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MOLECULAR CHARACTERIZATION OF ERG11 GENE IN TRIAZOLE RESISTANT CANDIDA ALBICANS ISOLATED FROM A TERTIARY CARE HOSPITAL

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Abstract

Treatment options for Candidiasis include polyenes, azoles, echinocandins, nucleoside analogues, and allylamines, but drug resistance is a growing problem. Some species, like C. krusei and C. glabrata, exhibit high resistance to fluconazole and azoles. Accurate identification of Candida species and their susceptibility patterns is crucial for effective treatment. Mutations in the ERG11 gene, responsible for lanosterol 14α-demethylase enzyme production, lead to azole drug resistance, reducing their efficacy. This study focuses on identifying ERG11 gene mutations, offering prognostic and therapeutic significance in preventing drug resistance emergence.

The triazole resistant Candida albicans isolates were selected and the DNA is extracted. ERG11 gene is amplified and sequenced to find point mutations and the substituted amino acids by using ABI 3500 XL genetic analyzer.

A total of 83 mutations in the ERG11 gene of 10 isolates of C. albicans which were resistant to Fluconazole, Voriconazole and Itraconazole were detected in this study. Of the 83 mutations, 56 mutations resulted in change in amino acid (missense mutations) and 27 were silent mutations where the change in nucleotide sequence did not result in any change in the amino acid. D116E, E266D and G464S mutations occurred 6 times each in the 10 isolates. K128T, I147T mutations were found in 4 isolates. A114S, S405F, T229A, G465S, K143E mutations were found in 3 isolates. R467K, R523G, V488I, W520C mutations were found in 2 isolates.

This study further establishes that the ERG11 gene point mutations are one of the major causes of azole antifungal drug resistance in Candida albicans.

Key words : Candida albicans, Candidiasis, ERG11, Mutations

Introduction

There has been a substantial increase in the incidence of Candidiasis in the recent past. This has lead to increased morbidity and mortality. Candida albicans and Non-Albicans Candida (NAC)

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species like C. tropicalis, C. paeapsilosis, C. galbrata, C. krusei and C. auris are some of the major causative agents of Candidiasis. Despite the

Candidiasis pose a huge risk to both individuals with compromised immune systems and those who are otherwise healthy[1]. Candida species are also a leading cause of nosocomial infections. They evade immune defenses, invade tissues, and cause severe infections, often facilitated by medical devices. Factors such as the use of broad-spectrum antibiotics, indwelling catheters, chemotherapy, and parenteral nutrition contribute to the growth and invasion of Candida. However, the emergence of multidrug-resistant strains, such as Candida auris, presents a significant challenge, especially for critically ill patients. It is worth noting that the distribution of Candida species can vary between multicenter studies and single-center studies.

The commonly used drug classes for treating Candidiasis include polyenes, azoles, echinocandins, nucleoside analogues, and allylamines. Candida species exhibit variations in virulence and drug sensitivity patterns. For example, C. krusei, one of the NAC, is inherently resistant to fluconazole as the affinity to ERG11 and fluconazole is low. Studies have shown high rates of resistance among C. krusei and C. glabrata to fluconazole and azoles, respectively[2]. It is crucial to identify the Candida species accurately before initiating treatment, as empirical therapy can prolong the disease and lead to increased morbidity and mortality. Determining the susceptibility pattern of Candida species to antifungal agents is essential for selecting the appropriate and targeted therapy.

As there are a limited number of antifungal drugs available to treat Candidiasis, the growing drug resistance poses a significant problem in the treatment. One of the major mechanisms which make the Candida spp. azole drug resistant is the mutations in ERG11 gene. This gene codes for the enzyme lanosterol 14α-demethylase which is required for ergosterol synthesis.

As the point mutations in the ERG11 gene result in a modified ERG11 protein (ERG11p), it reduces the efficacy of azoles leading to increased minimum inhibitory concentration or resistance[3]. Advancements in molecular biology now allow us for the identification and characterization of these mutations, providing insights into drug resistance mechanisms. This study aims to identify point mutations in ERG11 gene which are one of the major reasons for azole antifungal resistance. Such identification offers prognostic and therapeutic significance, enabling clinicians to select appropriate antifungal agents and prevent the emergence of drug resistance.

