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Original Article

Microbiology Section

Early Detection of Fluconazole Resistance Targeting Y132F Mutation in *ERG11* Gene Among *Candida Tropicalis* Clinical Isolates using Polymerase Chain Reaction: A Cross-sectional Study

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ABSTRACT

Introduction: *Candida tropicalis* is the most prevalent yeast associated with high mortality rates in candidaemia patients. The extensive use of fluconazole for prophylaxis has led to the emergence of resistance. The Y132F mutation occurs in the active site of the enzyme, making the organism resistant to fluconazole, and may serve as a significant marker.

Aim: To detect the presence of the Y132F mutation in the *ERG11* gene for early identification of fluconazole-resistant *C. tropicalis* isolates.

Materials and Methods: This cross-sectional study was carried out in the Department of Microbiology at Sri Ramachandra Institute of Higher Education and Research, Chennai Tamil Nadu, India, over a period of 19 months from May 2018 to December 2019. Blood samples received in the Microbiology laboratory that grew *Candida* during the study period were included. A total of 92 *Candida* isolates from candidaemia patients were studied. Among these, isolates identified as *C. tropicalis* by phenotypic and genotypic methods were subjected to Antifungal Susceptibility Testing (AFST) by the broth microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M27-A3). Subsequently, Polymerase Chain Reaction (PCR) assays were performed on fluconazole-resistant *C. tropicalis* isolates, targeting the Y132F mutation in the *ERG11* gene using in-house primers. Additionally, sequencing of the *C. tropicalis ERG11* gene was performed to confirm the presence or absence of the Y132F mutation in both resistant and susceptible isolates.

Results: Among the 92 candidaemia patients, 37 (40.2%) cases were caused by *C. tropicalis*, of which 10 (27%) were resistant to fluconazole. The PCR assay accurately detected all 10 fluconazole-resistant *C. tropicalis* isolates and also produced bands for control strains with the Y132F mutation. DNA sequencing further confirmed that the Y132F mutation was present in fluconazole-resistant isolates, with results consistent with the PCR assay.

Conclusion: This PCR-based detection of the Y132F mutation highlights the importance of molecular diagnostics in identifying fluconazole resistance in *C. tropicalis*. Such early detection enables healthcare providers to initiate appropriate treatment, reducing the risk of treatment failure and improving patient outcomes. Moreover, this assay could be a valuable screening tool to detect potentially resistant strains and monitor their spread.

Keywords: Amino acid substitution, Lanosterol 14-alpha demethylase, Molecular diagnostics

INTRODUCTION

Candida albicans is the primary cause of invasive candidiasis, followed closely by non-*albicans Candida* (NAC) species such as *Candida tropicalis*, *Candida parapsilosis, Candida glabrata,* and *Candida krusei*. Although *C. albicans* remains the most frequently isolated species, NAC species are increasingly reported and are associated with significant morbidity and mortality. Among them, *C. tropicalis* ranks as the first or second most common cause of candidaemia in many parts of the world [1-3]. In India, *C. tropicalis* is the most prevalent yeast among NAC species causing candidaemia [4-6].

Patients admitted to intensive care units are frequently infected with *C. tropicalis*, particularly those with cancer, long-term catheterisation, or who are receiving broad-spectrum antibiotics. It is also commonly associated with neutropenia and malignancy and has been increasingly reported in neonates [4,7]. According to clinical and experimental data, *C. tropicalis* infections are associated with morbidity and mortality rates 30-70% higher than those caused by *C. albicans* [2,8].

