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Effect of phenotypic switching on expression of virulence factors by *Candida albicans* causing candidiasis in diabetic patients

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ABSTRACT

A total of 110 strains belonging to seven species of *Candida* were isolated from various forms of candidiasis in diabetic patients. They were *Candida albicans* 53 (47%), *Candida tropicalis* 36 (33%), *Candida glabrata* 9 (8%), *Candida parapsilosis* 4 (4%), *Candida guilliermondii* 2 (2%), *Candida krusei* 5 (5%) and *Candida kefyr* 1 (1%). All 53 strains of *C. albicans* isolated were observed to express virulence factors such as cell surface hydrophobicity (CSH), adherence to human buccal epithelial cell (BEC) and proteinase activity (100%), while phospholipase activity was observed in 52 (98%). Phenotypic switching and its influence on the pathogenicity of *C. albicans* were studied. Two *C. albicans* strains isolated from oral and vaginal thrush, respectively, in diabetic individuals, and the control strain *C. albicans* NCPF 3153A were induced to undergo phenotypic switching by exposure to UV light and the degree of expression of virulence factors by the different morphological forms was determined. Three different morphological forms of *C. albicans* were obtained, namely Star (S), Wrinkled (W) and Ring (R) types from the original Smooth (O) variety. It was found that proteinase activity was greatest with the W type followed by the R type then the O type. The S type produced the least proteinase. The phospholipase activity was greatest with O type followed by R type. The W and S types produced the least phospholipase. Expression of CSH and adherence was greatest in the O type followed by the R and then the W type and finally the S type. Differential expression of virulence factors occurs with different phenotypic forms of *C. albicans* and this may provide a particular morphological type with a distinct advantage over other types in causing candidiasis.

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Efecto de las variaciones fenotípicas de cepas de *Candida albicans*, aisladas de pacientes diabéticos, en la expresión de factores de virulencia

RESUMEN

Palabras clave:

Candida albicans

Variaciones fenotípicas

Virulencia

Diabetes

Se aislaron 110 cepas que pertenecían a siete especies de *Candida* procedentes de pacientes diabéticos con distintas formas de candidiasis, encontrándose 53 aislamientos de *Candida albicans* (47%), 36 de *Candida tropicalis* (33%), 9 de *Candida glabrata* (8%), 4 de *Candida parapsilosis* (4%), 2 de *Candida guilliermondii* (2%), 5 de *Candida krusei* (5%) y 1 de *Candida kefyr* (1%). En las 53 cepas de *C. albicans* aisladas se estudió la expresión de factores de virulencia tales como la hidrofobicidad de la superficie celular (CSH), adherencia a células epiteliales bucales humanas (BEC) y actividad enzimática. La actividad proteolítica se detectó en el 100% de las cepas de *C. albicans*, mientras que la producción de fosfolipasa se detectó en 52 cepas (98%). Se estudió la variación fenotípica y su influencia en factores de patogenidad en dos cepas de *C. albicans*, procedentes de boca y vagina respectivamente, y en la cepa patrón *C. albicans* NCPF 3153A. Se les indujo la variación fenotípica mediante exposición a luz UV y se valoró el grado de expresión de los factores de virulencia por las diversas formas morfológicas obtenidas. Se obtuvieron tres variaciones morfológicas de *C. albicans*: forma de estrella (S), rugosa (W) y anular (R), a partir de la variedad lisa original (O). La actividad proteinasa fue mayor en el tipo W, seguida por el tipo R, y por el tipo O; el tipo S fue el de menor actividad proteolítica. La actividad fosfolipasa fue mayor en el tipo O, seguida por el tipo R; los tipos W y S presentaron una actividad fosfolipasa menor. La expresión de la CSH y de la adherencia fue superior en el tipo O, seguida por el tipo R y el tipo W, y finalmente el tipo S. Las variaciones fenotípicas de *C. albicans* presentan una expresión diferenciada de factores de virulencia y ello puede proveer a un tipo morfológico particular de ciertas ventajas, facilitando el inicio de una candidiasis.