Materials and Methods

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An observational cross sectional study was done from 2019-2022 at Rohilkhand Medical College, Bareilly. A total of ten Candida albicans isolates which are resistant to Fluconazole, Voriconazole and Itraconazole are obtained from clinical samples.

These isolates are further analyzed for mutations in the ERG11 gene. The yeast DNA was extracted using HiPuraYeast DNA kit according to the manufacturer's instructions. The extracted DNA is subjected to polymerase chain reaction (PCR) in order to amplify the ERG11 gene. The primers used for the amplification were Forward - 5′-CAAGAAGATCATAACTCAAT 3′ and Reverse - 5′-AGAACACTGAATCGAAAG 3′[4]. For the PCR reaction, each well is loaded with 15 µL of Big dye direct PCR master mix and 5 µL of extracted Candida DNA. The thermal cycling conditions were an initial holding period of 5 minutes at 96 °C, followed by denaturation at 94°C for 30 seconds, annealing at 62°C for 45 seconds and extension at 68°C for 45 seconds repeated for 35 cycles followed by a final extension at 72°C for 4 minutes[4]. The final PCR product size and quality were analyzed by 1.5% agarose gel electrophoresis.
The resultant amplified product is cleaned up and sequenced with ABI 3500 XL Genetic Analyzer (Applied Biosystems™) using Big Dye Terminator 3.1 cycle sequencing kit (Applied Biosystems). The sequences of the ERG11 genes were compared with the triazole sensitive ATCC MYA-2876 SC5314 sequence (GenBank X13296) with the help of Molecular Evolutionary Genetics Analysis software version 11.

Results

A total of 83 mutations in the ERG11 gene of 10 isolates of C. albicans which were resistant to Fluconazole, Voriconazole and Itraconazole were detected in this study. Of the 83 mutations, 56 mutations resulted in change in amino acid (missense mutations) and 27 were silent mutations where the change in nucleotide sequence did not result in any change in the amino acid. Refer Table 1, 2 and 3 for the list of amino acid changes, missense and Silent mutations respectively. D116E, E266D and G464S mutations occurred 6 times each in the 10 isolates.

Strain	Site of isolation	FLU MIC	VOR MIC	ITR MIC	Amino acid substitution

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		µg/mL	µg/mL	µg/mL	
CA01	Respiratory	8	2	1	D116E, K128T, K143E, T229A, I471T, G465S
CA02	Urine	16	4	2	A114S, K119L, Y257H, K344E, R523G, Y132H, S405F, I471T
CA03	Respiratory	4	1	1	D116E, E266D, G464S, G465S K119L, F145L
CA04	Respiratory	8	4	4	A114S, K128T, K344E, R523G, R467K, G464S
CA05	Blood	16	2	4	D116E, K128T, E266D, K143E, T229A, G464S, G465S
CA06	Respiratory	16	2	2	D116E, E266D, I471T, G464S
CA07	Respiratory	8	4	1	A114S, E266D, D116E, S405F, I471T
CA08	Blood	16	2	2	W520C, R467K, G464S, K128T
CA09	Blood	4	1	4	E266D, V488I, W520C, S405F
CA10	Blood	16	4	2	D116E, E266D, P375A, K143E, T229A, G464S

Table 1. List of amino acid changes in the C. albicans isolates along with their MIC to azoles

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Fig1. Point mutations in ERG11 gene isolated from Candida albicans in comparison with Reference strain X13296