Azoles, particularly fluconazole, are the most widely used antifungal agents for prophylaxis in hospitalised patients. Fluconazole inhibits ergosterol synthesis by binding to the lanosterol 14α-demethylase enzyme encoded by the *ERG11* gene. The extensive use of fluconazole has contributed to rising resistance and higher Minimum Inhibitory Concentration (MIC) values. Several mechanisms are implicated in fluconazole resistance in *C. tropicalis*, including drug efflux, *ERG11* gene overexpression, point mutations in the *ERG11* gene, and bypass pathway formation [4-7,9]. Among these, the Y132F amino acid substitution resulting from a point mutation (A395T) in the *ERG11* gene is the most significant and frequently reported mechanism [10-12]. This mutation is also found in other *Candida* species, including *C. albicans*, *C. parapsilosis*, and *C. auris* [13-15]. Therefore, the Y132F mutation could serve as an important marker for identifying fluconazole resistance in *C. tropicalis*, as has been demonstrated previously in *C. parapsilosis* [16].

Although AFST is routinely used, it has several drawbacks, including a long turnaround time, labour-intensive procedures and subjective interpretation of results. With the increasing reports of fluconazole resistance in *C. tropicalis*, there is a growing need for molecular

diagnostic techniques that allow rapid and reliable detection. Early and accurate identification of resistance can help reduce treatment failure and guide the optimal selection of antifungal therapy.

Therefore, present study aimed to develop and validate a simple conventional PCR assay for the early identification of fluconazole-resistant *C. tropicalis* isolates by targeting the Y132F mutation in the *ERG11* gene.

MATERIALS AND METHODS

This cross-sectional study was conducted in the Department of Microbiology at Sri Ramachandra Institute of Higher Education and Research, Chennai Tamil Nadu, India, over a period of 19 months (May 2018 to December 2019). Ethical approval was obtained from the institutional ethics committee (CSP/18/APR/68/106). Informed consent was not obtained because only clinical isolates were used.As this was a time-bound study, no sample size calculation was performed.

Inclusion criteria: All *Candida* isolates recovered from blood samples during the 19-month study period were included in the study.

Exclusion criteria: Repetitive *Candida* isolates from the same patient were excluded from the study.

Study procedure [16]: A total of 92 *Candida* isolates obtained from blood cultures during the study period were included. Since this was a time-bound study, all isolates available within the duration were considered. Identification of isolates was performed using phenotypic techniques such as Gram staining, culture characteristics, germ tube formation and colony colour on CHROMagar, with appropriate control strains. Further confirmation was done by genotypic identification using PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) [17].

DNA extraction: DNA was extracted from all *Candida* isolates using the phenol-chloroform method with minor modifications [17,18]. The extracted DNA was stored at -20°C for further molecular assays [16].

Polymerase Chain Reaction (PCR)

PCR Amplification of Internal Transcribed Spacer (ITS) Regions

PCR amplification of the ITS regions (ITS1 and ITS2) was carried out using universal fungal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR master mix consisted of 25 µL of PCR mix (Takara, Japan), 1 µL each of the forward (ITS1) and reverse (ITS4) primers, 5 µL of template DNA and sterile nuclease-free water to a final volume of 50 µL.

Amplification was performed in a thermal cycler (Veriti 96-well, Applied Biosystems, USA) under the following conditions: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec; followed by a final extension at 72°C for 10 min. After amplification, 10 µL of the PCR product was subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualised under UV illumination using the Bio-Rad Gel Documentation System (USA) [16].

Restriction Fragment Length Polymorphism (PCR-RFLP)

The PCR products were subjected to restriction digestion using the MspI restriction enzyme (Thermo Scientific, Lithuania), which cleaves DNA at specific sites, producing fragments of varying lengths [17,18]. Species identification was based on the resulting banding pattern.

The reaction mixture contained 10 µL of amplified PCR product, 2 µL of 10X enzyme buffer, 5 units of MspI restriction enzyme and sterile nuclease-free water to a final volume of 20 µL. The mixture was incubated at 37°C for 1 hour. Following incubation, 10 µL of the digested products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualised under UV illumination using the Bio-Rad Gel Documentation System (USA) [16].

