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Microbiology Section

Ibuprofen-Mediated Reversal of Fluconazole Resistance in Clinical Isolates of *Candida*

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ABSTRACT

Introduction: In view of the increasing prevalence of invasive Candidiasis in today's health-care scenario and the emergence of fluconazole resistance among clinical isolates of *Candida*, we sought to determine if Ibuprofen could elicit a reversal of fluconazole resistance and thereby offer a potential therapeutic breakthrough in fluconazole-resistant Candidiasis.

Materials and Methods: We selected 69 clinical isolates of *Candida*, which demonstrated an MIC of >32 µg/ml for fluconazole, and subjected them to broth microdilution in presence and absence of Ibuprofen.

Results: Forty two of the 69 isolates (60.9%) demonstrated reversal of Fluconazole resistance with concomitant use of Ibuprofen. This was characterized by significant species-wise variation (p=0.00008), with all the *C. albicans* isolates and none of the C. *glabrata* isolates demonstrating such reversal. Only 22.2% and 37.7% of C. *krusei* and C. *tropicalis* isolates respectively showed Ibuprofen-mediated reversal of Fluconazole resistance.

Conclusion: Since Ibuprofen is a known efflux pump inhibitor, our findings hint at the possible mechanism of Fluconazole resistance in most of our *Candida* isolates and suggest a potential therapeutic alternative that could be useful in the majority of Fluconazole-resistant clinical isolates of *Candida*.

Keywords: Antifungal treatment, *Candida*, Efflux pump, Fluconazole resistance, Ibuprofen

INTRODUCTION

Invasive candidiasis has emerged as the commonest form of opportunistic mycoses throughout the world. Apart from its widespread occurrence, it is often acutely progressive, difficult to diagnose and associated with increased hospital stay and high mortality rates [1-5]. Treatment of this condition has become further complicated owing to the relative rise in the proportion of non-*albicans Candida* isolates, which often demonstrate intrinsic resistance towards specific antifungal agents [6-11]. Moreover, there has been a documented increase in fluconazole resistance even among other species of *Candida*, including *C. albicans*, C. lusitaniae, C. *tropicalis* and C. *dubliniensis* [12-16], which has been partially attributed to the popular use of fluconazole as empirical antifungal therapy since the 1990s [17].

Azole resistance calls for the use of alternative antifungal drugs like echinocandins, voriconazole, posaconazole, ravuconazole and Amphotericin B. But constraints like high costs or adverse effects associated with these agents often limit their usage. Some recent pioneering studies have reported the modulating effect of verapamil, oestradiol, progesterone and ibuprofen on resistance of *Candida* isolates. While fluconazole MIC decreased in most strains after exposure to these modulators, this effect was particularly remarkable for Ibuprofen. The molecular basis of this reversal of resistance has also been demonstrated recently [18]. Thus, drugs capable of reversing fluconazole resistance might offer novel breakthroughs in the treatment of resistant *Candida* infections. As we have previously observed a high prevalence of fluconazole resistance among *Candida* isolates recovered in our centre [19], in this study we were interested in exploring if the resistant phenotype could be reversed in a proportion of these isolates by the use of Ibuprofen.

MATERIALS AND METHODS

The study was conducted over a period of 18 months, from December 2009 till May 2011, in a tertiary care teaching hospital in the Himalayan region of northern India. *Candida* species isolated from various clinical samples such as blood, CSF, ET secretions, indwelling devices, urine, sputum etc., were processed for mycological characterization and antifungal susceptibility testing by broth microdilution method recommended by CLSI [10]. Sixty nine isolates with MIC>32 µg/ml for fluconazole were included in the study. Standard ATCC strains, viz. *C. albicans* ATCC 5314 and C. *krusei* ATCC 6258, were used as controls.

