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Original

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.

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

Palabras clave:
Candida albicans
Variaciones fenotípicas
Virulencia
Diabetes

RESUMEN

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 patogenicidad 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 medium[10] supplemented with 70 μg arginine/ml and 0.1 μM $ZnSO_4$ at 25 °C for 24 h. The cells were harvested by centrifugation and suspended in sterile distilled water and counted using a haemocytometer. *Candida* (10^6) 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 1×10^3 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 Pearsall[7] 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 (1×10^5 cells/ml of PBS, 0.5 ml) and yeast cells (1×10^7 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 Hazen[4] 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. 9×10^8 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 (5×10^6 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 10^6 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