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Farnesol Sensitivity of Serum Induced Yeast to Hyphae Morphogenesis: A Study on Fifty Clinical Isolates of *Candida albicans*

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Authors' contributions

This work was carried out in collaboration between all authors. Author SMK designed the study and guided throughout, author JSR contributed to the susceptibility studies and wrote the first draft of the manuscript. Authors VSR, SSR and SRM managed the collection and identification of clinical isolates. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: Objective of this study was to examine farnesol sensitivity of yeast to hyphae dimorphism in clinical isolates of *Candida albicans*.
Study Design: Variations in virulence attributes contribute to variations in pathogenicity of *C. albicans*. Ability to switch from yeast to hyphae morphology is an important virulence factor. Farnesol, a quorum sensing molecule is known to play an important role in the regulation of *C. albicans* morphogenesis. Analysis of farnesol susceptibility of yeast to hyphae conversion may reveal a factor responsible for variation in pathogenicity among clinical isolates of *C. albicans*.
Place and Duration of Study: SCG Medical College & SGGS Memorial Hospital, and School of Life Sciences, SRTM University, Nanded, India. Duration of this study was, December 2008 to December 2010.
Methodology: Fifty clinical isolates of *C. albicans* were recovered from body fluids (such as, sputum, blood, urine, vaginal swab, tracheal swab, throat swab, feces, pus and cerebrospinal fluid, etc.) of patients with different clinical manifestations, in the tertiary care

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center hospital. Presumptive identification of *C. albicans* was done on HiCHROM agar-*Candida*, while confirmation was done by Germ tube formation assay, Carbohydrate assimilation and Corn meal agar test. Serum induced yeast to hyphae morphogenesis in *C. albicans* was performed in 96 well plates. Recent methodology of micro broth dilution was used for farnesol susceptibility testing in fifty clinical isolates.

Results: Farnesol prevented hyphae formation in a concentration dependent manner, in the range 25 to 400 µM. Inhibition of ≥ 50% hyphae was considered as significant reduction in morphogenesis. MIC_{70} for farnesol mediated inhibition of morphogenesis in *C. albicans* was at 200 µM. Mean values for percentage inhibition of morphogenesis in fifty strains was compared by analysis of variance (ANOVA). P = 0.05 was considered significant.

Conclusion: Susceptibility of yeast to hyphae morphogenesis to the quorum sensing molecule farnesol, varied significantly among clinical isolates of *C. albicans*. We hypothesize that variation in farnesol sensitivity may be a factor responsible for variable dissemination and infection ability of *C. albicans*.

Keywords: Candida albicans; biofilm; farnesol; morphogenesis; quorum sensing; sensitivity; virulence.

1. INTRODUCTION

Various virulence attributes enable *Candida albicans* as a successful fungal pathogen of the humans [1]. *Candida albicans* is the fourth most common cause of blood stream infections [2] and rank as the third cause of catheter related infections [3]. Ability to switch between yeast and hyphae forms of cells is considered as an important virulence factor in *C. albicans* [4]. The hyphae/filamentous forms facilitate invasion of tissues and establishment of infection. While, yeast form cells can easily disseminate through the blood stream and spread to different tissues [5]. Hyphae are considered as important components of *C. albicans* biofilms which are drug resistant [6]. Various environmental and nutritional factors such as oxygen availability, nitrogen, calcium, pH, temperature, sugar, amino acids as well as various metabolites of microbial and host origin, regulate morphogenesis in *C. albicans* [7]. Serum is one of the well known inducers of hyphae formation in *C. albicans* [8]. Quorum sensing molecules like farnesol and tyrosol, as well as autosignaling molecules like isoamyl alcohol, dodecanol, phenyl ethanol, ethyl alcohol and tryptophol are reported to modulate dimorphic switching [9,10].

