Increased expression of virulence attributes in oral *Candida albicans* isolates from human immunodeficiency virus-positive individuals

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Oral candidiasis caused by *Candida albicans* is recognized as one of the most frequent opportunistic infections in human immunodeficiency virus (HIV)-infected individuals. The overall severity and chronicity of oral candidiasis has been attributed exclusively to the HIV-induced immune deficiency of the affected individuals but not to the virulence factors of the pathogen, i.e. *C. albicans*. However, genotypic and phenotypic studies have suggested that HIV infection might be associated with preferential selection of *C. albicans* strains with altered virulence determinants, leading to colonization with *Candida* populations that are better able to cause disease in these immunologically compromised hosts. If this process of selection is indeed related to pathogenicity, it may be possible to measure alterations in different virulence factors produced by *C. albicans* in HIV-infected patients. To evaluate this hypothesis, the present work was undertaken to determine simultaneously the expression of five virulence factors in oral *C. albicans* isolates colonizing and infecting HIV-positive and -negative individuals. The significance of genotypes in the pathogenesis of oral candidiasis was also elucidated. Oral swabs were collected from 335 consecutive individuals (210 HIV-positive and 125 HIV-negative). Virulence factors and genotypes were determined for all the *C. albicans* strains isolated. The results showed significantly increased expression of proteinase, phospholipase and haemolytic activities, as well as a greater ability to adhere, in isolates from HIV-positive compared with HIV-negative individuals (*P* < 0.05). However, no significant differences in virulence factor expression in isolates colonizing or infecting HIV-positive individuals were seen. Genotype A was the predominant type (71.3%); however, a relationship could not be established between the genotypes and the virulence factors, or with clinical infection. These data support the concept of preferential *C. albicans* strain selection with altered virulence determinants in HIV-infected individuals and emphasize the need for further molecular genetic linkage studies that could be helpful in dissecting the molecular causes of preferential strain selection, which may lead to new approaches for therapeutic intervention.

**INTRODUCTION**

*Candida albicans* is recognized as one of the most common causes of opportunistic infections in human immunodeficiency virus (HIV)-infected individuals. Up to 90% of HIV-positive individuals suffer from mucosal candidiasis at least once in the course of their disease (Hung et al., 2005); this typically involves the oral cavity, with an occasional spread to the oesophagus. The overall severity and chronicity of oral candidiasis has been attributed exclusively to the HIV-induced immune deficiency of the affected individuals and not to the virulence factors of the pathogen (Ollert *et al.*, 1995). However, genotypic and phenotypic studies suggested that HIV infection might be associated with preferential selection of *C. albicans* strains with altered virulence determinants, resulting in replacement of the original commensal strains and leading to colonization with *Candida* populations that are better able to cause disease in these immunologically compromised hosts (Miyasaki *et al.*, 1992; Schmid *et al.*, 1992; Ollert *et al.*, 1995; Sweet *et al.*, 1995; Jain *et al.*, 2010). These virulence factors include adherence, production of hydrolytic enzymes, haemolytic activity and phenotypic switching, and identification of these could provide powerful insights into the pathogenic process (Calderone & Fonzi, 2001). If this process of selection is indeed related to pathogenicity, it may be possible to measure alterations in different virulence factors produced by *C. albicans* in HIV-infected patients. A few researchers have investigated this...
aspect, but generally this has involved a limited number of virulence factors (Ollert et al., 1995; Sweet et al., 1995; Jain et al., 2010). The present study simultaneously evaluated the expression of five important virulence factors, namely adherence to buccal epithelial cells (ABEC), proteinase activity, phospholipase activity, haemolytic activity and phenotypic switching in C. albicans isolates colonizing and infecting HIV-positive and -negative individuals. The epidemiology of C. albicans infection is complex. Several DNA-based molecular typing methods are now available for investigating species evolution and epidemiology. One such method is genotyping based on the presence or absence of the self-splicing group I intron in the large subunit (CaLSU) of the rRNA gene, which enables strain differentiation into three genotypes in the same genome (Sugita et al., 2002). Few workers have shown an association between certain genotypes and virulence factors (Sugita et al., 2002; Nawrot et al., 2008).

The present work was undertaken to determine the expression of virulence factors and to elucidate the significance of genotypes in pathogenesis of oral candidiasis in C. albicans isolates obtained from HIV-positive and -negative individuals.

**METHODS**

**Subjects.** A total of 335 consecutive individuals (210 HIV-positive and 125 HIV-negative) attending the National AIDS Research Institute clinic in Pune, India, during July 2007 to March 2009 were included in the study. The study was approved by the institutional ethics committee. Participants providing informed consent were included in the study. Participant recruitment criteria have been published previously (Mane et al., 2010).

