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Research article

Comparative Analysis of *CDR1* Gene Deletion Effects on Fluconazole Susceptibility in *Candida glabrata* and *Candida albicans*

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Abstract

Background: *CDR1* is an important component of ATP-binding cassette (ABC) transporters, functioning as an efflux pump that enables different *Candida* species to develop resistance to the antifungal drug fluconazole. **Aim:** To investigate the effect of *CDR1* gene deletion on fluconazole sensitivity in *Candida glabrata* and *Candida albicans*. **Methods:** Antifungal resistance was assessed using gene knockout assays. Broth microdilution techniques were applied, and IC_{50} values were calculated to evaluate changes in drug susceptibility. **Results:** *CDR1* deletion mutants in both species exhibited markedly increased sensitivity to fluconazole. However, resistance patterns differed between the two species. For *C. glabrata*, MIC decreased from >200 mg/L to 1.5–6 mg/L and IC_{50} reduced to ~2.5 mg/L. For *C. albicans*, MIC decreased from >200 mg/L to 0.5–1 mg/L and IC_{50} reduced to ~0.8 mg/L. Overall, susceptibility increased by more than 80-fold in *C. glabrata* and over 100-fold in *C. albicans*. **Conclusion:** CDR1 acts as a major determinant of fluconazole resistance in both *C. glabrata* and *C. albicans*. Its role varies between species, highlighting the importance of considering CDR1 as a key regulatory mechanism and a target for species-specific antifungal therapy.

Keywords: *Candida glabrata, Candida albicans, CDR1 gene, antifungal resistance, fluconazole, gene knockout*

Introduction

Candida species are the conventional fungal pathogens responsible for opportunistic infection globally in people with weakened immune systems (e.g., HIV/AIDS and organ transplants as well as chemotherapy treatment) (Pfaller and Diekema, 2007; Pappas *et al*., 2018). The invasive *Candida* species of *Candida albicans* and *Candida glabrata* are the two leading pathogens causing invasive candidiasis, and they lead to serious health complications and death as they complicate antifungal therapies (Whaley *et al*., 2017). Azole antifungals used by medical professionals are considered effective because of their beneficial pharmacokinetic properties and minimal toxic effects of fluconazole (Whaley *et al*., 2017). Emerging resistant strains bring dire clinical difficulties, as they reduce the efficacy of treatments relying on azole (Brun *et al*., 2004).

The principal mechanism of azole resistance functions through efflux pumps, which extrude the antifungals from fungal cells and decrease its internal level (Prasad and Kapoor, 2005, Prasad and Rawal, 2014). The *CDR1* gene (*Candida* Drug Resistance 1) encoding an ATP-binding cassette (ABC) transporter system is capable of delivering fluconazole from fungal cells (Gulshan and Moye-Rowley, 2007; Prasad *et al*., 2019). Although both species *C. albicans* and *C. glabrata* harbor the *CDR1* gene, their efflux systems demonstrate markedly different regulatory networks' function and operating redundancy. CDR1 functions as the principal mechanism allowing *C. albicans* to create resistance against antifungal treatments. *C. glabrata* has *CDR1*, as an interplay element in a vast system, which has many efflux pumps regulated by the Pdr1 transcription factor in tandem with various PDH1 and SNQ2 systems (Cannon *et al*., 2009; Miyazaki *et al*., 2010; Thomas *et al*., 2013; Vale-Silva and Sanglard, 2015; Czajka *et al*., 2023). Differences between these species suggest that *CDR1* functions differently between these species as two species carry the same gene (Brunke and Hube, 2013).

