 2020).

The study mainly focused on the role of efflux transporters and how their expression pattern changes when antifungal drugs are used. Another purpose of the study was to investigate today's prescribed drug efficacy by observing the inhibition zone and determining the lowest concentration of antifungal medication to suppress the fungal growth.

Methodology

Isolation and identification of *Candida krusei*

A total of 148 *Candida* isolates causing oral candidiasis, ten *Candida krusei* (CBS 573) clinical isolates were included in the present study, speciated by chromogenic agar medium (HiCrome Candida Differential Agar), as described in our previous studies (Haque 2022). The isolates were identified by PCR sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (White et al. 1990). The isolates were grown in yeast extract peptone dextrose (YEPD) broth (HiMedia, Mumbai, India) by incubating at 37°C for 24-48 hours in an incubator shaker at 150 rpm. Freshly grown fungal cells were used for subsequent experiments. Cells were counted using a hemocytometer.

Antifungal susceptibility testing (AFST)

Susceptibility of the isolates to antifungal drugs was carried out by the agar disk diffusion method (Rex 2009) and the broth microdilution method (PA 2002), based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI) guidelines. As a part of quality control, some reference strains of *Candida krusei* ATCC 6258 were used. Himedia Laboratories Pvt. Limited, Mumbai, India, was the institution from which all the culture media, antifungal disks and antifungal powder were attained.

Efflux pump activity

To assess the ABC-type drug transporter activity of fungal cells, we evaluated the glucose-induced efflux of rhodamine 6G (R6G) (Sigma-Aldrich, USA) assay previously described (Maesaki et al. 1999, Ben-Ami et al. 2017, Gbelska et al. 2017). We measured the R6G fluorescence of the samples at laboratory temperature using the fluorometer (the excitation wavelength of 515 nm; emission wavelength of 555 nm).

RNA extraction

The expression of efflux transporter genes known to be associated with azole resistance in *Candida* spp. was assessed by real-time reverse transcription-quantitative PCR (RT-qPCR) as previously described (Sanguinetti et al. 2005). After initial culture conditions, cells were enumerated in a hemocytometer and transferred into fresh YEPD with antifungal agents (concentrations of 16 µg/ml) or into drug-free YEPD at a final concentration of 10^7/ml. Yeast suspensions were incubated for 3 h at 37°C with shaking; this stage did not affect *Candida* spp. viability, as determined by quantitative subculture on Sabouraud's agar. Next, cells were pelleted, snap-frozen in liquid nitrogen, and thoroughly ground with 3-mm glass beads. RNA lysis buffer was added, and the lysate was further homogenized in a homogenizer (Homogenizer with Controller; DLAB; D-160; US). RNA was extracted with the SV Total RNA Isolation System kit (Promega, USA) according to the manufacturer's instructions. The quantity of isolated RNA was measured by using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). The isolated RNA (2 µg) was then converted into cDNA using TIANscript M-MLV reverse transcriptase.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

The synthesized cDNA products were subjected to real-time PCR to estimate the expression of the *CDR1*, *CDR2*, *TAC1*, and *ERG11* genes by isolates during FLC, VRC, MIC, CC or NS exposure (after 3 h of exposure) using specific primers (Table 1). Each reaction (10µL) was performed in triplicate, comprising GoTaq® qPCR Master Mix (2x) (Promega, USA) and using a real-time PCR machine (Eco™ Real-Time PCR System, Illumine®, USA). The $2^{-\Delta\Delta CT}$ method was used for the relative quantification of gene expression (Livak and Schmittgen 2001), and the data were normalized to the *ACT1* gene expression.

Table 1: List of primers in real-time PCR.