Antifungal Susceptibility Testing (AFST)

AFST was performed for all *C. tropicalis* isolates using the broth microdilution method according to the CLSI guidelines (M27-A3). The isolates were tested against fluconazole (0.25-64 µg/mL), itraconazole (0.06-16 µg/mL), voriconazole (0.06-16 µg/mL), and posaconazole (0.06-16 µg/mL) [19]. Growth control (inoculum without drug) and sterility control (RPMI-1640 medium without inoculum) were included in each run.

MIC values for all antifungal agents were determined after 24 hours of incubation [16]. Isolates with MIC ≥ 8 µg/mL were considered fluconazole-resistant, based on CLSI M27M44S-ED3:2022 [20]. *Candida tropicalis* ATCC 750 was used as the reference strain.

Standardisation of PCR for Detection of Y132F Mutation in the *ERG11* Gene

DNA extracted from all *C. tropicalis* isolates was used for detection of the Y132F mutation in the *ERG11* gene. PCR conditions were standardised using archived fluconazole-resistant and -susceptible *C. tropicalis* isolates, with the presence or absence of the Y132F mutation previously confirmed by sequencing.

Positive control: *C. tropicalis* isolates with Y132F mutation (KY290569.1, KY290568.1, KY290567.1)

Negative control: *C. tropicalis* isolates without Y132F mutation (KY290572.1, KY290571.1, KY290570.1)

Primer Designing

Primers were designed using archived fluconazole-resistant and -susceptible *C. tropicalis* isolates previously confirmed by sequencing. The nucleotide sequences of the *ERG11* gene of *C. tropicalis* were obtained from the NCBI database and aligned using MEGA11 software (version 11).

Primers were manually designed to target the Y132F mutation at nucleotide position 395 in the *ERG11* gene, where adenine (A) is replaced by thymine (T), resulting in a Y132F amino acid substitution in the protein. The designed primers were verified using the NCBI Primer-BLAST tool and further analyzed with the in-silico PCR software (http://insilico.ehu.es/user_seqs/). Primers were synthesized by Sigma-Aldrich Chemical Pvt. Ltd., Bangalore [16].

PCR Amplification

PCR was performed using CT 132FP (5'-TCCAGTTTTTGGTAAAGGTGTTATTTTT-3') as the forward primer and CT 132RP (5'-TGACCACCCATCAAAACACCA-3') as the reverse primer. The reaction mix contained 12.5 µL of PCR mix (Takara, Japan), 0.5 µL each of forward and reverse primers, 4 µL of DNA template, and sterile nuclease-free water to a final volume of 25 µL.

The PCR conditions for detection of the Y132F mutation in the *ERG11* gene were as follows: initial denaturation at 95°C for 10 min; 35 cycles of 95°C for 30 sec, 53°C for 30 sec, and 72°C for 30 sec; followed by a final extension at 72°C for 7 min. The PCR products were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide and visualised under UV illumination using the Bio-Rad Gel Documentation System (USA) [16].

Comparison of AFST and Y132F Mutation-Detecting PCR

The results of PCR for the rapid detection of fluconazole resistance in *C. tropicalis* isolates using in-house species-specific primers were compared with the antifungal susceptibility patterns obtained for all *C. tropicalis* isolates and were further validated by gene sequencing.

Amplification of the *ERG11* Gene

PCR amplification of the *ERG11* gene was carried out using CT *ERG11* 1F (5'-TCTGACATGGTGTGTGTGTG-3') and CT *ERG11* 3R (5'-CAAGGAATCAATCAAATCTCTC-3') primers [21]. The reaction

mix consisted of 15 µL of PCR mix (Takara, Japan), 0.5 µL each of forward and reverse primers, 4 µL of template DNA, and sterile nuclease-free water to a final volume of 30 µL.

Amplification was performed in a thermal cycler (Veriti 96-well, Applied Biosystems, USA) with the following conditions: initial denaturation at 95°C for 10 min; 35 cycles of 95°C for 45 sec, 53°C for 30 sec, and 72°C for 2 min 30 sec; followed by a final extension at 72°C for 7 min. The PCR products (10 µL) were resolved on 1.5% agarose gel, stained with ethidium bromide and visualised under UV illumination using the Bio-Rad Gel Documentation System (USA) [16].