Previous studies have shown that deletion of *CDR1* leads to a major increase in fluconazole susceptibility (Egner *et al*., 1998; Katsipoulaki *et al*., 2024). Studies to only a limited extent have assessed direct comparisons of *CDR1* deletion effects on *C. albicans* and *C. glabrata* via identical testing methods. However, the total impact of compensation by *C. glabrata* upon *CDR1* deletion phenotype was not well established (Holmes *et al*., 2006; Miyazaki *et al*., 2010). Therefore, the present study was aimed to determine the role of *CDR1* deletion in the resistance to fluconazole in *C. albicans* and *C. glabrata*

by testing functional effects of *CDR1* deletion. Gene knockout mutants for susceptibility testing were performed with standard assays (broth microdilution and IC_{50} estimation) through test methodologies. This study aimed also to examine the intercommunication between *CDR1* genes in both species and their evolutionary correlates to prove the different functional roles according to function. The investigators followed this integrated approach to develop a clearer understanding of the mechanisms by which *CDR1* performs differently for various species, a critical factor in formulating custom antifungal therapies.

Materials and methods

Strains and culture conditions

We compared wild-type parental strains using reference strains of *C. albicans*, the reference strain SC5314, and *C. glabrata*, the reference strain ATCC 2001. Table 1 summarizes the plasmids from *S. cerevisiae* to amplify the *URA3* gene used in this study. The laboratory cultured the strains on YPD agar plates having 1% yeast extract, 2% peptone, 2% glucose, and 2% agar, at a temperature of 30°C. YPD broth was chosen as growth medium for cells grown at 30°C on shaking at 200 rpm.

Amplification of the target gene and URA3 selectable marker by PCR for transformation

The laboratory generated gene disruptions via PCR product-based techniques via the Promoter-Dependent Disruption of Genes (PRODIGE), demanding 80-nucleotide primers for appropriate *URA3* genomic amplification and gene deletion products (Table 2). The primer sequences were designed to allow scientists to homologously recombine *C. glabrata* and *C. albicans* coding sequences (CDS) by adding selectable marker CDS. To this end, each primer was characterized by a homologous to *C. glabrata* (60 nucleotides, 5′ position), and *C. albicans* fragments located along the target CDS, and a ~20-nucleotide, 3′ position, complementary to the *S. cerevisiae URA3* sequence. The PCR reactions were performed with 25µl total volume, 12.5µl of Hot Start Taq 2X Master Mix, 1 µl of diluted plasmid or PCR product (estimated concentration 2.0–2.2 ng/µl), 0.5µl of forward primer (10nmol), 0.5 µl reverse primer (10nmol), and nuclease-free water. PCR amplification was carried out by the thermocycler with 35 cycles as operation method.

Transformation and genotype analysis

The LiAc protocol was used to create enough transformants from one experimental run. The *URA3*$^+$ transformants of *C. glabrata* and *C. albicans* underwent colony PCR testing to verify correct *URA3* integration through one oligonucleotide upstream of the target sequence and another complementary to *URA3* (Table 3).

Susceptibility assays

- Broth microdilution

After overnight incubation, according to CLSI guidelines, a minimum inhibitory concentration (MIC) is the lowest antimicrobial concentration that prevents visible growth (Standard, 2017). Fresh overnight cultures of single colonies were diluted (1:100) in liquid YPD medium and incubated for roughly 3–4 hours to determine MIC values. 100µL aliquots were transferred into a 96-well flat-bottom plate. Row A was the highest concentration of the test compounds (200mg/L fluconazole), which were serially diluted through rows B-G and row H was used as drug-free control. Wells were inoculated with wild-type *C. glabrata*, wild-type *C. albicans*, or mutant strains (*CDR1* Δ) and incubated overnight at 30°C, while growth was measured by OD600 using a POLAR star Omega Microplate Reader with un-inoculated medium and drug-free wells acting as 100% growth controls for background measurement. All the assays were performed in triplicate while repeating each assay at least three times independently. The MIC values were obtained from the growth inhibition curves.