Farnesol is the first quorum sensing molecule identified in eukaryotic microorganisms [11]. It is continuously secreted by the cells and acts in a cell density dependent manner. A threshold concentration of farnesol prevents induction of germ tubes (an intermediate stage in hyphae formation). *In vitro*, exogenously added farnesol was shown to inhibit hyphae as well as biofilm formation in *C. albicans* [12,13], hence being investigated for therapeutic potential [14]. However, few of the workers proposed that farnesol may act as a virulence trait *in vivo* [15]. Farnesol concentration required to exert its activity varies with inducers, growth media and growth phase of the inoculum [16-18]. Recently it was reported that the cells in exponential and stationary phase of growth respond to farnesol exposure in distinct manners [17,18]. In synthetic media, like RPMI-1640, inhibition of yeast to hyphae transition required only a few micro molar (2 to 10 µM) concentration. While, 250 µM farnesol was needed to inhibit serum (20%) induced filamentation. 300 µM concentration of farnesol was shown to be inhibitory to biofilm formation. Concentrations >300 µM may exert inhibitory effect on the growth [17]. It is evident from previous studies that the amount of farnesol produced and the concentration required for inhibition of hyphae, varied with different strains

and metabolic modification [16,19]. Hence, *in vivo* farnesol sensitivity of *C. albicans* may vary depending on strain variation in natural population [16]. However, this has not been studied with sufficient number of clinical isolates of *C. albicans*. In the present *in vitro* study, we have analyzed farnesol sensitivity of serum induced yeast to hyphae morphogenesis in fifty clinical isolates of *C. albicans*.

2. MATERIALS AND METHODS

2.1 Cultures

A standard strain of *Candida albicans*, ATCC 90028, was obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Clinical isolates were obtained from Fungal Biology Lab, School of Life Sciences, SRTM University, Nanded (MS), India. Fifty clinical isolates of *C. albicans* were recovered from body fluids (sputum, blood, urine, vaginal swab, tracheal swab, throat swab, faeces, pus and cerebrospinal fluid, etc.) of various patients with different clinical manifestations, in a tertiary care center hospital. As described previously [20], presumptive identification of *C. albicans* was done on HiCHROM agar-*Candida* (HiMedia Lab. Ltd., Mumbai, India). Confirmation was done by Germ tube formation assay, carbohydrate assimilation [20,21] and Corn meal agar test. Cultures were maintained on Yeast-Peptone-Dextrose (YPD) agar slants and stored at 4ºC.

2.2 Media/Chemicals

YPD broth was prepared by dissolving individual components- yeast extract 1%, peptone 2% and dextrose 2% in distilled water; pH was adjusted to 6.5 and sterilized by autoclaving. Phosphate buffered saline (PBS) was prepared by adding 2.7 mM KCl and 157 mM NaCl in 10 mM Potassium Phosphate Buffer pH 7.4 (All media components & chemicals were purchased from HiMedia Laboratories Ltd. Mumbai, India). 20% solution of Horse serum (HiMedia Laboratories Ltd. Mumbai, India) was made in sterile, deionized distilled water. t, t-farnesol was purchased from Sigma-Aldrich, India.

2.3 Activation of Culture, Harvesting and Washing of Cells

C. albicans colonies from YPD agar plate were inoculated in 25 ml YPD broth in 100 ml conical flask. The flasks were incubated at 30 ºC in an orbital shaker, at 100 rpm for 24 hours. Cells from activated culture were harvested by centrifugation at 2000 × *g* speed for 5 minutes. Cells were washed three times and resuspended in PBS. All the cells in the stationary phase of growth were in yeast form.

2.4 Yeast to Hyphae Transition and Farnesol Sensitivity

Formation of hyphae was studied using micro plate assay in 96 -well plates [9]. Cells from a stock suspension were inoculated in 20% horse serum solution, to get 1×10^6 cells ml^{-1}. The stock solution (10 mM) of farnesol was prepared in methanol. Various concentrations of farnesol (ranging from 25 µM to 400 µM) were prepared in the wells by double dilution method. Wells without farnesol were kept as a control. Final volume of the assay system in each well was 200 µL. The plates were incubated at 37ºC, in an orbital shaker at 200 rpm, for 2 hours. After incubation cells were observed microscopically. Every time 100 cells were counted and formation of ≥ 95 hyphae in control wells was considered significant. For each

concentration, mean of three different readings was noted. Percentage of hyphae formation in test well was calculated by comparing with the control. This specified the percentage inhibition of yeast to hyphae conversion. Experiments were repeated three times and standard deviation from the mean was calculated.

2.5 Statistical Analysis

Experiments were repeated three times and the values mentioned are mean with standard deviations. Values of the treatment and control groups were compared by using Student's 't' test. A 'P' value < 0.05 was considered significant.