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Candida is commensal yeast of oral, gastrointestinal and vaginal mucosa in healthy individuals. Candidiasis has emerged as a significant opportunistic disease with the increase in number of individuals with immunodeficiency and other predisposing conditions such as diabetes mellitus, prolonged antibiotic treatment, cancer treatment and invasive procedures. Some alteration of the host cellular defenses, physiology or normal flora as well as virulence factors must occur before colonization, infection and disease production by *Candida* can take place. The susceptibility of diabetic patients to candidiasis has been well documented.³ Several immune deficits have been described in diabetics and factors such as poor glycaemic control and poor innate immunity have been attributed with predilection to *Candida* infection.

The transition of *Candida* from a harmless commensal to an unrelenting pathogen is a fine line and one that is attributable to an extensive repertoire of virulence determinants selectively expressed under suitable predisposing conditions.¹⁶ Enhanced expression of virulence factors is seen in *Candida* strains isolated from diabetic patient.^{13,23} *Candida* spp. colonizing the vaginal and oral cavities of diabetics are subjected to selective pressures that may lead to the emergence of strains with altered genotypic/phenotypic characteristics. Most strains of *Candida albicans* are known to be capable of switching spontaneously, reversibly and at high frequencies between a number of general phenotypes distinguishable by colony morphology.^{21,22} Switching has been demonstrated to regulate a number of phenotypic characteristics involved in pathogenesis such as adhesion, expression of cell surface hydrophobicity (CSH) and secretion of proteinases and phospholipases.^{6,8,15,24}

The objectives of the present study were to isolate and identify *Candida* spp. from candidiasis in diabetic patients and to observe the relationship, if any, between high-frequency phenotypic switching and expression of virulence factors by *C. albicans*.

Material and methods

Organisms

Appropriate specimens were collected from 110 diabetic patients from hospitals of Kasturba Medical College, Mangalore, India, who were suspected of having different types of *Candida* infection. The specimens were processed and the *Candida* strains isolated were identified using standard method in the Department of Microbiology, Kasturba Medical College.⁹ *C. albicans* strains isolated were screened for the expression of various virulence factors and two strains expressing high levels of virulence factors were used in the study.

Three strains of *C. albicans*, namely *C. albicans* NCPF 3153A, CA-O58 and CA-V88, were used for phenotypic switching studies. *C. albicans* NCPF 3153A was procured from the National Collection of Pathogenic Fungi, Mycology Reference Laboratory, London, UK, and used as the control. *C. albicans* NCPF 3153A produces virulence factors such as proteinase, phospholipase, adherence, cell surface hydrophobicity and exhibit phenotypic switching.^{8,21,26} *C. albicans* CA-O58 was isolated from oral thrush and CA-V88 from vaginal thrush of diabetic patients.

Induction of phenotype switching

C. albicans was grown in liquid Lee's medium¹⁰ supplemented with 70 µg arginine/ml and 0.1 µM ZnSO₄ at 25 °C for 24 h. The cells were harvested by centrifugation and suspended in sterile distilled water and counted using a haemocytometer. *Candida* (10⁶) cells were taken and suspended in 100 ml sterile water in a

Petri dish and exposed to UV light (15 W with an emission wave length of 254 nm, total energy output 31 J m⁻²) for 5 s. An aliquot of 1 ml of irradiated cell suspension was taken and diluted in sterile water to obtain a final concentration of 1 × 10³ cells/ml. Hundred microliters containing about 100 cells were spread on plates containing Lee's medium with 2% agar and incubated at 25 °C for 7–14 days and observed for different colony morphological forms.¹⁴

Yeast cell suspensions in phosphate-buffered saline (PBS, pH 7.2) were prepared at 25 °C using a single colony from each of the different phenotypes of *C. albicans* grown on Lee's medium and used for the estimation of proteinase and phospholipase production, adherence to human buccal epithelial cell (BEC), expression of CSH and susceptibility to azoles. After each assay, the colony phenotype was verified by plating 10 µl of the cell suspension on to Lee's medium agar plates and incubating the plates at 25 °C for 7 days.

