



The Prevalence of Persister Cells and Heteroresistance Subpopulations in *Candida albicans* Isolates from HIV-Positive Patients with Oral Candidiasis: Emerging Challenges in Treatment

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Abstract

Background: Oral candidiasis remains a major challenge in managing HIV-infected patients, particularly in tropical regions such as Ahvaz. In addition to classical resistance, nonclassical mechanisms, including persister cells and heteroresistance, may contribute to antifungal treatment failure.

Objectives: This study aimed to determine the prevalence of persister cells and heteroresistance in *Candida albicans* isolates recovered from HIV-positive patients with oral candidiasis in Ahvaz, and to evaluate their potential associations with clinical and immunological parameters.

Methods: In this cross-sectional study, 101 clinical isolates of *C. albicans* were recovered from oral samples of HIV-positive patients with oral candidiasis who were referred to the Behavioral Diseases Counseling Center in Ahvaz, southwestern Iran. After obtaining written informed consent, comprehensive medical histories, including prior antifungal use, were collected. Oral samples were cultured on CHROMagar™ *Candida*, and species identification was performed using PCR-RFLP with the *MspI* enzyme. Antifungal susceptibility testing was performed according to the clinical and Laboratory Standards Institute (CLSI) M27-A3 and M27-S4 microdilution guidelines. Persister cells were identified by exposure to high-dose amphotericin B followed by regrowth analysis. Heteroresistant cells were assessed using population analysis profiling and area under the curve (AUC) calculations, with high heteroresistance defined as AUC $\geq$ 25.76 (75th percentile). Host immunological parameters (CD4 counts and viral loads) and antifungal susceptibility profiles (minimum inhibitory concentrations (MICs) of amphotericin B and fluconazole) were documented to evaluate potential clinical correlates.

Results: Among 101 patients, persister-cell phenotypes were distributed as follows: high persister, 1% ($n = 1$); low persister, 95% ($n = 96$); and non-persister, 4% ($n = 4$). Amphotericin B MIC values were higher in the low-persister group than in the non-persister group; however, the difference was not statistically significant ($P = 0.131$). Heteroresistance profiling identified high heteroresistance (AUC $\geq$ 25.76) in 21.78% ($n = 22$), low heteroresistance in 64.35% ($n = 65$), and no heteroresistance in 13.86% ($n = 14$). CD4 counts differed significantly among the heteroresistance groups ($P = 0.014$), and Bonferroni-adjusted pairwise comparisons showed a significant difference between the non-heteroresistance and low-heteroresistance groups. Fluconazole MIC and viral load did not differ significantly among the heteroresistance groups.

Conclusions: Persister cells and heteroresistant subpopulations were prevalent among *C. albicans* isolates from HIV-positive patients with oral candidiasis. The heteroresistance phenotype was significantly associated with the CD4 count; however, this cross-sectional association should be interpreted with caution. These findings support rigorous detection and monitoring of these resistant phenotypes, as well as continued viral-load surveillance, to improve clinical management.

Keywords: Oral Candidiasis, Human Immunodeficiency Virus (HIV), Persister Cells, Heteroresistance, Amphotericin B, Fluconazole

1. Background

Fungal infections, particularly oral candidiasis caused primarily by *Candida albicans*, represent a substantial public health challenge in immunocompromised

individuals (1-3). Oral candidiasis is a common mucosal infection reported in more than 90% of HIV-infected patients, particularly during the early and advanced stages of HIV infection (4). The risk may be exacerbated in regions

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with climatic conditions, such as those in Ahvaz, that favor fungal persistence and colonization (5). HIV compromises the immune system by targeting CD4 T-helper cells and impairing infection control. Consequently, HIV-infected individuals are vulnerable to opportunistic infections, including oral candidiasis (6, 7). Although azoles, particularly fluconazole, are widely used to manage oral candidiasis in HIV-positive patients, the emergence of resistant strains has become a major clinical concern. Recent surveillance data from Iranian HIV-infected populations indicate resistance rates to azoles, including fluconazole (32%), ketoconazole (28%), and clotrimazole (14%) (8). Antifungal resistance is therefore an important concern in this population (9), particularly because heteroresistance and persister cells may contribute to treatment failure and persistent or recurrent infection (10, 11).