Y132F Mutation Analysis

The *ERG11* gene of representative fluconazole-susceptible and -resistant isolates was sequenced (Eurofins Genomics India Pvt. Ltd., Bengaluru, Karnataka). The sequences obtained were verified using the Basic Local Alignment Search Tool (BLAST) and deposited in the National Centre for Biotechnology Information (NCBI, USA; http://www.ncbi.nlm.nih.gov) GenBank database, where accession numbers were assigned.

Nucleotide sequences were translated into amino acid sequences using the BioEdit tool with the standard genetic code as a reference. The sequences were aligned using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) and analysed for the Y132F amino acid substitution [16].

RESULTS

Among the 92 *Candida* isolates tested, *C. tropicalis* was the predominant species, accounting for 37 (40.2%), followed by *C. albicans* (24; 26.1%), *C. parapsilosis* (17; 18.5%), *C. glabrata* (7; 7.6%), *C. krusei* (4; 4.3%), and *C. auris* (3; 3.3%).

Interpretation of Antifungal Susceptibility Testing

Out of the 37 *C. tropicalis* isolates tested by the broth microdilution method, 10 (27%) were resistant to fluconazole, with MIC values ranging from 8 µg/mL to 64 µg/mL. Among these resistant isolates, 8 (21.6%) were resistant to fluconazole alone. One isolate was resistant to fluconazole, itraconazole (MIC=2 µg/mL), and voriconazole (MIC=4 µg/mL), while another was resistant to fluconazole and itraconazole (MIC=2 µg/mL). All isolates were susceptible to posaconazole. The remaining 27 (73%) *C. tropicalis* isolates were susceptible to all antifungal agents tested.

Detection of Y132F Mutation

The PCR assay using in-house Y132F mutation-detecting primers yielded a band at 558 bp for all fluconazole-resistant *C. tropicalis* isolates with the Y132F mutation (n=10), whereas no band was observed for fluconazole-susceptible isolates without the mutation (n=27). The in-house primers correctly identified the mutation in all fluconazole-resistant isolates [Table/Fig-1].

Comparison of AFST and PCR

The results of the PCR assay were comparable to those of AFST. Among the 37 *C. tropicalis* isolates, 10 were resistant and 27 were susceptible to fluconazole. This was in concordance with the PCR assay, which accurately detected the presence and absence of the Y132F mutation in resistant and susceptible isolates, respectively. The developed PCR assay successfully identified the mutation in all fluconazole-resistant *C. tropicalis* isolates.

ERG11 Gene Amplification

The *ERG11* gene from representative fluconazole-resistant and -susceptible isolates was amplified. The *ERG11* gene product was approximately 1500 bp in size, as shown in [Table/Fig-2].

[Table/Fig-1]: Detection of Y132F mutation in ERG11 gene of *Candida tropicalis*
*Lane 1: DNA marker (100bp), Lane 2: ATCC *C. tropicalis* 750, Lane 3: Fluconazole resistant *C. tropicalis* clinical isolate (with Y132F mutation), Lane 4:Fluconazole resistant *C. tropicalis* clinical isolate (with Y132F mutation), Lane 5:Fluconazole susceptible *C. tropicalis* clinical isolate (without Y132F mutation), Lane 6:Fluconazole susceptible *C. tropicalis* clinical isolate (without Y132F mutation)

[Table/Fig-2]: Amplification of *ERG11* gene of *C. tropicalis*
*Lane 1: DNA marker, Lane 2: Positive control (ATCC *C. tropicalis* 750), Lane 3: Negative control (Nuclease free water), Lane 4:Fluconazole resistant *C. tropicalis* clinical isolate, Lane 5:Fluconazole susceptible *C. tropicalis* clinical isolate