Adherence assay

The adherence assay described by Kimura and Pearsall⁷ was used with minor modification. Buccal epithelial cells (BEC) were obtained from the buccal mucosa of a single healthy donor, on the day of the assay. BEC were washed thrice in PBS (pH 7.2) and finally suspended in PBS. Standardized suspensions of human BEC (1 × 10⁵ cells/ml of PBS, 0.5 ml) and yeast cells (1 × 10⁷ yeast cells/ml in PBS, 0.5 ml) were mixed and incubated at 37 °C with gentle shaking for 45 min. Epithelial cells were then washed with PBS to remove unattached yeasts, collected by filtration, fixed by methanol on to a microscopic slide and stained by Gram's method. The number of adherent yeast cells on each of 100 epithelial cells was counted for each preparation.

Polystyrene microsphere assay for CSH

The CSH assay described by Hazen and Hazen⁴ was used with minor modification. Blue-dyed polystyrene microspheres (Sigma, USA) having a diameter of 0.8 ± 0.1 µm were used in the study. A working solution containing approx. 9 × 10⁸ microspheres/ml in ice-cold PBS was prepared from a stock of colloidal suspension of microspheres (10% solids). Equal volumes (200 µl) of microsphere suspensions and yeast cells (5 × 10⁶ cells/ml of PBS) were mixed, rapidly equilibrated to room temperature and vortexed for 30 s. Cell surface hydrophobicity was determined as the percentage of yeast cells (from at least 100) with three or more attached microspheres, when viewed by bright field microscopy at 400 × .

Assay of proteinase

For estimation of proteinase activity, cells from each of the different phenotype colonies were taken and suspended in 1 ml of sterile distilled water and counted with a hemocytometer. Erlenmeyer's flasks containing 10 ml Macdonald and Odds medium were inoculated with 10⁶ cells/ml and incubated at 25 °C for 7 days.¹¹ The broth culture was centrifuged and the supernatant was used for the estimation of extracellular proteinase. The sample of culture supernatant (0.2 ml) was mixed with 0.8 ml substrate (1% bovine serum albumin (BSA) in 0.025 M sodium citrate buffer, pH 3.2) and incubated at 37 °C for 3 h. The reaction was halted by the addition of 2.0 ml of 5% trichloroacetic acid (TCA), resulting in the precipitation of BSA. The tubes were kept at 4 °C overnight and centrifuged at 2000 rpm for 20 min. Proteolysis was determined by measuring the absorbance of the soluble peptides at 280 nm. For control, the substrate was added to the culture supernatant and immediately treated with TCA. The

absorbance of controls was subtracted from test samples to obtain values for enzyme activity.¹¹

Phospholipase assay

The phospholipase production by *C. albicans* was assayed according to Samaranayake et al.²⁰ with minor modification, using the egg yolk agar plate method of Price et al.¹⁹.

Sabouraud's dextrose agar plates containing 1 M NaCl, 0.005 M CaCl₂ and 8% sterile egg yolk emulsion was used. Ten microliters of yeast cells suspension containing 10⁶ cells/ml were spot inoculated on the surface of the egg yolk agar using a micropipette and incubated at 37 °C for 4 days in a humid chamber. Phospholipase activity (Pz) was determined as the ratio of the colony alone to the diameter of the colony plus precipitation zone. The lower the Pz value for a strain, the higher the phospholipase activity.

Antifungal susceptibility testing

The susceptibility testing of different phenotypes of *C. albicans* to fluconazole, itraconazole and ketoconazole was performed using CLSI guidelines (formerly NCCLS).¹⁸

Statistical analysis

The expression of virulence factors by the different morphological forms of *C. albicans* was compared using the Kruskal-Wallis Anova test.