Persister cells are a dormant fungal subpopulation that survives antifungal treatment through metabolic inactivity and drug tolerance (12-14). They may remain viable during therapy and reactivate after treatment cessation, leading to recurrence of infection (15). This mechanism is clinically relevant in HIV-infected individuals, whose compromised immunity and frequent antifungal exposure may favor persistence and recurrent disease (16). Heteroresistance refers to the survival of a minority subpopulation during antimicrobial exposure, while most of the population remains susceptible (17, 18). In *C. albicans*, small heteroresistant subpopulations may survive azole exposure and contribute to recurrent oral candidiasis and potential treatment failure in immunocompromised patients (19). Such phenotypes may arise from genetic alterations or environmental pressures during drug exposure (11, 20) and may reduce the effectiveness of standard antifungal therapy, thereby contributing to treatment failure, relapse, and disease progression (21, 22).

2. Objectives

This study aimed to investigate the prevalence and characteristics of persister and heteroresistant cell populations in *C. albicans* isolates obtained from HIV-positive patients with oral candidiasis in Ahvaz and to assess their associations with clinical and immunological parameters. We hypothesized that these phenotypes would be associated with treatment-related measures and immune status. To our knowledge, this is the first study to report the prevalence and clinical correlates of both

persister cells and heteroresistant subpopulations of *C. albicans* in HIV-positive patients with oral candidiasis.

3. Methods

3.1. Study Design and Participants

In this cross-sectional study, 101 clinical isolates of *C. albicans* were recovered from oral samples of HIV-positive patients with oral candidiasis who were referred to the Behavioral Diseases Counseling Center in Ahvaz, southwestern Iran. Comprehensive medical records were reviewed, with emphasis on prior antifungal treatment history.

3.2. Inclusion Criteria

Individuals with symptoms suggestive of oral candidiasis were eligible, including altered taste perception, xerostomia, and inflamed lesions of the oral mucosa or tongue characterized by red crusts, oral pseudomembranes or creamy-to-white plaques, chronic hyperplastic candidiasis, and erythematous candidiasis. Following culture on CHROMagar™ *Candida*, samples with ≥ 10 colonies were considered colonized and were included (23). *C. albicans* isolates were confirmed by macroscopic and molecular identification. To investigate the target resistance mechanisms, the study specifically included amphotericin B-susceptible isolates ($\text{MIC} \leq 1 \mu\text{g/mL}$) for persister-cell analysis (12) and fluconazole-susceptible isolates ($\text{MIC} \leq 2 \mu\text{g/mL}$) for heteroresistance characterization (24).

3.3. DNA Extraction and Molecular Identification

Genomic DNA from each strain was isolated using the boiling method (4, 25). The extracted DNA was amplified using the universal fungal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (26). The resulting PCR products were analyzed using the restriction enzyme MspI (Thermo Fisher Scientific, Waltham, MA, USA) in a PCR-RFLP protocol (27, 28).

3.4. Antifungal Susceptibility Assay

Because the persister-cell and heteroresistance assays required amphotericin B (AMB)- and fluconazole (FLU)-susceptible strains, antifungal susceptibility testing was first performed on all 101 *C. albicans* isolates in accordance with Clinical and Laboratory Standards Institute

documents M27-A3 and M27-S4 (29-31). *C. albicans* ATCC 10231 was used as the reference strain for quality control (32). The final concentration ranges tested were 0.016 - 8 µg/mL for AMB and 0.016 - 32 µg/mL for FLU (33).

3.5. Persister-Cell Assay

Isolates were initially cultured on Sabouraud dextrose agar at 35°C for 24 h. Single colonies were inoculated into 1000 µL of Sabouraud dextrose broth and incubated in a shaking incubator at 30°C and 100 rpm for 24 h. The cultures were centrifuged at 3000 rpm and 4°C for 3 min, washed twice with sterile phosphate-buffered saline (PBS), and resuspended in PBS to a final concentration of 10⁶ CFU/mL. Next, 100 µL of this suspension was mixed with 100 µL of RPMI 1640 in a 96-well flat-bottom microplate and incubated at 35°C for 24 h. After incubation, the medium was removed, and 200 µL of amphotericin B (Sigma-Aldrich, USA) diluted in RPMI 1640 to 100 mg/mL was added to each well. The plates were incubated at 35°C for 48 h (12, 34, 35). The wells were then washed once with 100 µL of PBS, and an additional 100 µL of PBS was added. The well bottoms were gently scraped with a pipette tip to ensure homogeneity. A 20-µL aliquot from each well was diluted 1:10 in PBS (180 µL PBS + 20 µL sample). From this dilution, 50 µL was plated on yeast peptone dextrose agar and incubated at 35°C for 48 - 72 h. Based on colony counts, samples were classified as high persister, low persister, or non-persister (29, 35, 36). For persister isolates, the amphotericin B MIC was determined according to Clinical and Laboratory Standards Institute M27-S4 guidelines using RPMI 1640 medium at 35°C for 24 - 48 h. Persister isolates were defined as those with an MIC < 2 µg/mL (37, 38).