- Calculation of half-maximal inhibitory concentration (IC_{50})

From our dose-response curve testing, we were able to quantify the fluconazole half-maximal inhibitory concentration (IC_{50}) of bacterial strains which we had isolated. We assessed bacterial growth with optical density at 600nm (OD600) after they tested different fluconazole concentrations from 0.1 to 200mg/L. IC_{50} calculations were performed in GraphPad Prism software version 8.0.2 by using four-parameter logistic regression modeling.

Statistical analysis

POLAR Star Omega Microplate Reader was used to measure the optical density at 600nm to conduct cell growth analysis and to calculate their growth estimation. The data were analyzed with GraphPad Prism 8 and sample effect of compound concentrations in multiple conditions compared (version 8.0.2; GraphPad Software, San Diego, CA, USA). IC_{50} numbers, representing antifungal agent concentration for 50% inhibition of fungal growth, were computable using GraphPad Prism 8 (version 8.0.2; GraphPad Software, San Diego, CA, USA) software. Fluconazole susceptibility test was used to assess *C. glabrata* wild types and *C. albicans* wild types and mutant strains (*CDR1*Δ) as determined from IC_{50} value changes.

Ethical approval

This study did not involve any human subjects or animal experiments. Instead, we utilized wild-type parental strains *C. albicans* (SC5314) and *C. glabrata* (ATCC 2001) to investigate the *CDR1* gene deletion effects on fluconazole susceptibility in *Candida glabrata* and *Candida albicans*.

Results

Bioinformatic analysis of transporter genes in C. albicans and C. glabrata

Multidrug resistance of the two main classes of efflux pumps in fungi is mediated by their two active pump systems and involve the ATP-binding cassette (ABC) transporters *CDR* family and the major facilitator superfamily (MFS) members. Antifungal agents and toxins are actively transported out of the cell by these pumps located on all cytoplasmic membrane locations (17). The *CDR1* gene, the most studied genetic element of *Candida* species, encodes an ABC transporter that controls azole resistance development in *Candida* species. All ABC transporter-encoding genes were

Table 1. Strains and plasmids used in this study.

Strain/plasmid	Source/reference
C. glabrata CBS138 (or ATCC2001)	American Type Culture Collection
C. glabrata MH100 ATCC2001 ura3Δ	Muller *et al.*, 2008
C. albicans SC5314(or ATCC MYA-2876	NCBI:txid237561
C. albicans SC5314(or ATCC MYA-2876 ura3Δ	American Type Culture Collection
pYEURA3	Prasad *et al.*, 1995
p416GPD	Mumberg *et al.*, 1995
pYHSRed1	Luu and Macreadie, 2018

Table 2. Primers are used for PCR (Primer sequences).

Primer type	Sequence	Tm (°C)	Size (nt)
CDR1-URA3 F *C. glabrate*	5`-TACTTACAGGAAAAAGAATTTACAACTCTTGATAT ATACAAAGTAGAAAAGTAACAATGTCGA AAGCTACATATAAGG-3`	68	80
CDR1-URA3 R *C. glabrate*	5`-TTTTCCGAATGCATTGTATTAATACAGAGCCAGA TTATGAGCGCAGGCTAAATAAATTAGTTTTGCTGG CCGCATC-3`	72	79
CaAlb_ CDR1 F	5`- TATGTCAGATTCTAAGATGTCGTCGCAAGATGAA TCTAAATTAGAAAAGGCAATTAGTCAATGTCGAAAGCT ACATATAAGG-3`	72	80
*CaAlb_ CDR1*_R	5`- TATTTCTTATTTTTTTTCTCTCTGTTACCCTTT GGAACTCTAGCTAACCAATAAATTTTGCTGGCCGCATCAGT-3`′	72	80

Table 3. Primers used for Genomic PCR.