Gene Symbol	Gene Name	Primer	Sequence	Ta (°C)
ACT1	β-actin	Forward Reverse	5′-T CGTCGGTAGACCAAGACACC-3′ 5′-CCCAGTTGGAGACAATACCGT-3′	60
CDR1	Candida Drug Resistance 1	Forward Reverse	5′-GCGTTTGACCATCGGAGTT -3′ 5′-TACCGCTGTTTGCGAATCT -3′	59
CDR2	Candida Drug Resistance 2	Forward Reverse	5′-TAGTCCATTCAACGGCAACATT -3′ 5′-CACCCAGTATTTGGCATTGAAA -3′	60
TAC1	Transcriptional activator of CDR genes	Forward Reverse	5′-TGGCAATGTATTTAGCAGATGAGG-3′ 5′-TGCTTGAACTGAGGTGAATTTTG -3′	60
ERG11	Lanosterol 14-alpha demethylase	Forward Reverse	5′-CTACTCCCAAAAAAAACCATA -3′ 5′-TAAACCTAATCCCAAGACATC -3′	60

Statistical analysis

The mean ± standard deviation (SD) of three independent biological replicates was used to express the data. All the graphs and analyses of the data were performed using Microsoft Excel 2013.

Results

Antifungal susceptibility testing

Antifungal susceptibility was assessed using the agar disk diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI) in document M44-A2 (Rex 2009) against five antifungal agents. The results demonstrated that fluconazole (FLC), voriconazole (VRC), miconazole (MIC), and clotrimazole (CC) exhibited strong antifungal activity against *C. krusei,* whereas nystatin (NS) showed no inhibitory effect (Fig. 1A). To find out

the MIC of five antifungal drugs, we used the broth microdilution. The MICs of the isolates were 1-2 µg/ml against FLC, 0.25-0.5 µg/ml against VRC, 0.5-1 µg/ml against MIC, 2-4 µg/ml against CC and >8 µg/ml against NS (Fig. 1B). The Fig. 1(A) shows the zone of inhibition (mm) for five antifungal agents— fluconazole (FLC), voriconazole (VRC), miconazole (MIC), clotrimazole (CC), and nystatin (NS)— following CLSI guidelines (document M44-A2) and demonstrated that FLC, VRC, MIC and CC exhibited the highest antifungal efficacy, while NS had no response. Fig. 1(B) shows that FLC (MIC ≤2 µg/ml), VRC (MIC ≤0.25 µg/ml), MIC (MIC ≤0.5 µg/ml) and CC (MIC ≤4 µg/ml) were the only antifungal drugs that were highly effective against all isolates. Notably, all of the fungal isolates had MIC values of at least 8 µg/ml for nystatin, which means they were completely resistant to this drug. Among the tested antifungal agents, FLC and VRC exhibited potent antifungal activity against all the isolates studied. The zone of inhibition (mm) and minimum inhibitory concentration (MIC, µg/ml) range are shown in Table 2.