3.6. Population Analysis Profiling Assay

Although no standardized method exists for detecting heteroresistance, the population analysis profiling (PAP) assay is considered one of the most reliable techniques for identifying heteroresistant subpopulations in *Candida* species (39). *C. albicans* isolates were subcultured on Sabouraud dextrose agar or yeast peptone dextrose agar at 35°C for 24 h. A single colony was then inoculated into 1000 µL of Sabouraud dextrose broth and incubated at 30°C with shaking at 100 rpm for 24 h. Cells were washed twice with PBS and resuspended in PBS to a density of 10⁶ CFU/mL. Only fluconazole-susceptible isolates, as confirmed by prior susceptibility testing, were used. Based on the experimental design requiring concentrations 10

times the MIC₅₀ of fluconazole, three concentrations were tested: 32, 64, and 128 µg/mL. From the 10⁶ CFU/mL suspension, four serial 10-fold dilutions (10⁵ to 10² CFU/mL) were prepared in PBS. Then, 5 µL of each dilution was spotted onto agar plates containing each fluconazole concentration and onto drug-free control plates. All plates were incubated at 30°C and examined at 24 and 48 h (24, 30, 40). All experiments, including antifungal susceptibility testing, persister-cell assays, and PAP, were performed in duplicate to assess reproducibility.

3.7. Statistical Analysis

Statistical analyses were performed using R software (version 4.3.3) and SPSS (version 26). The normality of continuous variables was assessed using the Kolmogorov-Smirnov test, which indicated nonnormal distributions ($P < 0.05$); therefore, data are presented as medians with interquartile ranges. Comparisons among the three heteroresistance groups were performed using the Kruskal-Wallis test followed by Bonferroni-corrected pairwise comparisons for post hoc analysis. The Mann-Whitney U test was used to compare quantitative variables between the low-persister and non-persister groups. Data visualization was performed in R using ggplot2. Histograms were generated to examine the distribution of area under the curve (AUC) values, and line plots illustrated changes in mean colony counts across increasing antifungal concentrations. Associations between persister and heteroresistance phenotypes were evaluated using Fisher's exact test.

4. Results

A total of 101 *C. albicans* isolates were analyzed for persister-cell formation. Four isolates were classified as non-persisters, 96 as low persisters, and 1 as a high persister. Because the high-persister group included only 1 isolate, statistical comparisons were performed only between the non-persister and low-persister groups. The median AMB MIC was higher in the low-persister group (0.25 [0.12 - 0.25] µg/mL) than in the non-persister group (0.07 [0.03 - 0.21] µg/mL), although this difference was not statistically significant ($P = 0.131$). CD4 count ($P = 0.356$) and viral load ($P = 0.289$) also did not differ significantly between the non-persister and low-persister groups (Table 1).

Heteroresistance patterns were evaluated by recording colony counts at fluconazole concentrations of 32, 64, and

Table 1. Comparison of MIC-AMB, CD4 Count, and Viral Load Measurements Between Non-Persister and Low-Persister *Candida* Isolates From HIV-Positive Individuals^a

Variables	Non-persister (n = 4)	Low-persister (n = 96)	P-Value
Minimum inhibitory concentration of amphotericin B ($\mu\text{g/mL}$)	0.07 (0.03 - 0.21)	0.25 (0.12 - 0.25)	0.131
CD4 (cells/mm ³)	412.00 (319.75 - 667.75)	324.50 (153.75 - 534.25)	0.356
VL (copies/mL)	25.20 (0.00 - 102.60)	97.40 (0.00 - 18767.40)	0.289

^a Values are expressed as median (interquartile range). P-values were calculated using the Mann-Whitney U test.