Results

A total of 110 strains belonging to seven species of *Candida* were isolated from various forms of candidiasis in diabetic patients. They were *C. albicans* 53 (47%), *Candida tropicalis* 36 (33%), *Candida glabrata* 9 (8%), *Candida parapsilosis* 4 (4%), *Candida guillermondii* 2 (2%), *Candida krusei* 5 (5%) and *Candida kefyr* 1 (1%). Of the 53 strains of *C. albicans*, all expressed greater adherence to BEC, high hydrophobicity and increased proteinase activity, whereas phospholipase was observed in 52 (98%) strains.

Three different morphological forms of *C. albicans* were obtained, namely Ring (R), Star (S) and Wrinkled (W) types, which differed from the original Smooth (O) variety. The frequency of switch of Smooth type to Ring, Star and Wrinkled was 1.4%, 1.1% and 0.95%, respectively.

It was observed that the adherence of clinical isolates CA-058 and CA-V88 to BEC was significantly greater than ($P<0.001$) the

Table 1

Expression of virulence factors by different morphological forms of *C. albicans*.

<i>C. albicans</i> strain	Colony type	Virulence factor			
		Adherence (no. of yeast cells/100 BEC)	Cell surface hydrophobicity (%)	Proteinase (OD ₂₈₀ /ml)	Phospholipase (P _z value)
CA-058	Smooth	494±28	76±13	2.182±0.131	0.60±0.05
	Ring	474±37	71±11	2.591±0.122*	0.64±0.07
	Wrinkled	328±32 [#]	52±9 [#]	2.815±0.193 [#]	0.76±0.06*
	Star	255±41 [#]	34±7 [#]	1.668±0.143 [#]	0.79±0.04*
CA-V88	Smooth	480±49	73±12	2.036±0.115	0.63±0.05
	Ring	462±36	66±10	2.451±0.163*	0.66±0.03
	Wrinkled	329±40 [#]	45±8 [#]	2.647±0.138 [#]	0.80±0.04*
	Star	268±27 [#]	30±6 [#]	1.480±0.146 [#]	0.87±0.03*
NCPF 3153A	Smooth	281±23	84±9	1.915±0.126	0.53±0.08
	Ring	248±17	83±13	2.240±0.166*	0.56±0.04
	Wrinkled	194±13 [#]	59±15 [#]	2.374±0.179 [#]	0.64±0.04
	Star	151±29 [#]	42±7 [#]	1.621±0.084*	0.68±0.05*

All readings are the mean (\pm SD) of four independent assays. * $P<0.05$, $^{#}P<0.01$ when compared to that of Smooth type.

control strain *C. albicans* NCPF 3135A. Also, the adhesion of 'O' type cells to BEC was significantly greater than the adhesion of 'W' type or 'S' phenotype, but not significantly greater than the adhesion of 'R' type (Table 1). This type of adhesion pattern was observed in all three strains tested.

The cell surface hydrophobicity of 'O' type cells was significantly greater than ($P<0.01$) CSH of 'W' or 'S' type, but not significantly greater than the CSH of 'R' phenotype (Table 1).

Proteinase activity of each phenotype, expressed as OD₂₈₀, varied dramatically among colony phenotypes (Table 1). While there was not much difference in the activities of all three strains tested (2.182 by CA-058 to 1.915 by *C. albicans* NCPF 3135A for 'O' phenotype, statistically not significant), there was significant difference ($P<0.01$) in proteinase activity between switch phenotypes of the same strain. Proteinase activity was highest with the 'W' phenotype (2.815) of *C. albicans* CA-058, followed closely by the 'R' phenotype (2.591) and then 'O' phenotype (2.182). The 'S' phenotype was observed to produce least proteinase activity when compared to the parent 'O' phenotype of *C. albicans*. This pattern of proteinase production was similar to all three strains of *C. albicans* tested.

Phospholipase activity of each phenotype, expressed as Pz, varied dramatically among colony phenotypes (Table 1). While there was not much difference in the activities of all three strains tested (0.60 by CA-058 to 0.53 by *C. albicans* NCPF 3135A for 'O' phenotype, statistically not significant), there was significant difference ($P<0.01$) in phospholipase activity between switch phenotypes of the same strain. Phospholipase activity was highest with the 'O' phenotype (0.60) of *C. albicans* CA-058, followed closely by the 'R' phenotype (0.64). The 'W' and 'S' phenotype was observed to produce least phospholipase activity when compared to the parent 'O' phenotype of *C. albicans*. This pattern of phospholipase activity was similar to all three strains of *C. albicans* tested.

