

## Extensive *ERG11* mutations associated with fluconazole-resistant *Candida albicans* isolated from HIV-infected patients

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### ABSTRACT

**Background and Purpose:** Azoles are preferred antifungal agents given their inexpensiveness, limited toxicity, and potentiality of oral administration. However, the extensive use of prophylactic azole therapy for chronic infections, especially in immunocompromised patients, has led to an increase in azole resistance, thereby rising health care costs. Fluconazole resistance is associated with poor clinical outcomes and the emergence of new infections. The present study aimed to investigate the mutations of *ERG11* gene in fluconazole-resistant *Candida albicans* isolates.

**Materials and Methods:** This study was conducted on 80 clinical samples collected from HIV-infected patients with suspected candidiasis in Tagore Medical College Hospital and Government Hospital of Thoracic Medicine, Chennai, India, for a period of 18 months (May 2016-December 2017). The antifungal susceptibility pattern was determined by agar diffusion and broth dilution techniques as per the Clinical and Laboratory Standards Institute guidelines. The *ERG11* gene of the known fluconazole-resistant strains of *C. albicans* was amplified by polymerase chain reaction (PCR). In addition, the samples were subjected to sequencing and mutation analysis.

**Results:** A total of 60 *Candida* species were isolated from HIV patients and were speciated using standard, conventional, and molecular methods. *Candida albicans* comprised 28.3% (n=17) of the *Candida* isolates, 59% (n=10) of which were resistant to fluconazole. Sequencing of the amplified product of *ERG11 C. albicans* gene isolates showed that they were highly mutated and included many nonsense mutations which were not reported earlier.

**Conclusion:** The molecular characterization of *ERG11* gene showed many missense and nonsense mutations. Such mutations, which were unique to the geographical area under investigation, could be concluded to account for the development of resistance to fluconazole.

**Keywords:** AIDS, Antifungal resistance, *Candida* species, Candidiasis, *ERG11*, Fluconazole, Mutation

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## Introduction

*Candida* species is a commensal and important opportunistic human pathogen which causes common ailments, such as oral thrush, vaginitis, and invasive infections, in immunocompromised patients. Presently, *Candida* species is the fourth most common cause of nosocomial bloodstream infections (BSIs). Moreover, it is the third leading causative agent of catheter-associated bloodstream infection in the United States with a mortality rate of up to 49% [1-5]. In the European and North American Intensive Care Units, it is the most important fungal infection, ranked after *Staphylococcus aureus* [6]. Furthermore, *Candida* is the first among the top ten bloodstream pathogens despite regional differences [7]. Extensive use of prophylactic azole therapy for chronic infections,

especially in patients under long-term antibiotic therapy, as well as steroids or chemotherapy, has resulted in an increase in azole resistance.

Azoles are preferred antifungal agents given their inexpensiveness, limited toxicity, and potentiality for oral administration. Azoles inhibit the target enzyme lanosterol 14 $\alpha$ -demethylase, resulting in impaired ergosterol synthesis, thereby disrupting the fungal cell membrane. The *Erg11p*, a member of cytochrome P450 superfamily, is essential for ergosterol synthesis. It has 528 amino acids with 13  $\alpha$  helices from A to M and several  $\beta$  pleated sheets. The *Erg11p* active center is located deep inside the protein, near the heme chromophore between helices I and L. The substrate interacts with a long access channel and is then demethylated [8-12]. Azoles block this process and

inhibit ergosterol synthesis. The *ERG11* contains 1851 bp, with the transcription start codon being located at 148-150 bp and stop codon at 1732-1734 bp [13].

There are multiple mechanisms for azole resistance. The first is based on the changes in the molecular configuration of the target enzyme 14 $\alpha$ -demethylase (*Erg11p*) due to mutations in the encoding gene *ERG11*. It can reduce the affinity between azole and protein, often resulting in azole resistance. Point mutation occurrence is associated with an increase in the minimum inhibitory concentration (MIC) of azoles. In addition, the upregulation of *ERG11*, *Candida* drug resistance genes (i.e., *CDR1* and *CDR2*), and multiple drug resistance genes (e.g., *MDR1*) can effectively decrease intracellular drug accumulation. In addition, biofilm is another mediator for antifungal resistance. Sequestration of fluconazole within intracellular vacuoles can be another novel mechanism of resistance. With this background in mind, the present study was conducted to investigate the molecular characterization of *ERG11* gene of certain fluconazole-resistant *C. albicans* and identify mutations that may account for fluconazole resistance.