128 $\mu\text{g/mL}$. The AUC was calculated using the trapezoidal rule across the concentration gradient. The 75th percentile ($Q_3 = 25.76$) served as the threshold. Isolates were classified as non-heteroresistant ($\text{AUC} = 0$), low heteroresistance ($0 < \text{AUC} < 25.76$), or high heteroresistance ($\text{AUC} \geq 25.76$). Cutoff points for individual drug concentrations were 0.47 at 32 $\mu\text{g/mL}$, 0.30 at 64 $\mu\text{g/mL}$, and 0.13 at 128 $\mu\text{g/mL}$.

Figure 1 presents the AUC distribution and changes in mean colony counts across increasing antifungal concentrations. Most isolates ($n = 65$) showed low heteroresistance ($0 < \text{AUC} < 25.76$), whereas 22 showed high heteroresistance ($\text{AUC} \geq 25.76$) and 14 showed no heteroresistance.

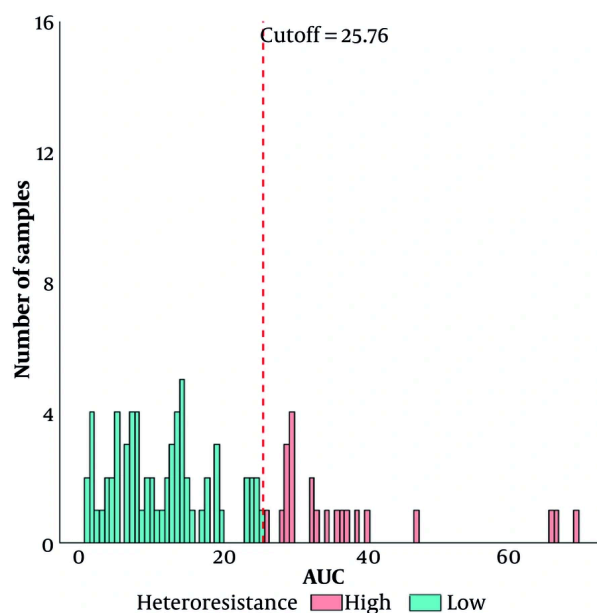
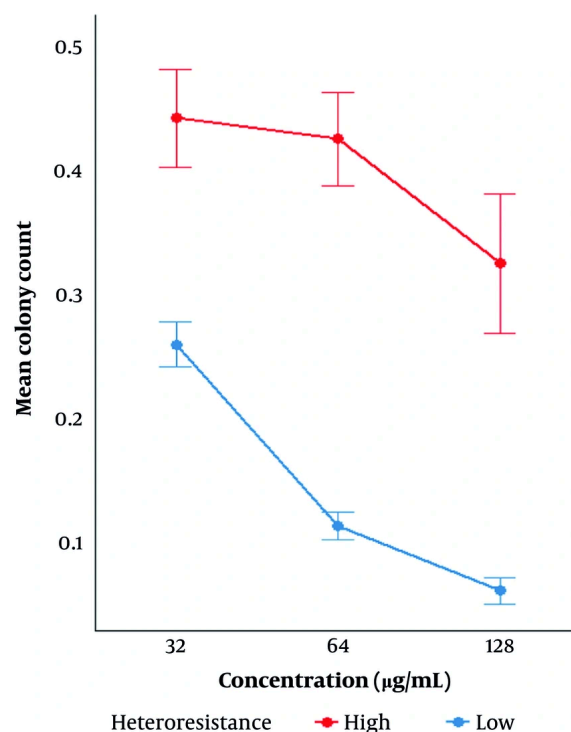
**Figure 1.** AUC Distribution of Heteroresistance in *Candida* Isolates. The red dashed line indicates the cutoff of 25.76 used to classify isolates into the low-heteroresistance group ($0 < \text{AUC} < 25.76$; $n = 65$) and high-heteroresistance group ($\text{AUC} \geq 25.76$; $n = 22$).

Figure 2 compares mean colony counts between the high- and low-heteroresistance groups across the three tested fluconazole concentrations. In both groups, colony growth declined progressively as the drug concentration increased.

**Figure 2.** Colony Counts by Heteroresistance Group and Drug Concentration. Error bars represent the mean $\pm$ standard error of the mean (SEM).