The MIC values of fluconazole for *C. albicans* strains CA-058, CA-V88 and NCPF 3135A were 5, 1 and 0.25 µg/ml, respectively, for itraconazole 0.25, 0.5 and 0.125 µg/ml, respectively, and for ketoconazole 0.5, 0.5 and 0.0625 µg/ml, respectively. Although there was significant difference ($P<0.001$) in MIC values for the three azole drugs between strains of *C. albicans* tested, there was no difference in the susceptibility of different phenotypes of each strain.

Discussion

In the present study, the most common species isolated were *C. albicans* followed by *C. tropicalis* and *C. glabrata*. However, a previous study on candidiasis in diabetic patients has shown

C. albicans and *C. glabrata* followed by *C. tropicalis* as the most common species causing infections in diabetic patients.¹² All strains of *C. albicans* isolated were able to express all virulence factors studied except for phospholipase production.

The present study indicates that the degree of expression of virulence factors such as CSH, adherence, proteinase and phospholipase does vary significantly among the different phenotypes of *C. albicans*. It was observed that proteinase activity was greatest with the Wrinkled (W) phenotype; followed by the Ring (R), Smooth (O) and the Star (S) phenotypes. A previous study has shown that switching in laboratory strains of *C. albicans* plays an important role in regulating the transcription of certain secreted aspartyl proteinase genes SAP1 and SAP3.⁵ Therefore, the variation in secretion of proteinases by the different morphological types observed might be due to the differential regulation of proteinase genes.

Phospholipase activity was greatest with the Smooth (O) phenotype, followed by the Ring (R) phenotype and least with Star (S) and Wrinkled (W) phenotypes. Phospholipases are usually present at the periphery of yeast cells and are secreted into the external environment, whereas phospholipase is concentrated only at the tips of the hyphal forms of *C. albicans*.² As the smooth colony phenotype has been shown to contain the greatest percentage of yeast cells and the Wrinkled/Star types the least, the above finding may explain the observations of our study.²⁵ But our results are in contrast to the findings of a previous study which showed the Stipple phenotype as exhibiting greater phospholipase activity than the wild, smooth variety.⁸ Previous studies have also shown that differential expression of phospholipase genes in *C. albicans* occurs in humans with active oral and vaginal infection and changes in the degree of phospholipase production due to phenotypic switching may facilitate a successful invasion by *C. albicans*.^{8,17}

Variation was also observed in the ability of the different phenotypes of *C. albicans* to adhere to BEC and in their expression of CSH. The generalized hierarchy of adherence to BEC was as follows: Smooth (O)>Ring (R)>Wrinkled (W)>Star (S). The expression of CSH by the different phenotypes also exhibited a similar pattern. Changes in the adhesion and CSH due to phenotypic switching have been reported in earlier studies.²⁵

C. albicans is known to exhibit both yeast form and hyphal form in culture during phenotypic switching. It has been demonstrated that the percentage of yeast cells in a particular population has a significant effect on the adherence of *C. albicans*. Such phenomenon occurs more frequently with the Smooth (O) phenotype, which has been shown to adhere better to mucosal epithelia, probably due to the formation of germ tubes rather than a population of *C. albicans*, which has a greater percentage of hyphal forms (Star type).²⁵

High-frequency phenotypic switching and differential expression of virulence have been shown to occur at an elevated level in *C. albicans* causing infection in HIV-infected individuals.^{1,24} This can occur in diabetic individuals also, which might not only result in the emergence of variant phenotypes expressing different levels of virulence determinants but also, presumably, exhibiting very different combinations of virulence traits. Thus, a high level of spontaneous variability in such populations would provide them with the advantage of rapid adaptation and this might provide a particular morphological type with a distinct advantage over other types in causing candidiasis.

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