## Materials and Methods

### Isolates and type strains

The study was conducted after the approval of Institutional Ethics Committee (IEC24/March 2016) of Tagore Medical College Hospital and Government Hospital of Thoracic Medicine. In line with the ethical principles of research, informed consent was obtained from all participants. A total of 80 samples were included in the study, out of which *Candida* species (n=60) were isolated. The samples included oropharyngeal swabs, nail, sputum, and blood from patients with clinical indicator conditions for HIV. For the purpose of the study, *C. albicans* (MTCC3017), *C. glabrata* (MTCC3019), *C. krusei* (MTCC 9215), and *C. tropicalis* (MTCC3421) were obtained from Microbial Type Culture Collection, Chandigarh, India, to be used as control strains.

### Media, Drugs, and Reagents

Sabouraud's dextrose agar (SDA; HiMedia, India) was prepared according to the manufacturer's instructions. In this study, CHROM agar (HiMedia, India) was prepared as recommended by the manufacturer. In addition, species identification was accomplished using corn meal agar (CMA; HiMedia, India) with Tween 80, yeast nitrogen base (YNB; HiMedia, India) for carbohydrate assimilation, and liquid medium for sugar fermentation test with 2% carbohydrates solution of dextrose, maltose, sucrose, lactose, maltose, and urease. Mueller Hinton agar (MHA; HiMedia, India) with 2% glucose and methylene blue (0.5  $\mu$ g/ml) and Roswell Park Memorial Institute (RPMI) 1640 broth (with glutamine and without bicarbonate and with phenol red indicator; Himedia, India) were used for antifungal susceptibility

testing. Furthermore, yeast peptone dextrose (YPD; HiMedia, India) broth was utilized for DNA extraction.

In this study, fluconazole (25  $\mu$ g; Oxoid<sup>TM</sup>, India), voriconazole (1  $\mu$ g; Oxoid<sup>TM</sup>, India), caspofungin (e-strip; HiMedia EM119, India), fluconazole (e-strip; HiMedia EM072, India), and amphotericin B (Sigma Aldrich) powder were included. In addition, glucose, sucrose, lactose, maltose, trehalose, galactose, and xylose discs supplied from HiMedia were used for assimilation tests. The DNA extraction was performed using HiPuraYeast DNA kit made by Himedia.

### Isolate Identification

Clinical specimens were inoculated onto SDA slants. The isolates were speciated by CHROM agar (Himedia, India), germ tube formation at 37°C, production of chlamyospore in CMA, urease production at 25°C, growth at 45°C, sugar assimilation, and fermentation tests. Internal transcribed spacer (ITS) 1, 4 sequencings were accomplished for the identification of inconclusive strains using fungal primers for ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC -3') (Eurofins India Pvt Ltd), amplifying the ITS region of the ribosomal subunit [14].

### Antifungal Susceptibility Testing

Susceptibility testing was carried out according to the Clinical Laboratory Standards Institute (CLSI) M44. It is a reference document for antifungal disc diffusion test of yeasts [15]. Sensitivity to fluconazole and voriconazole was evaluated by disc diffusion assays in modified MHA. All the fluconazole-resistant strains were tested for MIC using fluconazole e-strips; additionally, the MICs of caspofungin were also evaluated by e-strips. Susceptibility pattern against amphotericin B was tested by broth dilution method in RPMI 1640 broth, according to the CLSI document M27 A3. In addition, the antifungal susceptibility testing of the yeasts was accomplished using the reference method for broth dilution [16]. In addition, the standard strains of *C. albicans* (MTCC 3017), *C. glabrata* (MTCC 3019), *C. krusei* (MTCC 9215), and *C. tropicalis* (MTCC 3421) were included as quality controls.

### DNA Extraction

Fluconazole-resistant *C. albicans* strains were grown on SDA at 37°C for 24 h. The strains were inoculated onto YPD broth and incubated for 18-24 h. Total genomic DNA from resistant *C. albicans* isolates was extracted using the HiPure yeast DNA kit according to the manufacturer's instructions. DNA concentrations and DNA/RNA ratio were measured by BioPhotometer D30 (ependorf).