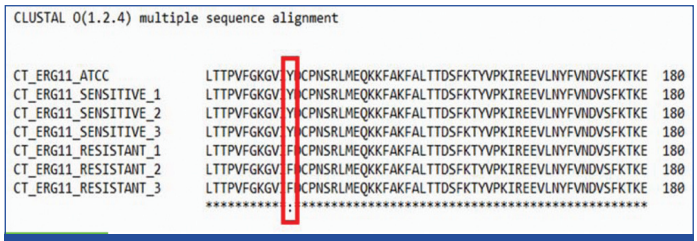
Y132F Mutation Analysis

The DNA sequences of representative fluconazole-susceptible and -resistant *C. tropicalis* isolates were compared with the *ERG11* gene sequence of *C. tropicalis* ATCC 750 (GenBank accession number M23673.1) and analysed for the Y132F amino acid substitution in the protein. In the mutation-detecting PCR, fluconazole-resistant *C. tropicalis* isolates that produced a band harboured the Y132F mutation, whereas fluconazole-susceptible isolates, which did not produce a band, lacked the mutation [Table/Fig-3,4].

GenBank Accession Numbers

The following are the GenBank accession numbers for the nucleotide sequences obtained in this study: PP069230, PP069231, PP069232, PP069233, PP069234, PP069235.

[Table/Fig-3]: Multiple sequence alignment of ERG11 gene of representative fluconazole susceptible and resistant *Candida tropicalis* clinical isolates.



[Table/Fig-4]: Y132F mutation analysis of *ERG11* gene of susceptible and resistant *Candida tropicalis* isolates.

DISCUSSION

In *C. tropicalis*, multiple mechanisms are involved in azole resistance, among which mutations in the *ERG11* gene represent the most significant mechanism. Of these, the missense mutation Y132F is the predominant factor responsible for fluconazole resistance and could serve as an important marker for detecting fluconazole resistance in *C. tropicalis*. Currently, the standard method for detecting fluconazole resistance in routine laboratory settings is AFST by the broth microdilution method. Detection of this mutation is generally performed by DNA sequencing, which is considered the gold standard. However, both methods are time-consuming, costly and require technical expertise for interpretation. Therefore, PCR-based identification of fluconazole resistance in *C. tropicalis* isolates by targeting the Y132F mutation in the *ERG11* gene is valuable for early detection and diagnosis.

Worldwide data indicate that the prevalence of candidaemia caused by *C. tropicalis* is higher in Asia (25.4%) compared with Europe and North America [22]. In Asian countries, including India, *C. tropicalis* is one of the most frequently isolated species from candidaemia patients, with a high mortality rate ranging from 40-70% [23]. In a recent study by Teo JQ et al., *C. tropicalis* was reported as the second most predominant species, accounting for about 30.4% of all candidaemia cases [24]. Similarly, an epidemiological study by Wang H et al., in the Asia-Pacific region reported that *C. tropicalis* is the most common *Candida* species found in India [25]. Consistently, a study conducted by Behera C et al., found that the majority of *Candida* isolates from the PICU of an Indian hospital were *C. tropicalis* (44.4%) [4]. Furthermore, a nationwide multicentric study on candidaemia from 27 Indian ICUs highlighted the high rate of *C. tropicalis* isolation (41.6%), making it the most prevalent of all *Candida* species [1]. Present study was consistent with these epidemiological findings, showing *C. tropicalis* as the most common cause of nosocomial candidaemia, representing 40.2% of *Candida* species isolated from blood cultures.

