DOI: 10.7860/JCDR/2025/75182.21541

Research Protocol

Microbiology Section

Molecular Mechanism of Fluconazole Resistance among Clinical Isolates of Non *albicans Candida* Species in a Rural Tertiary Healthcare Centre of Central India: A Research Protocol

DEEPENDRA MANISH[1], GARGI MUDEY[2], DIPIKA SHAW[3]

ABSTRACT

Introduction: The most common fungal infections are caused by *Candida* spp., and non *albicans Candida* (NAC) species are increasingly prevalent in India. This study protocol provides a concise summary of fluconazole resistance mechanisms in NAC species. Although fluconazole is fungistatic and resistance can be either intrinsic or acquired, it remains a mainstay of current treatment. Understanding species-specific molecular pathways is crucial because resistance mechanisms differ among species. Clarifying the mechanisms that drive fluconazole resistance is essential to preserve the effectiveness of this important antifungal agent.

Need of the study: The results of this study may inform public health initiatives and help shape policies to curb the emergence and spread of antifungal resistance, emphasising the need for targeted interventions and sustained surveillance programs.

Aim: To study the molecular mechanisms of fluconazole resistance among clinical isolates of NAC species.

Materials and Methods: This will be a cross-sectional study. A total of 78 clinically relevant NAC isolates obtained from specimens received between February 2024 and August 2026 in the Department of Microbiology will be tested for fluconazole resistance using disk diffusion and microbroth dilution methods. Polymerase Chain Reaction (PCR) will be performed to investigate molecular mechanisms of fluconazole resistance by examining mutations in *ERG11*. Data will be analysed using Student's t-test, Chi-square test and Fisher's exact test as appropriate. R and IBM Statistical Package for the Social Sciences (SPSS) Statistics version 29.0 will be used for statistical analysis.

Keywords: Fungistatic, Microbroth dilution, Molecular methods

INTRODUCTION

The NAC species are yeast-like fungi of the genus *Candida*. These organisms are common colonisers but can also cause nosocomial bloodstream infections. Common NAC species include *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. parapsilosis* [1]. Several countries, including India, have observed an epidemiologic shift from *C. albicans* toward NAC species. Indian studies report candidiasis rates of approximately 6-18%, with NAC species accounting for about 74% of isolates [2,3].

Among azoles (imidazoles and triazoles), fluconazole is the most widely used drug for treating *Candida* infections [4]. Resistance of NAC species to antifungal agents poses a major challenge for empirical, therapeutic and prophylactic strategies, with reported fluconazole resistance rates ranging from 0.5-13% [5]. Azoles act by inhibiting the cytochrome P450 enzyme lanosterol 14α-demethylase, encoded by *ERG11*, which is required for ergosterol biosynthesis [5]. Azoles bind the haeme iron of this enzyme via the nitrogen atom in the azole ring, preventing oxygen activation and blocking lanosterol demethylation. This interference leads to accumulation of methylated sterols in the fungal membrane, disrupting membrane function and inhibiting cell growth [6]. Resistance mechanisms to fluconazole, with particular emphasis on the *ERG11* gene, include gene alterations such as point mutations, overexpression, and modifications affecting the ergosterol biosynthesis pathway [7]. These mutations cause conformational changes that reduce the binding affinity of azoles to their targets [8].

REVIEW OF LITERATURE

Exploring the molecular pathways underlying fluconazole resistance in clinical isolates of NAC species from a rural tertiary healthcare centre in central India is the primary goal of this study. The findings may directly impact clinical practice by providing essential insights into the prevalence of resistance and the specific genetic factors contributing to it.

Chaudhary P et al., in their study, included screening tests for the detection of NAC species along with antifungal susceptibility testing, which showed varying azole resistance patterns [2]. According to the results of another systematic analysis by Paul S et al., protein homology modelling and molecular docking identified *ERG11* gene mutations that caused structural changes and reduced binding efficiency between *ERG11p* and ligands. A220C in *ERG11* and dual T503C and G751A mutations in UPC2 were observed in isolates with *ERG11* mutations. Azole resistance in clinical isolates of *C. tropicalis* is linked to non synonymous mutations in the *ERG11* gene and coordinated overexpression of many genes, including transcription factors, genes in the ergosterol production pathway, transporters and stress-responsive genes [7].