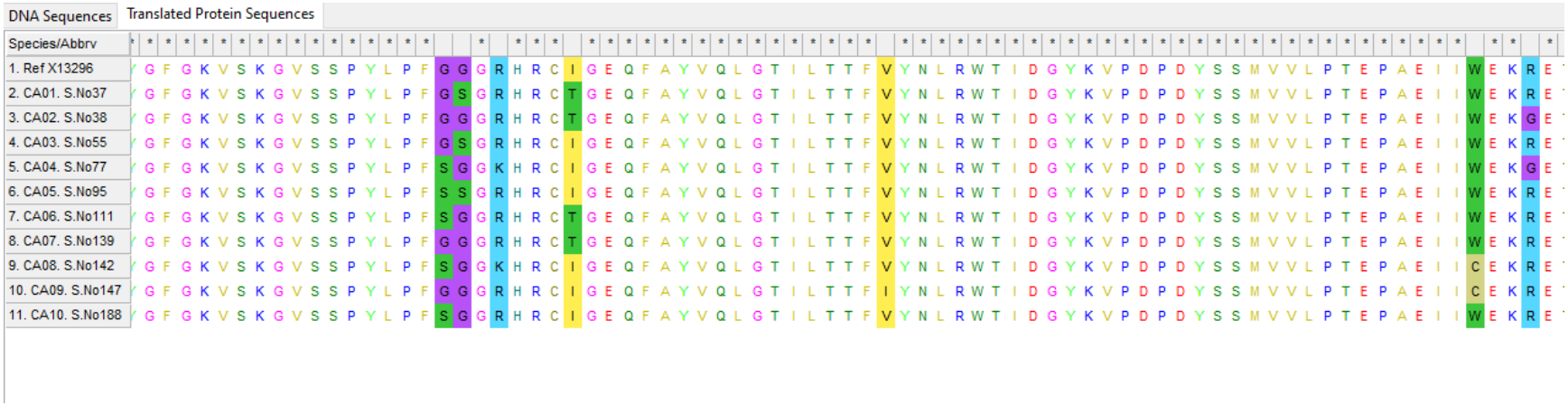

Fig 2. Amino acid substitutions in Erg11 protein in comparison with the reference strain X13296

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Isolate ID	Sample	Risk Factor	FLU MIC µg/ml	VOR MIC µg/ml	ITR MIC µg/ml	Site of nucleotide mutation	Nucleic Acid Mutation	Amino Acid Substitution	Change in ERG11 p
CA01	Respiratory sample	Surgery	8	2	1	348	GAT→GAA	Aspartate → Glutamate	D116E
						383	AAA→ACA	Lysine→Threonine	K128T
						427	AAA→GAA	Lysine→Glutamate	K143E
						1393	GGT→AGT	Glysine→Serine	G465S
						687	ACC→GCC	Threonine→Alanine	T229A
						1412	ATT→ACT	Isoleucine→Threonine	I471T
CA02	Urine	Pregnancy	16	4	2	340	GCT→TCT	Alanine→Serine	A114S
						769	TAT→CAT	Tyrosine→Histidine	Y257H
						1030	AAA→GAA	Lysine→Glutamate	K344E
						1567	AGG→GGG	Arginine→Glycine	R523G
						394	TAT→CAT	Tyrosine→Histidine	Y132H
						1214	TCT→TTT	Serine → Phenyl Alanine	S405F
						1412	ATT→ACT	Isoleucine→Threonine	I471T
CA03	Respiratory sample	Biomedical device	4	1	1	348	GAT→GAA	Aspartate→Glutamate	D116E
						1393	GGT→AGT	Glysine→Serine	G465S
						798	GAA→GAC	Glutamate→Aspartate	E266D
						1390	GGT→AGT	Glysine→Serine	G464S
						355	AAA→TTA	Lysine→Leucine	K119L
						435	TTT→TTA	Phenyl Alanine→Leucine	F145L