Primer Type	Sequence	Annealing Temperature (°C)	Size (nt)
*URA3*iR	5′-AGCAACAGGACTAGGATGAG-3′	59	21
Cg CDR1 UF.	5`-GCAGCTATGAGTTGAGGAAG-3`	52	20
*CaAlb_CDR1*_UF	5`-ATGACAGCTTGGAGTACGGA-3`	53	20

determined by genomic studies of *Saccharomyces cerevisiae* and *Candida albicans*, as well as a phylogenetic analysis that included the gene *CDR1*. Importantly, *C. glabrata* is more closely related phylogenetically to *S. cerevisiae* than to *C. albicans*, and this gives researchers the ability to perform functional studies on its multidrug resistance mechanisms linked to its *CDR1*-based pathways (Figure 1).

Homologues information of CDR1 between C. Albicans and C. glabrata

The examination of MDR-related ATP-binding cassette (ABC) and major facilitator superfamily (MFS) membrane transporter types of *C. glabrata* revealed that this organism, a close relative of *S. cerevisiae*, has a larger number of these transporters than any other *Candida* species genome. The study used phylogenetic analysis to show that *C. glabrata CDR1* exhibited a closer relationship with S. cerevisiae PDR5/YOR153W and C. albicans *CDR1* (C3_05220W) than with *C. glabrata* CDR2/PDH1 (CAGL0F02717g) according to his analysis of Jalview tools (Figure 2A). Clustal Omega tools indicated that *C. glabrata CDR2/PDH1* was more closely related to *C. glabrata CDR1* than other *C. glabrata* genes (Figure 2B).

In addition, by employing homolog data from the Candida Genome Database (CGD), they compared genetic data describing the sequence similarity of *CDR1* across species. The sequences of *C. glabrata CDR1p* present 74.06% similarity with *S. cerevisiae PDR5* and *C. glabrata CDR2/PDH1* demonstrates 73.35% similarity with *S. cerevisiae. C. glabrata CDR1* and *C. albicans CDR1* show 55% sequence identity, indicating that they share antifungal drug resistance functions but their evolutionary relationship is more distant.

The technology of gene targeting (Gene knockout)

In the past ten years, researchers improved gene targeting techniques and gene replacement methods. These emerging approaches provide researchers powerful tools to study multiple genes which have increasing applications in biotechnology. The PRODIGE (promoter-dependent disruption of genes) method enables scientists to achieve precise gene disruption results for *C. glabrata* and *C. albicans* while showing potential for application to other species (Edlind *et al.*, 2005; Vermitsky *et al.*, 2006; Culakova *et al.*, 2015).

After the PRODIGE technique was performed, the *CDR1* gene was removed from *C. albicans* and *C. glabrata* (as shown in figures 3 and 4). Primers were synthesized that enable the replacement of the coding sequence of the target gene through homologous recombination with the selectable *URA3* marker. The method has more specific disruption capability than standard techniques because the marker gene expression occurs mainly in cells that have completed successful recombination.

