Fluconazole Resistance in C. Tropicalis by Broth Microdilution Method and to compare the relative gene expression of erg11 gene in both Fluconazole Resistant and Sensitive C. Tropicalis

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ABSTRACT

Aim: To evaluate fluconazole resistance among C. tropicalis by broth micro dilution method and relative expression of ergosterol(ERG11) gene in fluconazole resistant and sensitive Candida tropicalis.

Methods: The study design was comparative study conducted in Microbiology Department of UHS. A sum up of 66 confirmed isolates of Candida tropicalis were attained from Jinnah Hospital, Lahore. The fluconazoleresistance of the isolates was determined by broth microdilution method and the relative gene expression of ERG11 gene was analyzed by real time PCR, in Microbiology Department of UHS.

Results: The antibiotic susceptibility testing of C. tropicalis showed fifty one fluconazole sensitive, eight susceptible dose dependent, and seven fluconazole resistant C. tropicalis. Relative gene expression of ERG11 gene showed an increase expression about 2.0 fold in C. tropicalis resistant isolates than C. tropicalis susceptible isolates. Globally, C. albicans was the most frequently isolated species but now there is a decline in trend with increase figures for non-albicans Candida species. These non-candida albicans are now emerging as resistant species and cause serious ailments. This study showed seven fluconazole resistant C. tropicalis among sixty six isolates by broth microdilution method. Further on molecular testing by relative gene expression of ERG11 gene it was noticed that these resistant isolates have 2.0 fold increased mRNA expression levels of this gene as compared to sensitive strains.

Conclusion: C. tropicalis was found to be resistant to fluconazole. Ergosterol expression was markedly raised in fluconazole resistant C. tropicalis in contrasted to drug sensitive C. tropicalis.

Keywords: C. tropicalis, fluconazole resistance, antifungal susceptibility testing, non-albicans Candida, broth microdilution method, relative gene expression.

INTRODUCTION

Fungal pathogens have serious burden on the number of morbidity and mortality cases in clinically diagnosed high risk patients. However, to counteract the menace timely diagnosis and management of patients plays pivotal roles. The mainstay anti-fungal drugs taken into account are azoles, polyenes, echinocandins and pyrimidine analogs. Fluconazole are the universally recommended antifungal medication to counter the fungal illnesses. It is used as therapeutic medicine for superficial and systemic fungal infections being least harmful, low risk than other azoles, with immediate absorption, available in market in both oral and intravenous formulations.

The azole antifungal drugs act by inactivating lanosterol 14α-demethylase which further inhibit the biosynthesis of ergosterol. Ergosterol is an important compound for cell integrity and functions of cell membrane. The decrease in ergosterol biosynthesis leads to concomitant increase in the number of intermediate metabolites. Furthermore, the changing trends of increasing number of fluconazole resistance among those Candida species which were once more susceptible such as C. tropicalis is worrisome.

The azole resistance is rendered to its continuous use or some intrinsic factors such as change in expression level or mutations of CDR1, CDR2, PDR5, ERG3, MDR1, FLU1 and ERG11 genes.

To visualize the characteristics of biofilms formation and antifungal resistance, a study was conducted in Brazil on C. tropicalis and reported that sessile cells overexpressed ERG11 and MDR1 genes. A study was carried out in China to detect the mechanisms of azole resistance. They take Fifty two clinical isolates of C. tropicalis and quantified CDR1, MDR1, CYTB and ERG11 genes and concluded that overexpression of ERG11 gene was responsible for resistance. Usually two major azole resistance mechanisms are mutations in ERG11 gene which encodes a target enzyme 14α-demethylase and multidrug efflux transporter genes i.e MDR/CDR genes.

Previously it has been reported that ERG11 and ERG3 genes encodes proteins which are critically involved in ergosterol biosynthesis and point mutations of these two genes may alter the susceptibility toazole drugs.

The objective of current study was to determine the fluconazole resistance among C. tropicalis by broth microdilution method and to compare the relative expression of ERG11 gene in fluconazole resistant and sensitive C. tropicalis.

MATERIALS AND METHOD

After approval by the ethical review board, the study was executed in the Department of Microbiology and Resource Laboratory of University of Health Sciences, Lahore. A total of sixty six confirmed clinical isolates of Candida tropicalis were taken from Jinnah Hospital, Lahore. With facilitation of broth microdilution procedure antifungal susceptibility was performed in microtitre plate as displayed in figure 1. Inoculum compositions are exhibited in figure 2. Ten distinct testing compositions of fluconazole (640μg/ml, 320μg/ml, 160μg/ml, 80μg/ml, 40μg/ml, 20μg/ml, 10μg/ml, 5μg/ml, 2.5μg/ml and 1.25μg/ml) were assembled. In concordance with the results, C. Tropicalis was classified into three groups as sensitive (S) (≤8μg/ml), susceptible dose dependent (SDD) (16–32μg/ml) and resistant (R) (≥64μg/ml).