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Isolate ID	Sample	Risk Factor	FLU MIC µg/ml	VOR MIC µg/ml	ITR MIC µg/ml	Site of nucleotide mutation	Nucleic Acid Mutation	Amino Acid Substitution	Change in ERG11 p
CA04	Respiratory sample	Steroids	8	4	4	340	GCT→TCT	Alanine→Serine	A114S
						383	AAA→ACA	Lysine→Threonine	K128T
						1030	AAA→GAA	Lysine→Glutamate	K344E
						1567	AGG→GGG	Arginine→Glycine	R523G
						1400	AGA→AAA	Arginine→Lysine	R467K
						1390	GGT→AGT	Glysine→Serine	G464S
CA05	Blood	Surgery	16	2	4	348	GAT→GAA	Aspartate→Glutamate	D116E
						383	AAA→ACA	Lysine→Threonine	K128T
						1393	GGT→AGT	Glysine→Serine	G465S
						798	GAA→GAC	Glutamate→Aspartate	E266D
						427	AAA→GAA	Lysine→Glutamate	K143E
						687	ACC→GCC	Threonine→Alanine	T229A
						1390	GGT→AGT	Glysine→Serine	G464S
CA06	Respiratory sample	Steroids	16	2	2	348	GAT→GAA	Aspartate→Glutamate	D116E
						383	AAA→ACA	Lysine→Threonine	K128T
						798	GAA→GAC	Glutamate→Aspartate	E266D
						1412	ATT→ACT	Isoleucine→Threonine	I471T
						1390	GGT→AGT	Glysine→Serine	G464S
CA07	Respiratory sample	Steroids	8	4	1	340	GCT→TCT	Alanine→Serine	A114S
						798	GAA→GAC	Glutamate→Aspartate	E266D
						348	GAT→GAA	Aspartate→Glutamate	D116E
						1214	TCT→TTT	Serine → PhenylAlanine	S405F
						1412	ATT→ACT	Isoleucine→Threonine	I471T

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Isolate ID	Sample	Risk Factor	FLU MIC µg/ml	VOR MIC µg/ml	ITR MIC µg/ml	Site of nucleotide mutation	Nucleic Acid Mutation	Amino Acid Substitution	Change in ERG11 p
CA08	Blood	Biomedical device	16	2	2	1464 1560 1400 1390	GTT→ATT TGG→TGC AGA→AAA GGT→AGT	Valine→Isoleucine Tryptophan→Cystiene Arginine→Lysine Glysine→Serine	V488I W520C R467K G464S
CA09	Blood	Biomedical device	4	1	4	798 1464 1560 1214	GAA→GAC GTT→ATT TGG→TGC TCT→TTT	Glutamate→Aspartate Valine→Isoleucine Tryptophan→Cystiene Serine → PhenylAlanine	E266D V488I W520C S405F
CA10	Blood	Biomedical device	16	4	2	348 798 1123 427 687 1390	GAT→GAA GAA→GAC CCA→GCA AAA→GAA ACC→GCC GGT→AGT	Aspartate→Glutamate Glutamate→Aspartate Proline→Alanine Lysine→Glutamate Threonine→Alanine Glysine→Serine	D116E E266D P375A K143E T229A G464S

FLU = Fluconazole VOR= Voriconazole ITR= Itraconazole A= Adenine T = Thymine G = Guanine C= Cytosine MIC= Minimum Inhibitory Concentration. Amino Acids are represented by the standard single letter codes

Table 2. List of point mutations and the corresponding amino acid changes in C. albicans resistant to Azoles.

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S.No	Isolate ID	Site of nucleotide mutation	Nucleic Acid Mutation	Amino Acid Substitution	Change in ERG11 p
1	CA01	558 1167	TCC→TCT TTA→TTG	Serine→Serine Leucine→Leucine	NC NC
2	CA02	--	--	--	--
3	CA03	--	--	--	--
4	CA04	462 558	TTT→TTC TCC→TCT	Phenyl Alanine→Phenyl Alanine Serine→Serine	NC
5	CA05	558 1167	TCC→TCT TTA→TTG	Serine→Serine Leucine→Leucine	NC
6	CA06	462 1143 504 805	TTT→TTC GTT→GTC AAA→AAG CTA→TTA	Phenyl Alanine→Phenyl Alanine Valine→Valine Lysine→Lysinr Leucine→Leucine	NC
7	CA07	462 504 1443 1449 805	TTT→TTC AAA→AAG GCC→GCT GCT→GCC CTA→TTA	Phenyl Alanine→Phenyl Alanine Lysine→Lysine Alanine→Alanine Alanine→Alanine Leucine→Leucine	NC
8	CA08	558 1443 805	TCC→TCT GCC→GCT CTA→TTA	Serine→Serine Alanine→Alanine Leucine→Leucine	NC
9	CA09	558 1143 1443 1449 1350	TCC→TCT GTT→GTC GCC→GCT GCT→GCC TAT→TAC	Serine→Serine Valine→Valine Alanine→Alanine Alanine→Alanine Tyrosine→Tyrosine	NC
10	CA10	462 558 1350 1143	TTT→TTC TCC→TCT TAT→TAC GTT→GTC	Phenyl Alanine→Phenyl Alanine Serine→Serine Tyrosine→Tyrosine Valine→Valine	NC