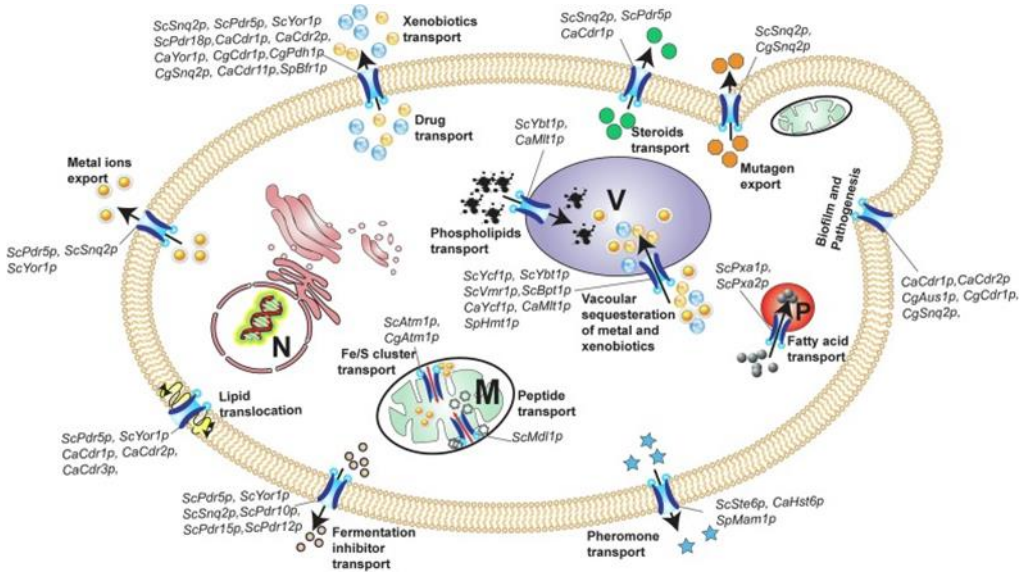

Figure 1. Subcellular localization of ABC proteins in *C. glabrata*, *C. albicans* and *S. cerevisiae* were predicted by LocTree3 and WoLF PSORT. Only membrane proteins are depicted. Obtained from Kumari *et al*., 2021.

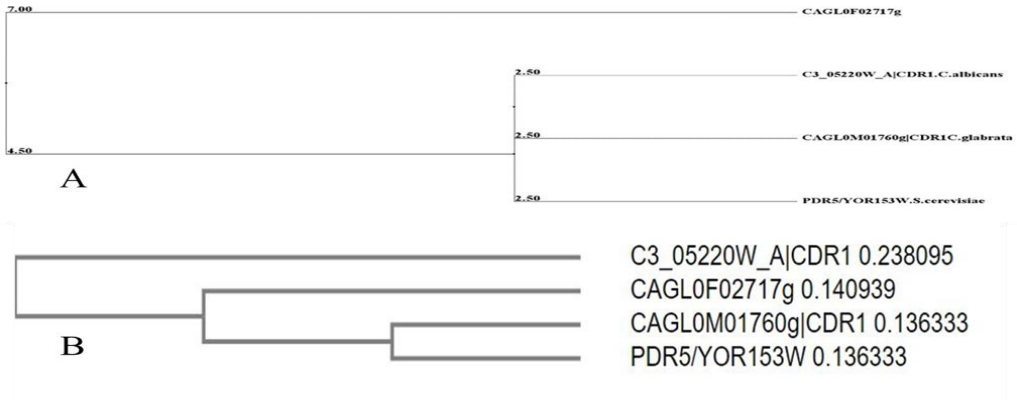

Figure 2. The phylogenetic tree for *C3_05220W CDR1 C. albicans*, *PDR5/YOR153W S. cerevisiae*, *AGL0M01760g CDR1 C. glabrata*, and *CAGL0F02717g CDR2/PDH1 C. glabrata* analyzed by Jalview tools (A) and Clustal Omega tools (B).

The results of plasmid polymerase chain reaction (PCR) testing show a band at the expected size of 924 base pairs confirming *URA3* marker amplification according to the data shown in figure 3. Results obtained by genomic PCR from transformed colonies show bands of the calculated sizes for gene knockout (478bp for *C. glabrata* and 455bp for *C. albicans*), demonstrating that we successfully deleted the *CDR1* gene from both species as indicated in their testing results shown in figure 4.

Susceptibility of deletion mutants to fluconazole

- Impact of deletion of CDR1 on fluconazole susceptibility

Figure 5 shows that the deletion of the *CDR1* gene in *C. glabrata* and *C. albicans* increased fluconazole sensitivity. The wild-type strain of *C. glabrata* demonstrated strong resistance with an MIC greater than 60mg/L, but the deletion of *CDR1* led to a dramatic MIC reduction which now ranged between 3-6mg/L, showing a substantial increase in drug susceptibility. Subsequent resistance was acquired through the co-expression of the ABC transporters, *PDH1* and *SNQ2*, which exert transcriptional control in collaboration with the *Pdr1* transcription factor. *C. glabrata*-ABC transporters make use of this regulatory pathway in order to maintain some of the fluconazole efflux capability after the deletion of *CDR1* protein, confirming previous findings regarding several other pathways for drug efflux (Vermitsky and Edlind, 2004; Torelli *et al.*, 2008; Miyazaki *et al.*, 2010; Thomas *et al.*, 2013; Paul *et al.*, 2014).