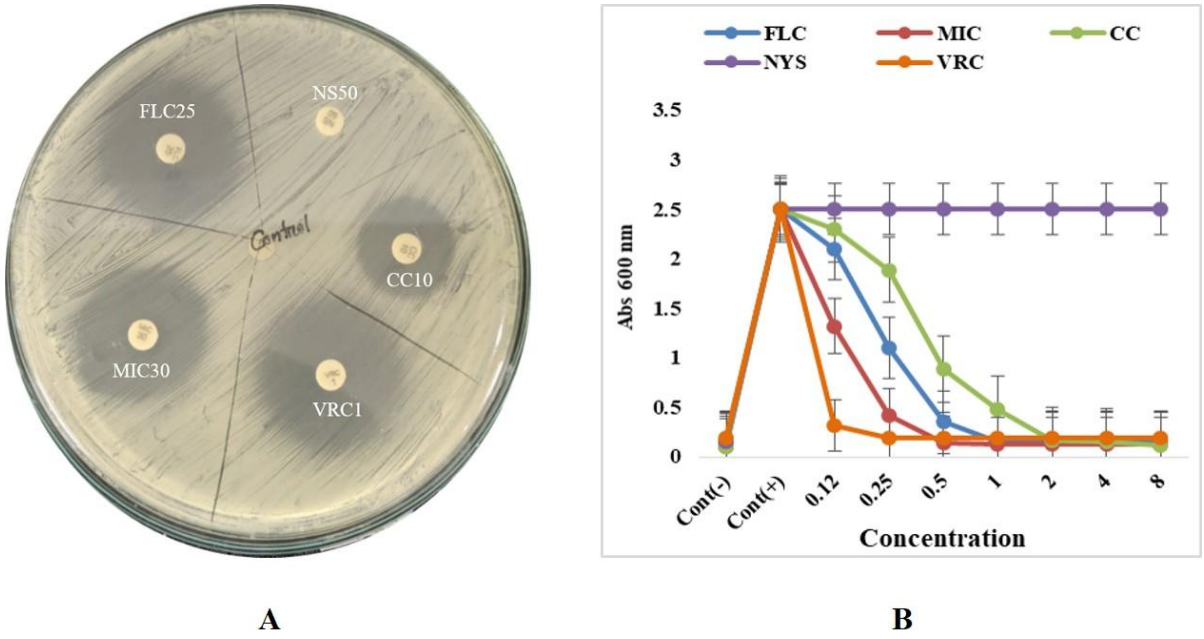

Fig. 1: Antifungal susceptibility profiles of *Candida krusei* (CBS 573). A) The agar disk diffusion method, and B) The broth microdilution method.

Table 2: Antifungal susceptibility profile of clinical isolates against five antifungal agents after 24 hours.

Antifungal agent	Zone of inhibition (mm) by agar disk diffusion	Minimum inhibitory concentration (MIC, µg/mL) by broth microdilution
Fluconazole (FLC)	29	1-2
Voriconazole (VRC)	33	0.25-0.5
Miconazole (MIC)	27	0.5-1
Clotrimazole (CC)	21	2-4
Nystatin (NS)	0	>8

Assessment of glucose-dependent efflux activity of ABC transporters

The glucose-dependent efflux activity of ATP-binding cassette (ABC) transporters in representative *Candida* isolates was evaluated using the rhodamine 6G (R6G) efflux assay, as previously described (Maesaki et al. 1999). As characteristic of ABC transporters, R6G efflux was assessed by measuring the relative fluorescence intensity in the supernatant, which reflects transporter-mediated extrusion activity. Initially, we measured baseline R6G efflux to assess intrinsic efflux pump activity in the isolates. Subsequently, the impact of various antifungal

agents- fluconazole (FLC), voriconazole (VRC), miconazole (MIC), clotrimazole (CC), and nystatin (NS)- on glucose-induced efflux was examined. After the addition of glucose (2 mM) to the cell suspension, R6G efflux was quantified by monitoring fluorescence at specific time intervals (0, 10, 20, 30 minutes, etc.).

Our results demonstrated that nystatin induced a higher level of glucose-stimulated R6G efflux compared to FLC, VRC, MIC, and CC (Fig. 2). These findings indicate differential modulation of ABC transporter activity by antifungal agents, potentially contributing to variations in drug resistance mechanisms.

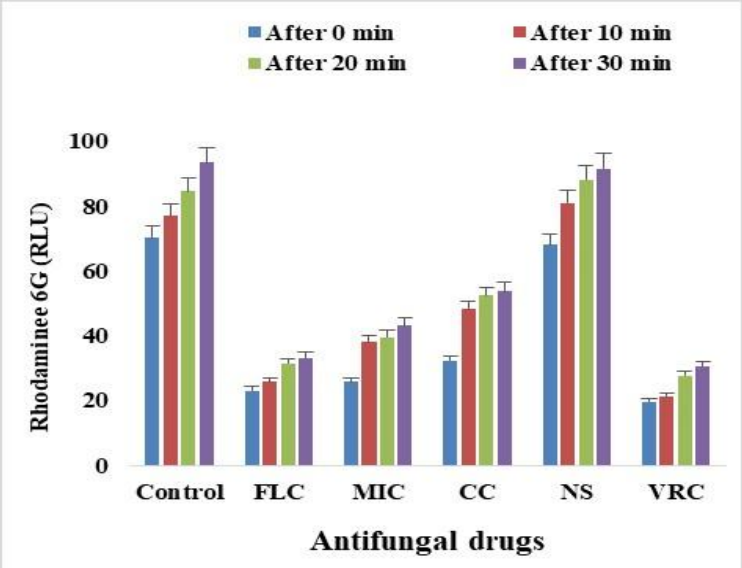

Fig. 2: Drug efflux activity in isolates.