Table 3. List of silent mutations in C. albicans resistant to azoles

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Discussion

Fungal infections have consistently posed a challenge for patients in hospital settings. However, over the past three decades, there has been a significant surge in fungal infections, particularly those attributed to Candida species other than the albicans variety[5]. The rising occurrence of iatrogenic Candida infections and infections in individuals with weakened immune systems can be attributed to a combination of fungal virulence factors and host vulnerability. In individuals with a normally functioning immune system, several factors can make them more susceptible to fungal infections, including extended use of antibacterial medications, corticosteroid treatment, breaks in the skin barrier due to intravenous or intra-arterial catheters, surgical procedures, inadequate nutrition, and metabolic disturbances[6].

D116E, E266D amino acid substitutions which were found in this study were previously reported by Marichal et al., 1999 [7]. Chau et al., 2004 [8] reported that these mutation are seen in both azole sensitive and resistant strains. This is also in agreement with a previous study of White et al., 2002 where they too reported a higher frequency of D116E and E266D mutations[9]. This mutation was found in 6 isolates in this study.

From the previous studies, it was known that the resistance to Fluconazole increased by 64 times with the G464S mutation[10]. This mutation was found in 6 isolates (CA01, CA03, CA04, CA05, CA06, CA08) in this study.

K128T is also not associated with azole resistance, but was found in both azole sensitive and resistant isolates in various other studies by Marichal et al., 1999 and Chau et al.,2004[7,8]. This mutation was found in 4 isolates (CA01, CA04, CA05, CA06) in this study.

I471T is associated with increased azole drug resistance and has a cumulative effect with Y132H. This combination was found in the CA02 isolate. This mutation has been previously studied and confirmed by Kakeya et al.,2004 and Xu et al., 2008[11,12]. I471T mutation results in increased MIC against azoles. This mutation was found in 4 isolates (CA01, CA02, CA06, CA07) in this study.

A114S was found in 3 isolates in this study. Jiang et al., 2006 and Xu et al., 2008 described this mutation as a cause for Fluconazole resistance in C.albicans [12,13] . This mutation was found in 3 isolates (CA02, CA04, CA07) in this study.

S405F mutation in Hotspot region III of ERG11 protein is a previously described cause for azole resistance in C.albicans by Chau et al., 2004; Favre et al., 1999 and Sanglard et al., 1998 [8,10,14]. It was one of the extensively studied mutations. It plays a major role in azole drug resistance by multiple mechanisms like increased MIC by site directed mutagenesis, over expression of ERG11 gene and decreased affinity of 14α-demethylase for azoles. This mutation was found in 3 isolates (CA02, CA07, CA09) in this study.

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S405F has a cumulative effect with Y132H, K108E, K143R, E266D, V437I, F126L, R467K thereby increasing the resistance to azoles [15].

T229A mutation role in azole resistance is still under study, but there are studies that indicate K143E significantly increase Fluconazole MIC when combined with T229A [14]. This mutation was found in 3 isolates (CA01, CA05, CA10) in this study. In all the 3 isolates, T229A is accompanied by K143E.

Marichal et al., 2004 first described G465S amino acid substitution in the hotspot region III of ERG11 protein. In combination with Y132H and S279F it is able to cause increased MIC to Fluconazole. G465S in combination with G464S makes C. albicans resistant to Fluconazole, voriconazole and Itraconazole. The mode of action is by reducing the affinity of azoles to CYP51 [10,16]. This mutation was found in 3 isolates (CA01, CA03,CA05) in this study.

K143E is a known mutation which significantly contributes to increased MIC to azoles. In combination with T229A, the C.albicans shows reduced susceptibility to azoles [14]. This mutation was found in 3 isolates (CA01, CA05, CA10) in this study. The isolate CA01 had both K143E and T229A mutations.