Deletion of *CDR1* in *C. albicans* produced a stronger impact than any other genetic modification. The wild-type strain had an MIC greater than 200mg/L, while *CDR1*Δ showed an MIC range of 0.5 to 1mg/L which

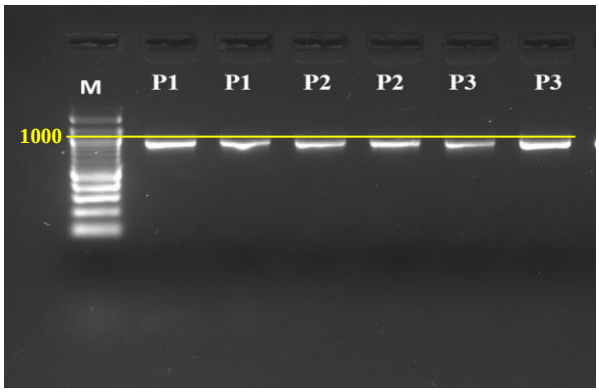

Figure 3. The gene disruption and replacement (plasmid PCR), a band of the correct size for amplification of the *URA3* marker 924bp was produced. M: Marker 1000bp, P1: p416GPD plasmid, P2: pYHSRed1 plasmid, P3: pYEURA3.

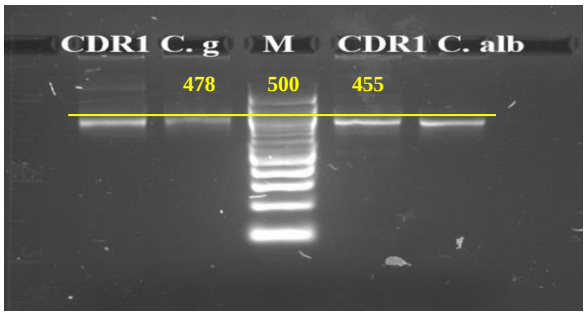

Figure 4. Genomic PCR products from transformation colonies, the correct size of the band (478bp of *C. glabrata* and 455bp of *C. albicans*) fragment that calculated for knock out of the gene. M: Marker 1000bp.

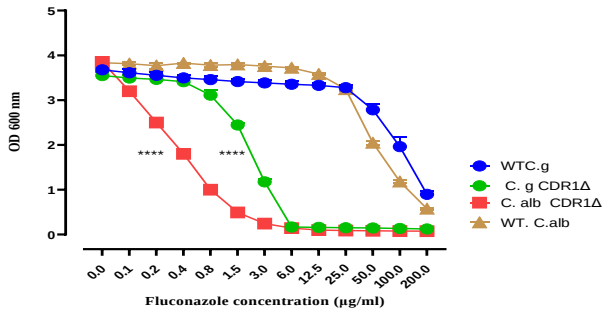

Figure 5. Growth curve (OD600 nm) of *C. glabrata* and *C. albicans* wild-type and *CDR1*Δ mutant cells treated with fluconazole. The mean of five independent replicates (one biological repeat with five technical repeats) is shown with the standard error of the mean (± SEM). Statistical significance was calculated by comparing the growth of each strain in the absence and presence of fluconazole in different concentrations. The Two-way ANOVA test (Dunnetts multiple comparison test) was used (****p<0.0001).

resulted in more than 200 times reduction. The remarkable decrease illustrates that *CDR1* is the principal efflux pathway in *C. albicans* due to the absence of most alternative efflux mechanisms in the organism (Egner *et al.*, 1998; Gulshan and Moye-Rowley, 2007; Osset-Trénor *et al.*, 2023).