### Polymerase chain reaction amplification and gene sequences of *ERG11* gene

The *ERG11* genes were amplified by polymerase

chain reaction (PCR; Bio-Rad) using the DNA templates of fluconazole-resistant *C. albicans* which were isolated from primer sequences, namely forward 5'GTT GAA ACT GTC ATT GAT GG 3' and reverse 5'TCA GAA CAC TGA ATC GAA AG 3' (Eurofins, India, Pvt Ltd) [13]. For all PCRs, a 25- $\mu$ L mixture contained 12.5  $\mu$ L PCR master mix, 2.5  $\mu$ L genomic DNA, 2.5  $\mu$ L of each both primer and 5  $\mu$ L deionized water. The thermocycling conditions for PCR reactions included 30 cycles at 92°C for 3 min, 92°C for 1 min, 55°C for 2 min, and 72°C for 1 min, followed by an extension step at 72°C for 10 min.

The PCR product size was analyzed preliminarily by agarose gel electrophoresis (1.5% concentration). Consequently, they were visualized and analyzed in Gel Doc XR+ (Bio Rad). The amplified products of the isolates were purified and sequenced with an ABI 3730 XL DNA analyzer (Applied Biosystems) using standard protocols (Big Dye Terminator v3.1 cycle sequencing Kit-Applied Biosystems) and previously designed primers [14]. For each strain, the entire *ERG11* open reading frame sequence was compared with *ERG11* sequences from the National Center for Biotechnology Information (NCBI).

## Results

### Species identification

A total of 60 *Candida* species were isolated from 80 clinical specimens. Among the 60 isolates, 16 (26.7%) cases were *C. albicans*, and the remaining 44 (73.3%) isolates were non-*albicans Candida* species. *Candida tropicalis* was identified as the predominant non-*albicans Candida* species (30%). The distribution of *Candida* isolates is presented in Table 1.

### Antifungal susceptibility test

In this study, 73.3% (n=44) of the *Candida* isolates were resistant to fluconazole. The sensitivity pattern differed among isolates. *Candida glabrata* and *C. tropicalis* showed increased MICs. The antifungal sensitivity patterns of the *Candida* isolates are shown in Table 2. All *C. albicans* isolates showed 100% sensitivity to amphotericin B and caspofungin. The MIC breakpoints of the fluconazole-resistant strains

ranged from > 8 to > 256  $\mu$ g/ml. All the isolates were sensitive to amphotericin B, and only 3 non-*albicans Candida* isolates were resistant to caspofungin. The MICs of amphotericin B and caspofungin had a range of 0.25-1 to 0.012-2  $\mu$ g/ml, respectively.

Over the last few decades, fungal infections are increasingly alarming, posing great challenges to healthcare professionals. Increase in the number of immunocompromised patients has been significantly contributed to a rise in candidiasis. Due to the variable clinical entities of candidiasis, it is highly important to identify this pathogen in all routine culture specimens received at the laboratory, irrespective of clinician's suspicion. There are also some reports regarding the emergence of non-*albicans Candida* species as an important pathogen. Hence, the identification of species level, along with antifungal susceptibility, becomes essential as species differ in antifungal susceptibility pattern.

In the present study, 75% of the HIV-seropositive patients suffered from one or other entities of candidiasis. Earlier studies also reported a high incidence of candidiasis in HIV-seropositive patients [17-22]. Although in most studies, *C. albicans* remains the predominant species, in this study, non-*albicans Candida* species outnumbered *C. albicans* in concordance with earlier investigations [23, 24].

### Molecular characterization of *ERG11* gene of *Candida albicans*

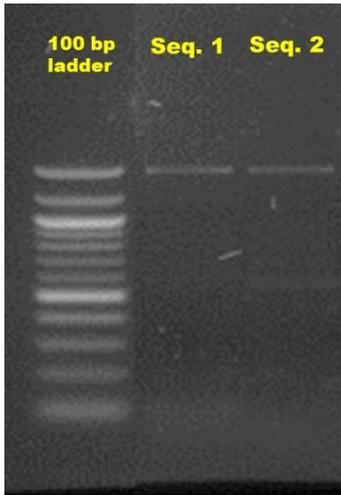
In the current study, the *ERG11* gene of fluconazole-resistant *C. albicans* was amplified. All the resistant isolates showed mutations in *ERG11* gene, in particular, two sequences exhibited extensive mutations. The *ERG11* coding region amplified by