A total of 101 isolates were classified as non-heteroresistant ($n = 14$), low heteroresistance ($n = 65$), or high heteroresistance ($n = 22$). MIC-FLU ($P = 0.452$) and viral load ($P = 0.384$) did not differ significantly among the

Table 2. Distribution of MIC-FLU, CD4 Count, and Viral Load Measurements Among Non-Heteroresistant, Low-Heteroresistant, and High-Heteroresistant *Candida* Isolates From HIV-Positive Individuals ^a

Variables	Non-heteroresistance (n = 14)	Low-heteroresistance (n = 65)	High-heteroresistance (n = 22)	P-Value
Minimum inhibitory concentration of fluconazole (µg/mL)	0.25 (0.25 - 0.50)	0.25 (0.25 - 0.50)	0.25 (0.25 - 0.31)	0.452
CD4 (cells/mm ³)	534.50 (208.75 - 601.25)	786.00 (458.00 - 1094.50)	580.50 (337.50 - 970.75)	0.014 ^b
VL (copies/mL)	166.50 (0.00 - 864898.20)	50.40 (0.00 - 657.65)	318.00 (0.00 - 126375.52)	0.384

^a Values are expressed as median (interquartile range). P values were calculated using the Kruskal-Wallis test.

^b Bonferroni-adjusted comparisons showed a significant difference between the non-heteroresistance and low-heteroresistance groups.

Table 3. Distribution of Persister and Heteroresistance Phenotypes Among HIV-Positive Individuals ^a

Persister phenotype	Non-heteroresistance	Low heteroresistance	High heteroresistance	P Value
Non-persister	0.0 (0.0)	2.0 (3.1)	2.0 (9.1)	0.217
Low persister	14.0 (100.0)	63.0 (96.9)	19.0 (86.4)	
High persister	0.0 (0.0)	0.0 (0.0)	1.0 (4.5)	

^a Values are expressed as No. (%). P values were calculated using Fisher's exact test.

groups. CD4 counts differed significantly among the three groups ($P = 0.014$), and Bonferroni-adjusted pairwise comparisons showed a significant difference between the non-heteroresistance and low-heteroresistance groups (Table 2).

The distribution of persister and heteroresistance phenotypes among individuals with HIV is shown in Table 3.

5. Discussion

Persister cells and heteroresistance in *C. albicans* may reduce antifungal efficacy and contribute to treatment failure and recurrence. These concerns are particularly relevant in HIV-infected individuals, who are at increased risk for opportunistic oral candidiasis. This study assessed the prevalence of persister cells and heteroresistance among *C. albicans* isolates from HIV-positive patients with oral candidiasis in Ahvaz and examined their associations with clinical and immunological parameters.

5.1. Prevalence of Persister Cells

Persister cells may remain latent within biofilms despite apparent drug susceptibility on minimum inhibitory concentration testing and may contribute to treatment failure, relapse, and chronic infection (35, 41). According to the classifications used by LaFleur et al. and

Aboualigalehdari et al., isolates with a survival rate $> 6\%$ were classified as high persisters, those with a survival rate $< 6\%$ as low persisters, and those with no survival as non-persisters. In this study, cells were exposed to a lethal concentration of amphotericin B (100 times the MIC), and viable cells were counted after 48 h. One *C. albicans* isolate was classified as a high persister, 96 as low persisters, and 4 as non-persisters, consistent with previous reports (12, 42).

Cohen et al. (36) described microbial persistence as an important form of non-genetic drug tolerance. Unlike genetically resistant cells, persister cells do not necessarily exhibit specific resistance-associated genotypic alterations; rather, their survival may reflect dormancy, reduced metabolic activity, and the inactivation of cellular processes targeted by antimicrobial agents (36, 43). In immunocompromised individuals such as HIV-positive patients, prolonged fungal exposure may be associated with a higher prevalence of persister cells and treatment nonresponse (44).

The MICs of persister isolates may not differ substantially from those of the primary population; however, persister cells can survive lethal drug exposure and may subsequently contribute to recurrence (45). Aboualigalehdari et al. (7) reported *Candida* colonization and associated factors among HIV-infected patients in southwestern Iran. In the present study, the persister phenotype was not significantly associated with CD4 count

or viral load ($P > 0.05$). Larger cohorts are needed to determine whether clinically meaningful associations exist.