In facilitation with commercial kit, RNA was drawn out and the amount was evaluated by Nano drop. The drawn out RNA was transformed into cDNA by using cDNA synthesis kit. Thermal cycling conditions of polymerase chain reaction (PCR) for cDNA synthesis are exhibited in figure 3. To make sure that all RNA samples have been transformed to cDNA, conventional actin PCR was performed. Actin PCR thermal cycling conditions are shown in

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figure 4. The synthesized cDNA (2µl) was also subjected to real
time polymerase chain reaction (RT-PCR). Actin was used as an
internal control in RT-PCR for relative gene expression of ERG11.
The primers used for the expression analysis of ERG11 gene was
ERG11-Forward primer:5’-TGCTGAAAGCCTTACCC-3’ and
ERG11-Reverse primer:5’-CAAGAATCAAATCTCTC-3’ while the primers used for ACT (β-actin) were ACT Forward primer: 5’-TCTGTGTGCTCCTGTGC-3’ and ACT Reverse primer: 5’-GGAGGCTGACATCCTG-3’. SPSS 20 (Statistical Package for Social Sciences) was used for data evaluation; qualitative data was shown in frequencies and percentages as displayed in figure 5 and figure 6. Mean±SD was computed for quantitative variable e.g. MIC as shown in table 1. Relative gene expression of ERG11 (C. tropicalis ERG11) gene of two types (sensitive and resistant C. tropicalis) were assessed by Mann Whitney U test. The median of sensitive group was 1.0 (IQR=1.1) and median of resistant group was 1.8 (IQR=2.7-1.5). There was statistically significant contrast between two groups and the p value was < 0.001.

RESULTS

The antifungal susceptibility testing, performed in microtitre plate
by broth microdilution method revealed that out of sixty-six clinically
isolated samples of C. tropicalis to fluconazole disclosed that 51
(77.3%) C. tropicalis were fluconazole receptive, 8 (12.1%) were
dose related responsive and 7 (10.6%) were fluconazole resistant
as shown in figure 5. MIC results of C. tropicalis (n=66) to fluconazole
are shown in table 1. Mean ± SD for quantitative variable e.g. MIC
are shown in table 1. The MIC results were categorized into three
groups according to CLSI document M27-A2. The result shown
were ≤8 Candida isolates were sensitive, 16-32 of Candida
isolates were in SDD (susceptible dose dependent) category and
≥64 was counted as resistant.

After MIC and gene optimization as shown in fig 6, Real
Time PCR was performed to find out the ERG11 gene expression
in resistant isolates as compared to sensitive strains by using gene
specific primers as shown in table and by considering Actin as
housekeeping gene. In the present study, fluconazole treatment
induces an increase in ERG11 gene expression by two fold in
resistant isolates than the susceptible C. tropicalis isolates
as shown in figure 5. It suggests that fluconazole treatment over
expressed the ERG11 gene mRNA in our resistant isolates. This
increased expression of ERG11 gene leads to increased
ergosterol production in the fungal cell membrane and decreased
fluconazole susceptibility. So, in current study this overexpression
played a significant role in mediating resistance in C. tropicalis
isolates.

Relative gene expression of ERG11 gene showed 2.0 fold
higher mRNA expression level in C. tropicalis resistant isolates
than one fold increase in C. tropicalis susceptible isolates
as shown in figure 5. Figure 6 shows gel electrophoresis for
optimization of PCR amplification for gene ERG11.
DISCUSSION

In our study, inhibition of azole antifungal to C. tropicalis was observed, the root cause of resistance of C.tropicalis azole group could be rational application and limited side effects of these antifungals. There is sufficient literature across the world that highlights the fact that high level of Candida species relucance to usage of efflux pump or modification in the expression of ergosterol. It has been brought in horizon, up regulation of ERG11 gene is involved in the resistance phenotype of Candida species. The current study demonstrated that relative gene expression of ERG11 gene in azole sensitive levels (sensitive and resistant C. tropicalis) were notably distinct. The up regulation of C. tropicalis ERG11 was correlated with high expression of fluconazole. A previous study of He et al. displayed the up regulation of ERG11 and ABC2 perhaps for the acquired itraconazole resistance in clinical isolates of C. krusei.

Similar to our study, the interpretation of Wang et al. exhibited relative expression of ERG11 gene in drug resistance group was greater as comparable to the susceptible group. Fungal infections remain a major source of mortality in non albicans compared to other infectious diseases. Fungal infections remain a major concern for public health. Antifungal drug resistance in non albicans is an alarming state. In our study, the root cause of resistance of C.tropicalis is possibly due to the absence of drug susceptibility. There is a need to recognize new drug targets and also to discover novel antifungal drugs against resistant organisms.

Practical implication: As limited data is available in Pakistan on Candida species antifungal susceptibility pattern, the molecular mechanisms involved in antifungal drug resistance. Our study will bring into limelight, the emerging C. tropicalis infections, their antifungal susceptibility patterns and the role of the molecular mechanism in antifungal resistance. This study will also help clinicians for the treatment of fungal infections accurately and facilitate in better management of patients.

Limitation: The confines of the current study concerns the sample size and mapping of other molecular techniques of ergosterol in rendering resistance to C. tropicalis. Thus the upcoming studies must entail large sample size and cloning and induction of non-synonymous mutation in ERG11 gene to indicate the modes of azole resistance.

Conflict of interest: Nil

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