**Sample collection and processing.** Oral swabs were obtained by firmly swabbing the lesion site with sterile cotton swabs in the case of symptomatic individuals, whilst for asymptomatic individuals, a strong thorough swabbing of the lesion site with sterile cotton swabs in the case of symptomatic individuals, whilst for asymptomatic individuals, a strong thorough swabbing of the lesion site was performed. The swabs were collected per patient. One swab was inoculated immediately onto Sabouraud dextrose agar (SDA) with chloramphenicol (0.05 g l⁻¹) and incubated at 37 °C for 2 days and for an additional 7 days at 37 °C before being considered negative. All isolates were identified and confirmed by standard mycological techniques (Mane et al., 2010). The second swab was used for determining primary phenotypic switching, described below. All isolates were maintained on SDA slants at 2-4 °C and fresh subcultures were used for determination of virulence factors.

**Virulence factors**

For determination of virulence factors, each isolate was tested in duplicate in each assay and two assays were carried out for each isolate on separate occasions. The mean of the four values obtained in this way was considered for analysis.

**ABEC.** An adherence assay was performed as described by Al-Abide et al. (2004). An overnight culture of Candida was grown at 37 °C in 0.67 % (w/v) yeast nitrogen base supplemented with 2.5 % (w/v) glucose. Flasks containing 50 ml of the same medium were inoculated with 1 ml overnight culture and grown for 24 h in a shaking water bath at 37 °C. The cells were harvested by centrifugation (1200 g, 10 min) and washed twice with sterile PBS. Yeast cells were suspended in PBS to a concentration of 1 × 10⁵ cells ml⁻¹. Buccal epithelial cells (BECs) were collected from healthy laboratory staff (two females and two males) aged 25–31 years. They had no signs or symptoms of oral candidiasis or other oral pathologies and were not receiving any antibiotic treatment at the time of study. BECs were collected at the same time of day each morning before performing the assay by gentle rubbing of cheeks with a sterile cotton swab. These BECs were dislodged in PBS, washed twice and collected by centrifugation. The BECs were suspended in PBS to a concentration of 1 × 10⁶ cells ml⁻¹. Yeast suspension (2 ml) was mixed with 2 ml PBS suspension. The mixture was shaken at 37 °C for 2 h and then passed through a 20 μm filter to remove non-adherent yeast cells. The BECs on the filter were washed in 5 ml vols sterile PBS and finally suspended in 5 ml of the same buffer. A drop of this suspension was spotted onto a glass slide and Gram staining was carried out. Adherence was determined microscopically by counting the mean number of yeast cells adhering to every 100 BECs. Each assay was carried out in duplicate on two separate occasions. C. albicans ATCC 90028 was used as the control strain.

**Proteinase production.** Proteinase production was determined using BSA agar plates (Aoki et al., 1990). Sixty millilitres of solution containing 0.04 g MgSO₄·7H₂O, 0.5 g K₂HPO₄, 1 g NaCl, 0.2 g yeast extract, 4 g glucose and 0.5 g BSA was prepared, and the pH was adjusted to 3.5 with 1 M HCl. The solution was sterilized by filtration and mixed with 140 ml molten agar. Twenty millilitres of this medium was poured into Petri dishes. Standard inocula of test and control Candida isolates [5 μl containing 10⁶ yeast cells (ml saline)⁻¹] were spot-inoculated onto the plates. A further 5 μl saline without yeast cells was also spot-inoculated. The plates were incubated at 37 °C for 7 days. Plates were stained with 0.5 % Coomassie Brilliant Blue R250 (Pierce Biotechnology) in 10% (v/v) acetic acid and 45 % ethanol for 20 min at room temperature and destained three times with destaining solution (10 % acetic acid, 45 % ethanol) for 20 min at 37 °C and once with water for 20 min at 37 °C (Borst & Fluit, 2003). The diameter of the colonies was measured before Coomassie staining and the diameter of the clear zones (in mm) was measured after staining. Proteinase activity (Prz) was expressed as the ratio of the colony diameter to the colony diameter plus the zone of precipitation. All isolates were tested in duplicate on two separate occasions. C. albicans ATCC 10231 and Candida kefyr ATCC 2512 were used as positive and negative controls, respectively. Prz values were interpreted as: 1, negative/no activity; 0.64–0.99, positive; <0.64, strongly positive. Thus, a low Prz indicated a stronger enzyme activity.