In another study by Fan X, et al., it was concluded that *ERG11* missense mutations were the major mechanism responsible for azole resistance in *C. tropicalis* isolates, but overexpression of *ERG11*, CDR1 and MDR1, as well as reduced expression of CYTb, also contributed to resistance [8].

On a broader scale, these results may guide public health initiatives and shape policies to mitigate the rise and spread of antifungal

resistance, emphasising the need for targeted interventions and sustainable surveillance programmes.

Objectives

- To isolate and identify *Candida* species from various clinical samples using conventional and automated methods.
- To study the antifungal sensitivity pattern for azoles among NAC species isolates by the disc diffusion method.
- To perform the antifungal sensitivity test for fluconazole among NAC species by the broth microdilution method.
- To explore the molecular mechanism of fluconazole resistance by studying mutations in the *ERG11* gene.

MATERIALS AND METHODS

The cross-sectional study will be conducted in the Department of Microbiology, Jawaharlal Nehru Medical College, a tertiary care centre in the state of Maharashtra, India, from February 2024 to August 2026. Approval was obtained from the Institutional Ethics Committee (IEC) of the Datta Meghe Institute of Higher Education and Research, Sawangi, Wardha, Maharashtra, with approval no. DMIHER (DU)/IEC/2024/136 dated 30/01/2024. CTRI registration on 25/04/2024 with registration no CTRI/2024/04/066326. As the study involves isolates recovered from specimens received by the Department of Microbiology, informed consent will be obtained from all study participants.

Inclusion criteria: All clinical samples (blood, urine, bronchoalveolar lavage, endotracheal tube secretions and vaginal swabs) received by the Department of Microbiology for isolation of *Candida* species will be included, across all ages and both genders.

Exclusion criteria: Clinical samples yielding growth other than *Candida* species will be excluded from the study.

Samples: The study will be carried out on all clinical isolates of NAC species that are isolated from specimens received from February 2024 to August 2026 in the Department of Microbiology.

Sample size:

- Population size in ICU patients: 100
- Estimated margin of error: 5%
- Estimated prevalence of candidaemia: 35% [9,10]

Using the formula below, the minimum sample size was calculated.

$$n \geq \frac{NZ^2_{1-\frac{\alpha}{2}}P(1-P)}{d^2(N-1)+Z^2_{1-\frac{\alpha}{2}}P(1-P)}$$

With the values of: Alpha (α)=0.05

Estimated proportion (p)=0.35

Estimated error (d)=0.05

Population size (N)=100

Minimal sample size needed will be=78

Isolation and Identification of *Candida* Species

Samples will be processed by conventional microbiological methods, including culture on Sabouraud dextrose agar, germ tube test, CHROMagar, Dalmau plate technique, carbohydrate assimilation methods and automated methods (VITEK2 Compact) to isolate and identify *Candida* species, according to standard protocols using Clinical and Laboratory Standards Institute (CLSI) M27-A3 guidelines and ICMR SOP for Mycology (2nd edition, 2019) [11,12].

Antifungal Susceptibility Test (AFST)

Disc diffusion method: AFST will be performed using the disc diffusion method for the following antifungals: fluconazole (25 μg), voriconazole (1 μg), posaconazole (5 μg), itraconazole (30 μg) and ketoconazole (5 μg). Mueller-Hinton agar supplemented with 2% glucose and 0.5 μg/mL methylene blue (GMB) will be used [11,12].

For quality control, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 will be used.

Microbroth dilution for fluconazole: For all isolates, the CLSI Broth Microdilution (BMD) method and interpretation according to CLSI (M27-A3, M27M44S) were used to determine the Minimum Inhibitory Concentrations (MICs) of fluconazole. The MICs were interpreted as susceptible, susceptible-dose dependent (SDD) or intermediate, and resistant [Table/Fig-1] [11,12].