Expression analysis of target genes

Since it seems that efflux pump activities are constitutively present in the isolates, we next examined the drug-induced expression of four genes: ergosterol synthesis genes (*ERG11*), drug efflux transporter genes (*CDR1, CDR2*), and transcriptional activator of *CDR* genes (*TAC1*). A pre-treatment was conducted 3 hours earlier with fluconazole, voriconazole, miconazole, clotrimazole, and nystatin before the RNA extraction as described in the protocol. The expressions of the genes were determined by RT-qPCR. The $2^{-\Delta\Delta CT}$ method was used for the relative quantification of gene expression. The β-actin gene was used as a housekeeping gene or as an internal control. The expression levels of *CDR1, CDR2, TAC1,* and *ERG11* genes were found to be modulated in response to antifungal treatment. Notably, the average inducible expression of these genes was markedly elevated in the isolates treated with nystatin; conversely, treatment with fluconazole, miconazole, voriconazole, and clotrimazole led to reduced expression of *CDR1, CDR2, TAC1*, and *ERG11,* except *CDR1*, which showed increased expression under voriconazole treatment (Fig. 3). These findings suggest that gene expression profiles vary with the specific antifungal agent used, highlighting the complexity of resistance mechanisms among *C. krusei*. The results were expressed as the mean ± standard deviation of three independent experiments.

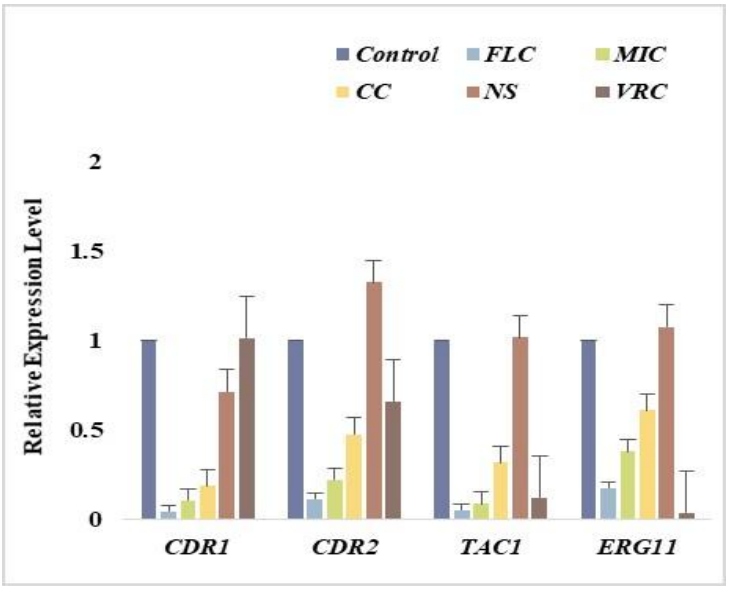

Fig. 3: Inducible expression of the *CDR1, CDR2, TAC1,* and *ERG11* genes in isolates.

Discussion

Oral candidiasis is a major infection caused by *Candida*, with *Candida krusei* being the most prevalent non-*albicans Candida* species. Antifungal drugs, primarily azoles and polyenes, are used to treat this fungal infection. However, over time, these drugs have developed resistance, leading to a decrease in their susceptibility. The emergence of antifungal resistance among *Candida* isolates has added a layer of complexity to the management of these infections, highlighting the need for a deeper understanding of the underlying mechanisms. The point of this study was to find out everything about antifungal susceptibility, drug efflux mechanisms, and gene expression patterns linked to resistance using a wide range of clinically important *Candida* species.

The study used HiCrome Candida differential media to identify *Candida* isolates quickly. The assessment of antifungal susceptibility revealed notable variations in the response of different *Candida* species to commonly used antifungal agents. Fluconazole and voriconazole demonstrated potent antifungal activity against the majority of isolates, corroborating previous reports (Greer 2003, Mandras et al. 2009). In contrast, nystatin has low efficacy in the treatment (Lyu et al. 2016). These findings underscore the dynamic nature of antifungal susceptibility and emphasize the need for ongoing surveillance to inform clinical decision-making (Pappas et al. 2016).