Quantitative assessment of survival after lethal drug exposure is necessary to identify persister subpopulations. Documenting persister levels together with MIC results may help characterize antifungal tolerance in specialized laboratory settings. Additional clinical studies are needed before such measurements can be incorporated into routine treatment decisions.

5.2. Prevalence of Heteroresistance

Heteroresistance, a mechanism that enables a resistant subpopulation to survive antifungal exposure, has attracted increasing attention. The present findings indicate that this phenotype is common among HIV-positive patients with oral candidiasis in Ahvaz (46, 47). Ben-Ami et al. (30) described fluconazole heteroresistance in *C. glabrata* as a continuously distributed phenotype rather than a binary trait. In that study, area under the curve ratio (AUCR) values ranged from 0.00012 to 13.74, higher heteroresistance levels were associated with elevated AUCR values, heteroresistance was reported in 57.6% of isolates, and the ATP-dependent efflux rate correlated with AUCR ($r^2 = 0.68$, $P < 0.0021$).

Gautier et al. (24) highlighted limitations of conventional methods for detecting heteroresistance and emphasized single-cell approaches and PAP for more accurate characterization. Posteraro et al. (48) reported that heteroresistance-related phenotypes in *Cryptococcus* may involve antifungal-resistance genes such as ERG11 and AFR1 and increased efflux-pump expression. Sionov et al. (49) analyzed more than 130 *Cryptococcus neoformans* isolates and reported intrinsic fluconazole heteroresistance, with resistance levels ranging from 4 to 128 µg/mL and resistant-subpopulation frequencies of 0.3%-10%.

Increasing heteroresistance has also been associated with increased virulence in animal models, and repeated fluconazole exposure may select for higher heteroresistance levels (49). Ferreira et al. (50) reported intrinsic itraconazole heteroresistance among *Cryptococcus gattii* isolates, with greater heteroresistance associated with lower animal survival and increased virulence. Zhai et al. (51) reported that heteroresistant *C. parapsilosis* isolates were associated with prophylaxis failure and breakthrough infection among transplant recipients.

Heteroresistance may influence antifungal treatment outcomes and the risk of recurrence or progression even when conventional susceptibility testing indicates susceptibility (24, 30, 52). In the present study, 21.78% of isolates were classified as highly heteroresistant ($AUC \geq 25.76$). These findings support further investigation of heteroresistance screening as a complementary approach to conventional susceptibility testing. Because heteroresistance exists on a continuum, quantitative measures such as AUC may help characterize its magnitude; however, the clinical utility of these measures requires further validation (49, 53).

5.3. Generalizability and Limitations

Because this study included HIV-positive individuals attending medical centers in Ahvaz, caution is warranted when generalizing the findings to other populations. Host characteristics, regional epidemiology, and antifungal-use patterns may influence the prevalence and intensity of persister and heteroresistance phenotypes (30, 35). Multicenter studies involving more diverse populations are needed to assess external validity.

The association between CD4 count and heteroresistance should also be interpreted cautiously. Because the study was cross-sectional, it cannot establish whether immune status directly influences heteroresistance. One possible explanation is that patients with better immune recovery had longer treatment histories and greater antifungal exposure, potentially selecting for heteroresistant subpopulations; however, this hypothesis requires longitudinal validation. Other limitations include reliance on in vitro assays that may not fully reflect in vivo dynamics, the single-center setting, and retrospectively collected prior-antifungal-exposure data that may be incomplete. Longitudinal and multicenter studies are needed to validate these findings.

5.4. Conclusions

Persister cells and heteroresistant subpopulations were common among *C. albicans* isolates from HIV-positive individuals with oral candidiasis. Amphotericin B MICs were higher in low-persister than in non-persister isolates, although the difference was not statistically significant. The heteroresistance phenotype was associated with CD4 count, with a significant post hoc difference observed between the non-heteroresistance and low-heteroresistance groups. These findings support further investigation of persister and heteroresistance

phenotypes, their molecular mechanisms, and their potential clinical relevance in antifungal treatment. Future longitudinal and multicenter studies should evaluate whether detection of these phenotypes improves therapeutic decision-making or patient outcomes.

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Footnotes

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Conflict of Interests Statement: The authors declare no competing interests.

Data Availability: The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Approval: The research protocol was reviewed and approved on September 25, 2024 by the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (Ethical Code: IR.AJUMS.REC.1403.034).

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