Species	Susceptible	Intermediate	Susceptible-dose Dependent (SDD)	Resistant
Candida albicans	<2	-	4	≥8
Candida glabrata	-	-	≤32	≥64
Candida parapsilosis	≤2	-	4	≥8
Candida tropicalis	≤2	-	4	≥8

[Table/Fig-1]: Fluconazole MIC breakpoints and interpretive categories (μg/mL) [11,12].
*MIC: Minimum inhibitory concentration

Molecular Analysis of Fluconazole Resistance

DNA extraction of the *ERG11* gene from fluconazole-resistant *Candida* isolates will be performed using the phenol-chloroform-isoamyl alcohol method [13,14].

PCR amplification of *ERG11* will be performed using an overlapping primer set (*ERG11* F1: 5′-TCACAGTTATAGACCCACAAGG-3′; *ERG11* R1: 5′-TCACCGCTTTCTCTTCTTCTCT-3′; *ERG11* F2: 5′-AAGGTTT CACCCCAATCAACTT-3′; *ERG11* R2: 5′-CGACTGAAACGTATACC GCGA-3′) [15].

SeqMan software (DNASTAR, USA) will be used to align multiple *ERG11* fragments to create a consensus sequence. Aligning the consensus sequence with the reference isolate *Candida* tropicalis MYA3404 (GenBank accession no. XM_002550939.1) allowed the detection of mutations associated with fluconazole resistance in the isolates [15].

Primary outcome: Identification of NAC species.

Secondary outcome: Detection of fluconazole-resistant isolates among NAC isolates, based on *ERG11* gene mutations.

STATISTICAL ANALYSIS

The data will be analysed using Student's t-test, Chi-square test, and Fisher's exact test as needed. R software and IBM SPSS Statistics v29 will be used for calculations.

Grant Information

Approved Intramural Grant from Datta Meghe Institute of Higher Education and Research, Sawangi, Wardha, Maharashtra (Ref. no. DMIHER (DU) R&D/2024/353). Approved ICMR R12019/26/2024-HR grant under the scheme component Financial Support of MD/MS/DM/MCh/DNB/DrNB/MDS thesis 2024 under the HRD Scheme of the Department of Health Research, with reference ID MDMS24JUN-0733.

Acknowledgement

Dr. Manoj Patil, Research Consultant, Department of Microbiology, JNMC, DMIHER, Sawangi (Meghe), Wardha, Maharashtra, India.

Ms. Aditi Warghade, PhD Scholar, Department of Microbiology, JNMC, DMIHER, Sawangi (Meghe), Wardha, Maharashtra, India.

Ms. Swati Kombe, Head Technician, Department of Microbiology, JNMC, DMIHER, Sawangi (Meghe), Wardha, Maharashtra, India.

REFERENCES

[1] Gómez-Gaviria M, Ramírez-Sotelo U, Mora-Montes HM. Non-*albicans Candida* species: Immune response, evasion mechanisms, and new plant-derived alternative therapies. J Fungi. 2022;9(1):11.

[2] Chaudhary P, Singla A, Mamoria VP, Mittal S, Bacchani D. Prevalence of non-*albicans Candida* and its antifungal susceptibility at a tertiary care hospital, Jaipur. Int J Contemp Med Res IJCMR. 2020;7(7):01-05.

[3] Oberoi JK, Wattal C, Goel N, Raveendran R, Datta S, Prasad K. Non-*albicans Candida* species in blood stream infections in a tertiary care hospital at New Delhi, India. Indian J Med Res. 2012;136(6):997-1003.

[4] Arendrup MC, Patterson TF. Multidrug-resistant *Candida*: Epidemiology, molecular mechanisms, and treatment. J Infect Dis. 2017;216(Suppl 3):S445-51.

[5] Berkow EL, Lockhart SR. Fluconazole resistance in *Candida* species: A current perspective. Infect Drug Resist. 2017;10:237-45.