K344E mutation role in azole resistance in fully understood. But studies by Wang et al., 2020; Yang et al., 2021 indicate that it might increase the MIC to azoles in combination with other mutations [17,18]. This mutation was found in 2 (CA02, CA04) isolates in this study.

R467K mutation located in the hotspot III of ERG11p reduces the CYP51 affinity for Fluconazole and increase the MIC. R467K has a cumulative effect with G464S [16,19]. This mutation was found in 2 isolates (CA04, CA08) in this study. In both the isolates, it was accompanied with G464S mutation.

The R523G substitution was identified in two resistant isolates, but the contribution of it to azole-resistance needs further confirmation. This mutation was also confirmed by Martel et al., 2010 [20]. This mutation was found in 2 isolates (CA02, CA04) in this study.

V488I mutation does not contribute to azole resistance. It was also confirmed by Sanglard et al., 1998, Chau et al., 2004[8,10]. This mutation was found in 2 isolates (CA08, CA09) in this study.

W520C is a novel mutation was detected in this study. This mutation was found in 2 isolates (CA08, CA09) in this study. No literature is available on the potential cause and effect of this mutation. W520G and W520R mutations were described by Sanglard and Bille, 2002 in azole resistant isolates[21].

F145L mutation in the ERG11p does not contribute to azole resistance as it was found in both azole susceptible and azole resistant isolates. Chau et al., 2001; Goldman et al., 2004 also did not find any evidence of this mutation contributing to azole resistance[8,22]. In the present study, only one isolate (CA03) has this mutation.

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K119L was previously described in Fluconazole, voriconazole and Itraconazole resistant isolates by Cernicka and Subik, 2006 [23]. In this study, one isolate (CA03)was found to have this mutation. K119N mutation was described by Xu et al.,2008 [12].

P375A mutation detected in one isolate (CA10) in this study was a novel discovery at least in the Indian context. No literature is available to confirm the role of this mutation in azole resistance.

Y132H mutation in the ERG11p is known to contribute to azole resistance. It has been proven in studies conducted by Chau et al., 2004 and Kakeya et al.,2000 [8,11]. Y132H along with S405F and R467K have a cumulative effect on azole resistance[15]. CA02 isolate in this study was having both Y132H and S540F mutations. The Y132H mutation is situated in the B–B′ helix cluster of the ERG11p. Mutations in the B–B′ helix cluster can cause 4-fold increases in fluconazole MIC as it decreases affinity between the target enzyme lanosterol 14a-demethylase and Fluconazole[24].

Y257H mutation was found in one isolate (CA02). In combination with G464S, it is known to have a cumulative effect on the increase of MIC against azoles according to Chau et al., 2004 and Xu et al., 2008 [8,12]

27 mutations were silent mutations which did not alter the amino acid sequence. These silent mutations, although do not alter the amino acid sequence, may further increase the chance of a missense mutation by a single nucleotide alteration[25,26]. cDNA 558 C>T (Serine→Serine) was the most common silent mutation which was found in 6 of the 10 isolates.

462 T>C (Phenyl Alanine→Phenyl Alanine) along with 1143 C>T (Alanine→Alanine) were the second most common silent mutations which were observed in four isolates each.

805 C>T (Leucine→Leucine) was the third most common silent mutation which was repeated thrice.

The point mutations at positions 504 A>G (Lysine→Lysine), 1167 A>G (Leucine→Leucine), 1350 T>C (Tyrosine→Tyrosine), 1443 C>T (Alanine→Alanine), 1449 T>C (Alanine→Alanine) were repeated twice.

In conclusion, we demonstrated that multiple amino acid substitutions in Erg11p were found frequently in triazole resistant C. albicans isolates. Early identification of these mutated strains help in the prevention of the spread of azole resistant C. albicans strain.

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DECLARATIONS

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Conflict of Interest

None.

Author's Contribution

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Funding

None.

Data Availability

All datasets generated or analysed during this study are included in the manuscript.

Ethics Statement

This study was approved by the institutional Ethics Committee, Rohilkhand Medical College, India with Reference number IEC:BIU/REG/PhD/722

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