- Calculation of half-maximal Inhibitory concentration (IC$_{50}$)

IC$_{50}$ values followed the MIC test results. The value of IC$_{50}$ decreased by more than 80 times for *C. glabrata* wild-type strain (IC$_{50}$ >200 mg/L) as compared to *CDR1*Δ mutant strain (IC$_{50}$ of ~2.5 mg/L), and *C. albicans* presented an even higher response to *CDR1* deletion with IC$_{50}$ decreasing from >200 mg/L in wild-type to approximately 0.8 mg/L (Table 5) resulting in more than 100x higher drug sensitivity following *CDR1* deletion.

The IC$_{50}$ values decreased because *CDR1* acted as a fluconazole resistance pathway. Nevertheless, the *C. glabrata CDR1*Δ mutant still showed partial resistance because its IC$_{50}$ value remained higher than the *C. albicans CDR1*Δ strain, which means that other efflux pumps such as PDH1 and SNQ2 that the Pdr1 transcription factor controls are compensating for lost *CDR1* function.

Discussion

The present study contains a full bioinformatics and functional analysis of ATP-binding cassette (ABC) transporters involved in the mechanism of antifungal drug resistance in *Candida albicans* and *Candida glabrata*. The two major efflux systems which enable fungi to develop multidrug resistance are the ABC transporter family and the major facilitator superfamily (MFS) that function to transport antifungal agents and toxic substances out of the cell through the plasma membrane (Del Sorbo *et al.*, 2000). The most detailed experiments have been performed on *CDR1* as the main factor which determines azole resistance.

The phylogenetic analysis also demonstrated that *C. glabrata CDR1* is more closely associated with *Saccharomyces cerevisiae PDR5* than with *C. albicans CDR1*, aligning with the confirmed evolutionary relationship of *C. glabrata* and *S. cerevisiae* (Tam *et al.*, 2015; Katsipoulaki *et al.*, 2024). Comparison of sequences revealed that *C. glabrata CDR1* was 74.06% similar to *S. cerevisiae PDR5*, while the comparison between *C. glabrata* and *C. albicans CDR1* revealed only 55% sequence identity. Although genetically different, the species maintain drug efflux function through their conserved functional domains, which preserve this ability.

However, these conserved functional domains indicate a preservation of effective drug efflux between species. Moreover, the relatively large sequence similarity between *C. glabrata CDR1* and *CDR2/PDH1* (73.35%) points out potential gene duplication and functional diversification events. Also, a significantly larger gene number of ABC transporters are also seen in *C. glabrata* which is in line with genomic analyses which have found this species to have more multidrug transporters than other *Candida* spp. (Prasad *et al.*, 2016). Such redundancy probably helps explain its intrinsic resistance to azole antifungals.

The promoter-dependent gene disruption method PRODIGE system allowed for the removal of the *CDR1*

Table 4. Antimicrobial activity: MIC and IC_{50} values.

Species	Strain Type	MIC (mg/L)	IC_{50} (mg/L)	Fold Increase in Susceptibility
C. glabrata	Wild-type	>200	>200	-
C. glabrata	*CDR1*Δ mutant	1.5–6	≈2.5	>80-fold
C. albicans	Wild-type	>200	>200	-
C. albicans	*CDR1*Δ mutant	0.5–1	≈0.8	>100-fold

gene from *C. albicans* and *C. glabrata*. The approach offers novel potential beyond previous gene targeting techniques by restricting marker gene expression to cells that have undergone successful recombination, therefore enhancing identification and operational performance (Vermitsky *et al.*, 2006; Culakova *et al.*, 2015). Plasmid PCR of *URA3* producing the expected 924bp amplification product and genomic PCR of *C. glabrata* fragments of 478bp and *C. albicans* fragments of 455bp confirm the complete disruption of the gene. The outcomes show successful homologous recombination and the PRODIGE system is a valid and reproducible method for functional genomics in fungi.