**Table 1.** Distribution of *Candida* isolates among HIV patients

Species	Total isolates
<i>C. albicans</i>	17 (28.3%)
<i>C. tropicalis</i>	18 (30%)
<i>C. krusei</i>	9 (15%)
<i>C. parapsilosis</i>	6 (10%)
<i>C. glabrata</i>	5 (8.3%)
Other <i>Candida</i> species	5 (8.3%)

**Table 2.** Antifungal susceptibility pattern of *Candida* species

<i>Candida species</i>	Fluconazole			Voriconazole			Amphotericin B			Caspofungin		
	S	SDD	R	S	SDD	R	S	SDD	R	S	SDD	R
<i>C. albicans</i>	1	2	14	10	2	5	16	0	1	15	1	1
<i>C. tropicalis</i>	1	5	12	12	3	3	17	1	0	17	0	1
<i>C. krusei</i>	0	0	9	5	2	2	8	1	0	8	1	0
<i>C. glabrata</i>	3	1	1	5	0	0	5	0	0	5	0	0
<i>C. kefyr</i>	0	0	1	1	0	0	1	0	0	1	0	0
<i>C. parapsilosis</i>	1	2	3	5	0	0	6	0	0	6	0	0
<i>C. dubliniensis</i>	0	0	1	1	0	0	1	0	0	1	0	0
<i>C. orthopsilosis</i>	0	0	1	1	0	0	1	0	0	1	0	0
<i>K. ohmeri</i>	0	0	1	0	0	1	1	0	0	1	0	0
<i>H. opuntiae</i>	0	0	1	1	0	0	-	-	-	1	0	0
<b>Combined Data</b>												
<i>C. albicans</i>	5	2	10	10	2	5	16	0	1	15	1	1
Non- <i>albicans Candida</i> species	5	8	30	31	6	6	40	2	0	41	1	1
<i>P</i>	>0.05			>0.05			>0.05			>0.05		



**Figure 1.** Agarose gel electrophoresis of polymerase chain reaction amplified products of *ERG11* gene

PCR was 1230 and 1160 bp long for sequences 1 and 2, respectively. Each PCR product showed a clear band on ethidium bromide that stained in agarose gel electrophoresis (Figure 1). The sequenced gene was also 1230 and 1165 bp long, respectively. A BLASTP (NCBI) search for both sequences was performed to find their identity. The sequences 1 and 2 showed a high similarity with the *ERG11* gene of *C. albicans* with 93% (E-value=0) and 92% (E-value=0) identity, respectively.

**Molecular analysis of *ERG11* gene mutations**

The *ERG11* gene sequences were subjected to multiple sequence alignment (MSA) using the online server tool Toffee with the wild type gene sequence GenBank accession number AF153844. The MSA of sequences 1 and 2 showed that they were highly mutated by possessing all types of mutations. Moreover, in all three translation frames, it possessed many stop codons (i.e., nonsense mutation). Frames 1, 2, and 3 of sequence 1 showed 37, 12 and 34 stop codons, respectively (Figure 2). Therefore, it is evident that gene can produce only a truncated polypeptide chain, thereby resulting in aberrant protein. The MSA of sequence 2 revealed that it also possessed many stop codons. In frames 1, 2, and 3, it showed 32, 36, and 10 stop codons, respectively (Figure 3). Hence, this gene-translated product also may result in a truncated protein.