Intriguingly, the study delved into the role of ABC-type drug transporters, crucial contributors to antifungal resistance. Glucose-induced Rhodamine 6G (R6G) efflux showed different patterns in each isolate against different treatments, which suggests that the transporter activity was different. These findings align with previous studies that have highlighted the significance of drug transporters in mediating resistance (Sanglard et al. 2009, Paul and Moye-Rowley 2014). This study used five drugs to observe the efflux pump activity. We compared their efflux pump effects with a control group that received no drug treatment. The results showed that fluconazole and voriconazole were effective at stopping R6G from leaving the cell and making it easier for it to gather inside. However, nystatin did not demonstrate any efflux control potential, indicating its inferior effectiveness. The observed differences in efflux activity between antifungal agents point to a possible interaction between how well a drug works and how well a transporter works.

Gene expression analysis provided valuable insights into the molecular mechanisms underlying antifungal resistance. Different antifungal treatments have different effects on the expression of these genes linked to resistance, like *CDR1, CDR2, TAC1* and *ERG11*. This demonstrates how biological reactions to drug exposure are always evolving. Two extensively researched ABC transporters, Cdr1p and Cdr2p, are essential for the development of azole resistance (Czajka et al. 2023). RT-qPCR analysis revealed differential gene expression responses to antifungal agents across all tested isolates. Treatment with fluconazole and voriconazole generally led to a

downregulation of the *CDR1, CDR2, TAC1*, and *ERG11* genes in most isolates. However, an exception was observed in the case of voriconazole, which induced a consistently high expression of *CDR1* across all isolates. In contrast, exposure to nystatin resulted in upregulation of all four genes (*CDR1, CDR2, TAC1*, and *ERG11*) in most isolates tested. The elevated or unaltered expression of genes associated with ATP-binding cassette (ABC) transporters, particularly *CDR1* and *CDR2*, suggests a possible mechanism of reduced susceptibility or resistance to these antifungal agents, as previously indicated by studies linking overexpression of efflux pump genes with decreased drug efficacy (Prasad and Rawal 2014). One study by Lyons and White found a connection between more mRNA in the *CDR* efflux pump gene family and increased azole resistance (Lyons and White 2000). Increased ABC transporter genes, *CDR1* and *CDR2* expression occurs when the transcription factor *TAC1* becomes hyperactive (Znaidi et al. 2007). *ERG11* encodes the lanosterol 14α-demethylase enzyme, a key target of azole antifungals. Overexpression of *ERG11* is a well-documented resistance mechanism in *Candida* species, leading to reduced azole susceptibility (Urbanek et al. 2022, Siqueira et al. 2025). The significant upregulation of *ERG11* in this study suggests that target site alterations may also contribute to the observed resistance. This shows that the isolates may be able to adapt and become resistant. These results are in line with earlier research that showed how these genes contribute to drug resistance. They also demonstrate the complexity of the molecular landscape that governs resistance mechanisms (Tavakoli et al. 2010, Prasad and Rawal 2014).

This study demonstrates how to target the molecular mechanism to inhibit *Candida* growth and facilitate their elimination with a more effective medication. The findings from this study have significant clinical implications. Understanding the interplay between antifungal susceptibility, drug transporters, and gene expression patterns can guide the development of targeted therapeutic strategies. Finding that susceptibility varies among species and that resistance mechanisms like drug transporters and differential gene expression are present makes it even more important to use individualized treatment plans. These insights could inform the selection of appropriate antifungal agents, dosages, and treatment regimens, optimizing patient outcomes and potentially mitigating the development of further resistance.

Conclusion

This study provides a comprehensive perspective on antifungal resistance mechanisms in clinically relevant *Candida* species causing oral candidiasis. This study helps us understand the problems caused by antifungal resistance by revealing the complex relationships between gene expression patterns, drug efflux, and antifungal susceptibility. This study also paves the way for the development of more effective therapeutic interventions. Continued research in this field is essential to stay ahead of the evolving landscape of antifungal resistance and to improve patient care in the face of oral candidiasis.

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Author contributions: AH and PH designed, supervised the study and finalized the draft. All the experiments were carried out by MNH, IS, DR and SBZ. DR and IS performed the analysis, interpretation of the data and drafted the manuscript. All authors agreed to the final manuscript. Both MNH and IS contributed equally as first authors.

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