For testing the modulation of fungal sensitivity to fluconazole with Ibuprofen, the fluconazole-resistant *candida* isolates were cultured with fluconozole (Sigma-Aldrich), in presence and absence of ibuprofen (Sigma-Aldrich). Briefly, the respective isolates were sub-cultured on Sabouraud's Dextrose Agar to ensure purity and viability and inoculum was prepared by picking five distinct colonies of approximately 1 mm diameter. Final inoculums size of 0.5 x 10^3 to 2.5 x 10^3 cfu/ml was achieved in sterile, 96-well, U-bottom microtiter plates, after dispensing 2X concentrations of fluconazole into the wells of columns 1 to 10 in 100 µl volumes. Serial doubling dilutions of fluconazole was used, ranging between 0.12 and 64.0 µg/ml. In growth-control wells 100 µl of sterile drug-free medium was added to the inoculums, while sterility-control wells contained drug-free medium only. In a separate microtiter plate, a similar plan of inoculation was used for monitoring the effect of Ibuprofen, except that 5 µg Ibuprofen drug solution (from a stock solution of 4.0 mg/ml) was dispensed immediately after addition of the fungal inocula. Following incubation for 48 hours at 35^{0}C, the microtiter plates were scored and growth in each well was compared with that of the growth control well. The MIC was defined as the lowest concentration of fluconazole in which a prominent decrease in turbidity was observed. MIC ≤ 8.0 µg/ml was considered to be sensitive, MIC between 16.0 and 32.0 µg/ml was considered to be sensitive-dose dependent and that ≥ 64.0 µg/ml was considered resistant.

STATISTICAL ANALYSIS

Chi-square test was performed to determine if the proportion of isolates demonstrating Ibuprofen- mediated reversal of fluconazole resistance was significantly different across the various species of *Candida*. p-value < 0.05 was considered significant.

RESULTS

A total of 69 fluconazole-resistant clinical isolates of *Candida* spp. were included in the study. Majority of the isolates were C. *tropicalis* (n=33), followed by C. *parapsilosis* (n=21), C. *krusei* (n=9), C. *glabrata* and *C. albicans* (3 isolates each). While 42 of the 69 selected fluconazole-resistant isolates (60.9%) demonstrated inhibition of growth with the concomitant use of Ibuprofen and Fluconazole, such reversal was characterized by significant species-wise variation (p=0.00008). All the isolates of *C. albicans* and 95.2% of *C. parapsilosis* demonstrated significant reduction in MIC after concomitant culture with fluconazole and ibuprofen. However, reversal of resistance was shown by only 37.7% of C. *tropicalis* isolates. On the other hand, no inhibition of growth was observed with the concomitant use of Ibuprofen among any isolate of C. *glabrata*. Similarly out of the 9 isolates of C. *krusei* tested for modulation of sensitivity to fluconazole, 7 isolates did not show any inhibition of growth with the use of Ibuprofen [Table/Fig-1].

Species	Resistant to fluconazole	Reversal after Ibuprofen (%)
C. albicans	3	3 (100)
C. *tropicalis*	33	17 (51.5)
C. *glabrata*	3	0 (0)
C. *parapsilosis*	21	20 (95.2)
C. *krusei*	9	2 (22.2)
Total	69	42 (60.9)
Chi-square	23.87	
Degrees of freedom	4	
p-value	0.00008	

[Table/Fig-1]: Species-wise distribution of clinical isolates of *Candida* demonstrating reversal of Fluconazole resistance with Ibuprofen

DISCUSSION

We have previously reported increased levels of fluconazole resistance among *Candida* isolates recovered from our centre [19]. Despite the rising prevalence of azole-resistance in *Candida* species, technical difficulties in the genetic manipulation of this diploid species have impeded the delineation of precise contributions of individual resistance mechanisms among clinical isolates of *Candida* [20]. However, this information is likely to be helpful in identifying potentially useful novel drug targets and in understanding their relative importance in clinical practice. Moreover, some of the mechanisms of antifungal resistance contribute to cross- resistance to other antifungal agents and, hence, knowledge of the same might aid clinical decision-making.