C. tropicalis isolates are usually susceptible to antifungal agents. However, an increasing number of candidaemia studies have reported a significant rise in azole-resistant *C. tropicalis*, with some even documenting pan-azole resistance [2,21,24,26]. Several studies have highlighted fluconazole resistance and azole cross-resistance in clinical isolates, particularly in the Asia-Pacific region [2,11,27]. For instance, Chakrabarti A et al., reported that 10.2% of *C. tropicalis* isolates were resistant to fluconazole [27]. In contrast, a more recent study on *Candida* bloodstream isolates by Teo JQ et al., found fluconazole resistance in 22% of *C. tropicalis* isolates [24]. Similarly, a study conducted in a Paediatric ICU (2017-2019) reported 25% fluconazole resistance in *C. tropicalis* [4]. In present study, approximately 27% of *C. tropicalis* isolates were resistant to fluconazole. Together, these findings highlight the emerging problem of fluconazole resistance in *C. tropicalis*.

In present study, one *C. tropicalis* isolate was resistant to all three azole antifungal agents (fluconazole, itraconazole and voriconazole). Similar cross-resistance between azoles has been reported from Latin America, Europe, North America and India [27,28]. The rising fluconazole resistance in *C. tropicalis* is significant because it is one of the most frequently isolated NAC species and tends to develop higher levels of drug resistance in patients undergoing fluconazole treatment or prophylaxis compared to *C. albicans*. Collectively, these findings demonstrate that *C. tropicalis* is not an innocuous, universally azole-susceptible species and must be detected early to enable better patient management.

Development of Fluconazole Resistance in *C. tropicalis*

The development of fluconazole resistance in *C. tropicalis* is caused by different mechanisms. Among these, point mutations in the *ERG11* gene have been responsible for a significant rise in fluconazole resistance among *Candida* species [29]. The Y132F mutation in the *ERG11* gene has been previously reported in fluconazole-resistant *C. albicans* and *C. tropicalis* [2,30-32]. Likewise, Flowers SA et al., observed a fourfold or greater increase in the fluconazole MIC in response to the Y132F amino acid substitution, demonstrating the significance of this mutation [33]. According to Teo JQ et al., about 47% of *C. tropicalis* isolates showed Y132F amino acid substitution in fluconazole-resistant strains [24]. Consistent with these findings, we also detected the Y132F amino acid substitution in all of our fluconazole-resistant (27%) *C. tropicalis* isolates.

In a study by Paul S et al., rapid detection of *ERG11* polymorphisms associated with azole resistance in *C. tropicalis* was achieved using T-ARMS-PCR, RSM and HRM. These approaches could detect resistant isolates with both A395T and C461T mutations. However, a limitation of this method is its inability to differentiate resistant isolates from susceptible isolates carrying only the A395T mutation. Moreover, not all resistant isolates carry both mutations, and importantly, only the A395T mutation contributes to fluconazole resistance, whereas C461T does not [29].

Therefore, in the present study, authors designed in-house primers targeting the Y132F mutation in *C. tropicalis* isolates to detect fluconazole resistance by a simple conventional PCR assay. This method accurately detected *C. tropicalis* isolates that showed resistance to fluconazole by the broth microdilution method. The total turnaround time for this PCR assay was 3 hours. Hence, this rapid and precise molecular method can be considered a predictive marker for detecting fluconazole resistance among *C. tropicalis*. The ability to rapidly detect fluconazole resistance enables timely initiation of the appropriate antifungal treatment, reduces the risk of transmission and helps prevent outbreaks with resistant strains. However, present study was limited to a single centre and a relatively small number of samples. A multicentre study with a larger sample size would help in further optimisation and validation of this PCR assay.

Limitation(s)

The main limitation of present study was that fluconazole resistance mechanisms other than the Y132F mutation cannot be detected by this method. Additionally, the in-house primers used in this study are species-specific, meaning they can detect fluconazole resistance only in *C. tropicalis* isolates, but not in other *Candida* species.

CONCLUSION(S)

Detection of the Y132F mutation by conventional PCR can be considered a simple, inexpensive and early method for identifying fluconazole resistance among *C. tropicalis* isolates. This method can be implemented in a basic molecular biology laboratory with a

mycology setup. Overall, early detection of fluconazole resistance in *C. tropicalis* facilitates targeted therapy, leading to improved patient care and reduced morbidity and mortality rates.

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