[6] Casalinuovo IA, Di Francesco P, Garaci E. Fluconazole resistance in *Candida albicans*: A review of mechanisms. Eur Rev Med Pharmacol Sci. 2004;8(2):69-77.

[7] Paul S, Kannan I, Mohanram K. Extensive ERG11 mutations associated with fluconazole-resistant *Candida albicans* isolated from HIV-infected patients. Curr Med Mycol. 2019;5(3):01-06.

[8] Fan X, Xiao M, Zhang D, Huang JJ, Wang H, Hou X, et al. Molecular mechanisms of azole resistance in *Candida tropicalis* isolates causing invasive candidiasis in China. Clin Microbiol Infect. 2019;25(7):885-91.

[9] Tak V, Mathur P, Varghese P, Gunjiyal J, Xess I, Misra MC. The epidemiological profile of candidemia at an indian trauma care center. J Lab Physicians. 2014;6(02):096-101.

[10] DiNubile MJ, Lupinacci RJ, Strohmaier KM, Sable CA, Kartsonis NA. Invasive candidiasis treated in the intensive care unit: Observations from a randomized clinical trial. J Crit Care. 2007;22(3):237-44.

[11] Bhargava B. Standard operating procedures for fungal identification and detection of antifungal resistance. In: Indian Council of Medical Research [Internet]. Division of Publication and Information; 2019. Available from: https://www.icmr.gov.in/icmrobject/custom_data/pdf/resource-guidelines/Mycology_SOP_2nd_Ed_2019.pdf.

[12] Rex JH, Alexander BD, Andes D, Arthington-Skaggs B, Brown SD, Chaturvedi V, et al. Reference method for broth dilution antifungal susceptibility testing of yeasts: Approved standard - third edition. Vol. 28, Clinical and Laboratory Standards Institute. 2008. 1-25 p.

[13] Jin L, Cao Z, Wang Q, Wang Y, Wang X, Chen H, et al. MDR1 overexpression combined with ERG11 mutations induce high-level fluconazole resistance in *Candida tropicalis* clinical isolates. BMC Infect Dis. 2018;18(1):01-06.

[14] Choi MJ, Kwon YJ, Byun SA, Kim MN, Lee WG, Lee J, et al. Molecular and clinical features of fluconazole non-susceptible *Candida albicans* bloodstream isolates recovered in Korean multicenter surveillance studies. Ann Lab Med. 2023;43(6):614-19.

[15] Paul S, Shaw D, Joshi H, Singh S, Chakrabarti A, Rudramurthy SM, et al. Mechanisms of azole antifungal resistance in clinical isolates of *Candida tropicalis*. Sturtevant J, editor. PLoS One. 2022;17(7):e0269721.

PARTICULARS OF CONTRIBUTORS:

1. Junior Resident, Department of Microbiology, Datta Meghe Institute of Higher Education and Research, Wardha, Maharashtra, India.
2. Professor and Head, Department of Microbiology, Datta Meghe Institute of Higher Education and Research, Wardha, Maharashtra, India.
3. Assistant Professor, Department of Microbiology, Datta Meghe Institute of Higher Education and Research, Wardha, Maharashtra, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Gargi Mudey,
Professor and Head, Department of Microbiology, Jawaharlal Nehru Medical College, Datta Meghe Institute of Higher Education and Research, Sawangi, Wardha, Maharashtra, India.
E-mail: gargi.microbiology@dmiher.edu.in

PLAGIARISM CHECKING METHODS: [Jain H et al.]

- Plagiarism X-checker: Sep 16, 2024
- Manual Googling: May 03, 2025
- iThenticate Software: May 05, 2025 (19%)

ETYMOLOGY: Author Origin

EMENDATIONS: 6

AUTHOR DECLARATION:

- Financial or Other Competing Interests: As declared above
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Sep 14, 2024**
Date of Peer Review: **Jan 23, 2025**
Date of Acceptance: **May 07, 2025**
Date of Publishing: **Sep 01, 2025**