3. RESULTS AND DISCUSSION

Farnesol showed inhibition of serum induced yeast to hyphae morphogenesis in a concentration dependent manner from 25 to 400 µM. In control i. e. 20% serum without farnesol, isolates showed ≥ 95% of germ tube formation in 2 h of incubation at 37^{o}C. No variable rates of germ tube formation were observed. 25 µM and 50 µM farnesol had no significant effect on serum induced yeast to hyphae transition. At these concentrations >90% of cells of different isolates were found capable of yeast to hyphae dimorphism. Farnesol mediated prevention of morphogenesis in presence of 100 µM farnesol varied from 16 to 66%.

Significant ($p < 0.05$) inhibition of yeast to hyphae transition was observed in 20% of the clinical isolates (Table 1). At 200 µM concentration, induction of hyphae varied from 28% to 85% in different isolates (Fig. 1; Table 1). Serum (20%) induced morphogenesis in >70% of the isolates was sensitive to 200 µM farnesol (Fig. 1), and considered as MIC_{70} for farnesol mediated inhibition of serum induced morphogenesis. The standard strain (ATCC 90028) was found to be sensitive to farnesol's dimorphism inhibitory activity. Complete (100%) prevention of serum induced yeast to hyphae conversion in all the clinical isolates of *C. albicans* was exhibited at 400 µM concentration of farnesol (data not shown). Formation of hyphae in response to serum as well as sensitivity to farnesol in various isolates did not show any correlation to its site of isolation in the body.

The options of antifungal drugs available to treat *C. albicans* are limited. Moreover, adverse side effects and emergence of drug resistance are other challenges [22]. Search for novel molecules which would target virulence factors instead of killing the pathogen, has emerged as a new strategy. Quorum sensing molecules are being investigated for their potential as effective antimicrobials. It would avoid the selection of resistant strains and development of resistance among populations of pathogenic microorganisms [23]. Yeast to hyphae morphogenesis is an important virulence factor in *C. albicans*, hence an attractive antifungal target [4]. *In vivo*, *C. albicans* can form hyphae in response to serum and invade through host tissues to establish infection. The quorum sensing molecule, farnesol, was reported to block serum induced germ tube formation at around 250 µM concentration in a few standard strains of *C. albicans* [16]. However, its activity was not studied in a sufficient number of clinical isolates. Our *in vitro* study with 50 clinical isolates showed that 400 µM farnesol completely prevented serum induced hyphae formation. However, 400 µM concentration of farnesol is too high to be produced in *C. albicans*. It was reported that farnesol up to 300 µM, do not have adverse effects on *C. albicans* growth when the cells are in stationary phase of growth [17- 19]. Concentrations above 300 µM are known to adversely affect *C. albicans*

growth [24]. Earlier studies have reported that *C. albicans* produce 40 to 45 times more farnesol in specific metabolic conditions, when sterol synthesis is blocked [19].

Table 1. Variation in sensitivity of serum induced yeast to hyphae morphogenesis to farnesol (100 µM and 200 µM), in fifty clinical isolates of *Candida albicans*

Isolate/Strain No.	Inhibition (%) of yeast to hyphae morphogenesis by farnesol		Isolate/Strain No.	Inhibition (%) of yeast to hyphae morphogenesis by farnesol	
	100 µM	200 µM		100 µM	200 µM
1	47 (± 2)[a]	76 (± 2)	27	43 (± 1)	71 (± 4)
2	43 (± 1)	73 (± 1)	28	45 (± 3)	67 (± 2)
3	34 (± 1)	84 (± 2)	29	41 (± 0)	58 (± 3)
4	21 (± 1)	54 (± 2)	30	19 (± 2)	33 (± 5)
5	46 (± 2)	76 (± 1)	31	48 (± 2)	67 (± 2)
6	43 (± 2)	76 (± 1)	32	24 (± 1)	70 (± 1)
7	53 (± 1)	84 (± 1)	33	42 (± 3)	66 (± 2)
8	43 (± 2)	76 (± 2)	34	58 (± 3)	71 (± 3)
9	45 (± 2)	69 (± 2)	35	34 (± 2)	66 (± 3)
10	66 (± 2)	85 (± 1)	36	66 (± 1)	76 (± 2)
11	48 (± 1)	66 (± 2)	37	17 (± 2)	30 (± 2)
12	39 (± 1)	77 (± 1)	38	62 (± 1)	83 (± 3)
13	31 (± 5)	61 (± 1)	39	20 (± 1)	33 (± 3)
14	32 (± 2)	55 (± 2)	40	52 (± 1)	74 (± 2)
15	39 (± 2)	64 (± 2)	41	32 (± 1)	38 (± 1)
16	16 (± 1)	54 (± 1)	42	25 (± 1)	41 (± 1)
17	51 (± 2)	72 (± 2)	43	29 (± 3)	44 (± 1)
18	37 (± 1)	69 (± 1)	44	22 (± 3)	28 (± 2)
19	39 (± 1)	73 (± 2)	45	28 (± 1)	42 (± 3)
20	60 (± 2)	61 (± 2)	46	32 (± 1)	45 (± 2)
21	45 (± 2)	75 (± 1)	47	34 (± 3)	46 (± 2)
22	40 (± 5)	73 (± 3)	48	23 (± 3)	36 (± 1)
23	23 (± 1)	36 (± 5)	49	25 (± 1)	35 (± 3)
24	33 (± 1)	56 (± 3)	50	45 (± 1)	67 (± 2)
25	28 (± 2)	50 (± 2)	ATCC 90028	42 (± 1)	56 (± 3)
26	45 (± 2)	68 (± 2)			