The proper functional coding sequence of the *ERG11* gene of both strains was prepared by trimming and translated to protein sequence in Expsay online translate server tool. These two protein sequences were subjected to BLASTP. They showed similarity with lanosterol 14 $\alpha$ -demethylase, the gene product of *ERG11* with 94% identity (E-value=1e<sup>-156</sup>) and 93% (E-value=2e<sup>-154</sup>) for sequences 1 and 2, respectively. The missense mutations of both translated products were also analyzed. Table 3 shows various missense mutations present in the translated products of both

```

5'3' Frame 1
RGRAT MetQL MetINFL StopDDFLFSAQNGIFRFIKKI Stop SSIIVLLDSLVLV Stop FCSFKWTTTL Stop IFRI MetSSKVV Stop CIFI Stop VIRENYDGL
FRSKRS StopICFQC StopII Stop CFC StopRCL Stop TFNYSSFR Stop RGYL Stop LSKF Stop INGTKKIC Stop ICFDY Stop FI Stop KICS Stop D Stop RRN
FELFCY Stop Stop KFKIERKNSRGCCQYENSTRNYFHFCKFIW Stop Stop NEKNF Stop PFICSIIF Stop FR Stop RFPY Stop FCFP Stop FTFSTL
LET Stop CCSKENLGYLYERN Stop TEKRPCDIDPNRDLIDSLIHSTYKDGVK MetTDQKLLIF Stop LVFLWVNNLLLLLVTFSEKPHLQ
MetSFIKSCDYEESGDLND Stop L Stop DYKIPSVIHL Stop KL Stop MetH MetPYFYLERYYHKNP Stop TIIVQRSYVKSRLCSYYAEFDT Stop DLHIY
ESLAKPIWFITSREKL

5'3' Frame 2
AGELRCN Stop Stop SISCR MetTFYSVLR MetEYLDLRLRYRAPLLFYWIPWFDASAASNGQQPYEFLESCPKYGDVFSRLLGKI MetTVYLG
KGFHEFVNAKLDVSAEDAYKHLTPVFGKGVYDPCNSRL MetEQKFAKALTTDSFKRYVPKIREILNYFVTDESFKLKEKTHGVANV
MetKTOPEITIFTASRSLFGDE MetRRIFDRSFAQLYDLDKGFPIINFVFPNPLPHYWRRDAAQKKISATY MetKEIKLRDRVILIQIV Stop LI
PY Stop FIQLIK MetV Stop K Stop LIRNC Stop SFNWYSYGWSTYFCYFCLFLHLVKNLIYRCHLSRVVI MetRKVV Stop MetIDYEITKYHQSYYT
NSRCICHTS Stop KGTTRILEQ Stop LSKGHTLSPGYAHT MetQLIPEI Stop ST MetNRSLSQSGS Stop PLERS

5'3' Frame 3
RASYDAIDDQFPVG Stop LSIQCSEWNI Stop IH Stop ENIELHYCFIGFLGLILQLQ MetDNNL MetNF Stop NHVLKS MetV MetV FHLGY Stop GKL Stop
RFI Stop VQKV MetNLF MetL NYL MetFLK MetLINI Stop LLQFSVKGLF MetIVQILD Stop WNKKNLLNLL Stop LLIHLKD MetFLRLEKKE Stop IILL
MetKVSNI Stop KKKLTGLP MetL Stop KLNQKLLFSLLDLYLV MetK Stop EEFLLVHLLNYILI Stop IKVLP LLLIFSLIYLYLIIGDV MetLLKRKSLLL
Stop KKLN Stop EETV Stop Y Stop SKS Stop FN Stop FLIDSFNL Stop RWCEND Stop SEIANLLIGIL MetGGQHTSASTSACSCYI Stop Stop KTSFTDVI
YQEL Stop L Stop GKW Stop FE Stop LT MetRLQNTISHTLIETLDAYAILLFRKVLV Stop ESLNNCNPKVIR Stop VQV MetLILCRI Stop YLRFDPL Stop I
AR Stop ANLVHNSREV
    
```

**Figure 2.** Translated sequences in three different frames in Expsay Translate tool of sequence 1 showing stop codons

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5'3' Frame 1
EWNI Stop IH Stop ENIELHYCFIGFLGLILQLQ MetDNNL MetNFWNHVLKS MetV MetV FH Stop GY Stop GKLGRFI Stop VRKV MetNVFS MetLNYL Met
FLK MetLINI Stop LLQFSVKGLF MetIVQILD Stop WNKKNLLNLL Stop LLIHLKD MetFLRLEKKE Stop IILL MetKVSNI Stop KKKLTGLP MetL Stop
KLNQKLLFSLLDLYLV MetK Stop EEFLLVHLLNYILI Stop IKVLP LLLIFSLIYLYLIIGDV MetLLKRKSLLL Stop KKLN Stop EETV Stop Y Stop SK
S Stop FN Stop FLIDSFNL Stop RWCEND Stop SEIANLLIGIL MetGGQHTSASTSACSCYI Stop Stop RTSFTDVIYQEL Stop L Stop GKW Stop FE Stop L
MetRLQNTISHTLIETLDAYAILLFRKLLP Stop ESLNNCNPKVIR Stop VQV MetLILCRI Stop YLRFDPL Stop IAR Stop ANLVHNSREV