Depletion of *CDR1* caused significantly elevated fluconazole susceptibility in both species reaching $p<0.0001$ by two-way ANOVA and Dunnett's multiple comparison tests. It should be emphasized that *CDR1* in this study acts as the main mechanism by which the cells acquire resistance against azole drugs.

Using *C. glabrata* method, minimum inhibitory concentration (MIC) dropped from >200 mg/L in wild type strains to 1.5 to 6 mg/L in the *CDR1*Δ mutant (mean susceptibility 30–130 fold). Likewise we observed a drop in the IC_{50} value to about 2.5 mg/L from >200 mg/L (>80-fold reduction). In contrast, the MIC-value drop in *C. albicans* was even higher, from >200 mg/L to 0.5 to 1 mg/L (>200-fold decrease), and IC_{50} values to around 0.8 mg/L (>100-fold decrease). These findings show that *CDR1* is indeed central in *C. albicans* resistance to fluconazole, corresponding with previous studies (Egner *et al.*, 1998; Gulshan and Moye-Rowley, 2007).

C. glabrata demonstrated decreased resistance after *CDR1* gene deletion because its remaining IC_{50} value stayed above the level of *C. albicans*. The findings suggest that other resistance mechanisms must account for this mechanism to maintain the organism defensive performance. ABC transporters, mediated by *PDH1 (CDR2)* and *SNQ2* under *Pdr1* control, facilitate formation of this regulatory system whereby *C. glabrata* can carry on drug efflux without *CDR1* present (Vermitsky and Edlind, 2004; Torelli *et al.*, 2008; Miyazaki *et al.*, 2010; Thomas *et al.*, 2013; Paul *et al.*, 2014). This redundancy contrasts with *C. albicans*, where resistance appears to rely more heavily on a limited number of dominant efflux pumps, particularly *CDR1*. These findings are consistent with previous data, indicating that *C. glabrata* exerts resistance via several genes whereas *C. albicans* relies on a single gene for resistance (Garnaud *et al.*, 2015; Spettel *et al.*, 2019).

The statistical analysis showed that phenotypic differences observed between groups present strong evidence for their existence. The two-way ANOVA and Dunnett's post hoc test in this study proved that fluconazole led to significantly reduced growth in all tested concentrations. The growing validity of the research data is due to the fact that MIC values match IC_{50} values. The fundamental biological function of *CDR1* accounts for the way, in which major fold changes in susceptibility are responsible for key antifungal resistance development. The variable response exhibited by species to antifungal treatments highlights the importance of creating organism-based resistance mechanisms to identify and develop appropriate antifungal therapy.

Conclusion

In conclusion, this study demonstrates that *CDR1* is the major factor for azole resistance in *C. albicans* and *C. glabrata*. A deletion of this gene leads to an increase of fluconazole sensitivity of the organism that is statistically significant. However, different systems of efflux pump redundancy and distinct regulatory network structures lead to variable effects in the species. Furthermore, these findings suggest that antifungal therapy against *C. glabrata* infections needs to have a multiple approach that targets efflux transporters and their regulatory pathways as a result. Integrated bio-informatics assessment, genetic assessment and phenotypic assessment provide a complete panorama through which to view pathogenic fungi that evolve resistance to numerous drugs.

Author contributions

Faten F. Laswad: Study design, experimental supervision, investigation (experimental performance), data analysis, result interpretation, and manuscript drafting and revision. Salheen M. Salheen: Sample collection, investigation (experimental performance), data analysis, result interpretation, and manuscript drafting. Mohammed F. Alajamia: Study design, experimental assistance, and manuscript drafting and revision. Mohamed T. Elfouly: Manuscript revision and intellectual content review and data analysis and validation.

Conflict of interest

The authors declare no conflict of interest related to the publication of this work.

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