[a]*Values in parenthesis indicate standard deviation from mean.*

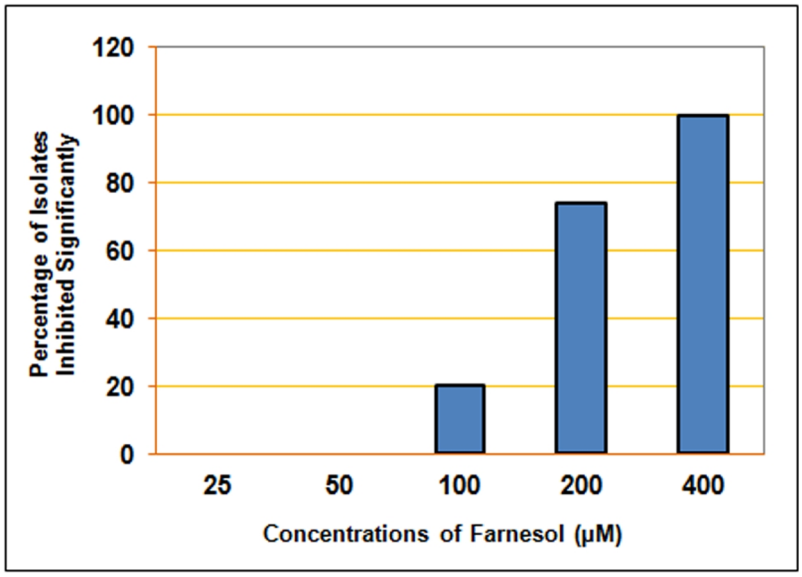

Fig. 1. Percentage of *C. albicans* isolates in which formation of hyphae was significantly (≥ 50%) inhibited in presence of various concentrations of farnesol.

In biofilm microenvironment farnesol may accumulate to reach concentrations in the range of 200 µM [17]. Considering this we analyzed effects in presence of 200 µM concentration of farnesol. Our results indicated that 200 µM of farnesol effectively prevented yeast to hyphae conversion in most of the *C. albicans* isolates. It was established as MIC_{70} (i. e. minimum concentration which inhibits at least 70% isolates) for serum induced morphogenesis. *C. albicans* were in the stationary phase of growth. Hence, farnesol's effect on morphological transition was independent of growth inhibition. Although farnesol acts as a quorum sensing molecule to inhibit morphogenesis, it may act as a virulence factor *in vivo*. A study in mouse model proposed that farnesol acts as a virulence factor and enhance pathogenicity [15]. Our study showed that clinical isolates of *C. albicans* vary in their sensitivity to farnesol. This should be taken into consideration while investigating farnesol as a virulence factor.

4. CONCLUSION

Susceptibility of yeast to hyphae morphogenesis to the quorum sensing molecule farnesol, varied significantly among clinical isolates of *C. albicans*. We hypothesize that variation in farnesol sensitivity may be a factor responsible for variable dissemination and infection ability of *C. albicans*.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors VR and JSR have equally contributed to the work and preparation of the manuscript. The authors declare that no competing interests exist.

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