5'3' Frame 2
NGIFRFIKKI Stop SSIIVLLDSLVLV Stop FCSFKWTTTL Stop IFGI MetSSKVV Stop CIFI VIREN Stop DGLRSRERS Stop MetCFLC Stop II Stop CFC
Stop RCL Stop TFNYSSFR Stop RGYL Stop LSKF Stop INGTKKIC Stop ICFDY Stop FI Stop KICS Stop D Stop RRN FELFCY Stop Stop KFKIERNNSRGC
CCYENSTRNYFHFCKFIW Stop Stop NEKNF Stop PFICSIIF Stop FR Stop RFPY Stop FCFP Stop FTFSTLLET Stop CCSKENLGYLYERN Stop L
EKRPCDIDPNRDLIDSLIHSTYKDGVK MetTDQKLLIF Stop LVFLWVNNLLLLLVTFSEKPHLQ MetSFIKSCDYEESGDLND Stop L Stop
DYKIPSVIHL Stop KL Stop MetH MetPYFYLESYYHKNP Stop TIIVQRSYVKSRLCSYYAEFDT Stop DLHIYESLAKPIWFITSREKL

5'3' Frame 3
MetEYLDLRLRYRAPLLFYWIPWFDASAASNGQQPYEFLESCPKYGDVFSRLLGKI RTVYLGPKGHECVFYAKLSDVSAEDAYKHLTPV
FGKGVYDPCNSRL MetEQKFAKALTTDSFKRYVPKIREILNYFVTDESFKLKEKTHGVANV MetKTOPEITIFTASRSLFGDE MetRRIF
RSFAQLYDLDKGFPIINFVFPNPLPHYWRRDAAQKKISATY MetKEIKLRDRVILIQIV Stop LIPY Stop FIQLIK MetV Stop K Stop LIRNC Stop
SFNWYSYGWSTYFCYFCLFLHLVKNLIYRCHLSRVVI MetRKVV Stop MetIDYEITKYHQSYYTNSRCICHTS Stop KATTIRILEQ Stop LFK
GHTLSPGYAHT MetQLIPEI Stop ST MetNRSLSQSGS Stop PLERS
    
```

**Figure 3.** Translated sequences in three different frames in Expsay Translate tool of sequence 2 showing stop codons

**Table 3.** Missense mutations of *Candida albicans* in the translated product of *ERG11* sequences 1 and 2

<b>Seq1</b>	Y18D, D23Y, V28L, G36D, Y41N, F49L, R53P, M63R, E243D, 245-283 missense mutations
<b>Seq2</b>	Y3D, D8Y, V13L, G21D, Y26N, F34L, R38P, F47L, M48G, M54R, F65C, N68Y, K142T, E229D

sequences.

### Nucleotide Sequence Accession Number

In this study, the *ERG11* gene sequences of two *C. albicans* clinical isolates were deposited in the GenBank database under the accession numbers of MF155016 and MF175064.

### Discussion

The purpose of the molecular characterization of *ERG11* gene in the present study was to understand the molecular mechanism of fluconazole resistance in *C. albicans* isolated from two HIV-infected patients. The sequences of the *ERG11* gene of two *C. albicans* species in the present study showed that they are highly mutated and possess all types of mutations; therefore, the mutation may be responsible for the development of fluconazole resistance. Many similar investigations addressing *ERG11* gene mutation reported different types of point mutations [12, 25]. However, these studies showed only a limited number of mutations, especially missense mutations.

However, in the present study, the *ERG11* gene of both strains of *C. albicans* demonstrated many nonsense mutations, along with a huge number of missense mutations. Hence, it is clear that the *ERG11* of these two strains produces a defective enzyme and contributes to fluconazole resistance property [13, 17]. The sterol 14 alpha-demethylase enzyme produced by mutated *ERG11* gene has an altered conformation which makes the enzyme to lose its ability to bind to azoles [26]. It can also be considered as a screening tool to identify resistant strains.

### Conclusion

The molecular characterization of *ERG11* gene showed an extensive mutation that may contribute to the resistance of *C. albicans* to fluconazole. Furthermore, the high prevalence of fluconazole-resistant strains of *C. albicans* in this particular geographical area in HIV patients indicating the presence of *ERG11* mutated strains.

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### Author's contribution

S. P. performed sample collection and molecular characterizations. I. K. carried out the bioinformatics work. Study design was conducted by K. M.

### Conflicts of interest

The authors report no conflicts of interest. The

authors alone are responsible for the content and writing of the paper.

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