In view of earlier studies demonstrating ibuprofen to be a potential efflux pump inhibitor, [18-21] we inferred that the reversal of fluconazole resistance with ibuprofen, as observed by us, hints at the possible role of efflux pumps mediating azole resistance in majority of our isolates. Similar to our study, several other authors have also observed an in vitro synergistic effect of ibuprofen with fluconazole in the pathogenic yeast *C. albicans* [18-22]. In one such study, Pina-Vaz et al., studied the modulating effect of verapamil, oestradiol, progesterone and ibuprofen on azole-resistance in *Candida*. They included 42 clinical isolates of *Candida* (38 fluconazole resistant, 2 ATCC type strains and *C. albicans* strains with known mechanisms of fluconazole resistance). Incubating these strains with sub-inhibitory concentrations of different modulators they observed that fluconazole MIC decreased in most strains after exposure to modulators, including control strains with documented over-expression of efflux pump. This modulatory effect was particularly remarkable for ibuprofen. Resistance to itraconazole and voriconazole was also reverted with the help of these modulators. However no significant MIC variation was observed for *C. krusei* isolates [18]. In another study by the same group, synergistic interaction between ibuprofen and fluconazole was analysed in 62 clinical isolates and 5 control strains of *C. albicans*. It was observed that resistant isolates that reverted to susceptible after incubation with ibuprofen showed over-expression of genes encoding efflux pumps, viz. CDR1 and CDR2. Conversely, strains that did not revert displayed a remarkable increase in expression of the azole target gene, ERG11, along with CDR genes [21]. Moreover, in view of the importance of prostaglandins in fungal colonization, additional in vivo therapeutic benefit with ibuprofen can also result from its inhibitory effect on prostaglandin synthesis [23].

Our study revealed a varying effect of ibuprofen among the different species of *Candida*, which is indicative of heterogeneity in the mechanism of azole resistance among the various species. Previous studies have also described species-wise differences in the molecular mechanisms responsible for azole resistance in *Candida*. Vandeputte et al., investigated the mechanism of acquired azole resistance in a clinical isolate of *C. tropicalis* and observed over-expression of the gene coding for target enzyme, lanosterol 14 α-demethylase, together with a missense mutation in this gene, though no over-expression of efflux protein gene CtMDR1 was found [24]. On the other hand, Sanguinetti et al., reported up-regulation of the CgCDR1-, CgCDR2-, and CgSNQ2-encoded efflux pumps in a set of fluconazole resistant and susceptible dose- dependent clinical isolates of C. *glabrata*, while no mutation or upregulation of the target enzyme was observed in any of these isolates [25]. Analysing the molecular mechanisms of resistance in clinical isolates of *C. albicans* recovered from an HIV-infected patient with recurrent oropharyngeal candidiasis, Martinez et al., noted over-expression of MDR and CDR genes encoding efflux pumps in isolates with decreased fluconazole susceptibility. However, over-expression of ERG11 gene encoding for the target enzyme of azoles was not observed in these isolates [26].

Antifungal resistance, as measured in vitro by MIC determination, is a function of the several genetic resistance mechanisms operating in any strain [20]. Since efflux pumps are encoded in Candida species by 2 gene families of transporters, viz. the CDR genes of the ATP-binding cassette super family and the MDR genes of the major facilitators class [25,27], we need to validate our findings in future studies by demonstrating the effect of ibuprofen on the quantitative level of expression of these genes in clinical isolates of Candida exposed to fluconazole.

CONCLUSION

Our study presents a simple phenotypic assay that is adaptable to the workload of a clinical microbiology laboratory and hints at the mechanism of fluconazole resistance in a majority of clinical isolates of *Candida*. We need to validate our findings in future studies by demonstrating the effect of ibuprofen on the quantitative level of expression of genes encoding efflux pump system in clinical isolates of *Candida* exposed to fluconazole.

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