**Phospholipase production.** Phospholipase production was assayed using an egg yolk agar plate method (Kumar et al., 2006). SDA plates containing 1 M NaCl, 0.005 M CaCl₂ and 8% sterile egg yolk emulsion were used. Standard inocula of test and control Candida isolates [5 μl containing 10⁶ yeast cells (ml saline)⁻¹] were spot-inoculated onto the plates. A further 5 μl saline without yeast cells was also spot-inoculated. The plates were incubated at 37 °C for 5 days after which the diameter of the zone of precipitation around the colony (in mm) was determined. Phospholipase activity (Pz) was expressed as the ratio of the colony diameter to the colony diameter plus the zone of precipitation. All isolates were tested in duplicate on two separate occasions and the results were expressed as the duplicate mean. Pz values were interpreted as for Prz. C. albicans ATCC 10231 and C. kefyr ATCC 2512 were used as positive and negative controls, respectively.

**Haemolytic activity.** The haemolytic activity was determined by a blood agar plate assay method (Luo et al., 2001). The medium was prepared by adding 7 ml fresh sheep blood to 100 ml SDA supplemented with 3 % glucose, giving a final concentration of 7 %. The pH of the medium was 5.6 ± 0.2. Standard inocula of test and control Candida isolates of 5 μl containing 10⁶ yeast cells (ml saline)⁻¹ were spot-inoculated onto the plates. A further 5 μl saline without yeast cells was also spot-inoculated. The plates were incubated at 37 °C for 5 days. Plates were stained with 0.5 % Coomassie Brilliant Blue R250 (Pierce Biotechnology) in 10% (v/v) acetic acid and 45 % ethanol for 20 min at room temperature and destained three times with destaining solution (10 % acetic acid, 45 % ethanol) for 20 min at 37 °C and once with water for 20 min at 37 °C (Borst & Fluit, 2003). The diameter of the colonies was measured before Coomassie staining and the diameter of the clear zones (in mm) was measured after staining. Haemolytic activity (Hz) was expressed as the ratio of the colony diameter to the colony diameter plus the zone of precipitation. All isolates were tested in duplicate on two separate occasions. C. albicans ATCC 10231 and Candida kefyr ATCC 2512 were used as positive and negative controls, respectively.
saline)−1 were spot-inoculated onto the plates. A further 5 µl saline without yeast cells was also spot-inoculated. The plates were incubated at 37 °C in 5% CO2 for 48 h. The presence of a distinct translucent halo around the inoculum site when viewed with transmitted light indicated positive haemolytic activity (H2). H2 was expressed as the ratio of the colony diameter to the translucent zone of haemolysis (in mm). All isolates were tested in duplicate on two separate occasions and the results were expressed as the duplicate mean. H2 values were interpreted as for Pr, C. albicans ATCC 90028 and Candida parapsilosis ATCC 2201 were used as positive and negative controls, respectively.

Phenotypic switching. Primary phenotypic switching was assessed. The second swab obtained from the patient was used to determine the phenotypic switching. The cotton end of each swab was inserted into 0.5 ml sterile water in a polystyrene test tube and mixed vigorously for 30 s with a vortex mixer. A 0.15 ml sample of the swab wash was spread onto each of two agar plates containing supplemented Lee’s medium (Lee et al., 1975; Morrow et al., 1989) and incubated for 7 days at 25 °C (Vargas et al., 2000). The plates were verified for the different phenotypes such as smooth white, irregular wrinkle, star, ring, myceliated and heavily myceliated.

Determination of genotypes. Genomic DNA from C. albicans strains was extracted using a Qiagen DNA Mini kit following the manufacturer’s instructions. The primers used were CalSU-F (5’-GTATACCATCATCGGTGTCAC-3’) and CalSU-R (5’-GTT-TCTGGCCAGTGTGCTCTG-3’) (Sugita et al., 2002), which spanned the region that includes the site of transposable group I intron of the TCCTGCCCAGTGCTCTG-3 sequence. Amplification was performed by initial denaturation at 94°C for 10 s, followed by 30 cycles of 15 s at 94°C, 30 s at 56°C and 1 min, with the remaining volume made up with sterile distilled water. Amplification was performed by initial denaturation at 94°C for 1 min, followed by 30 cycles of 15 s at 94 °C, 20 s at 65 °C and 30 s at 72 °C, with a final extension at 72 °C for 10 min in a thermal cycler (GeneAmp PCR System 9700; AB Biosystems). All reaction products were separated by electrophoresis on a 2% agarose gel for 1 h at 100 V at room temperature in TAE buffer, stained with ethidium bromide and visualized using a gel documentation system (Bio-Rad). A 100 bp DNA ladder was run as a size marker. C. albicans can be classified into three genotypes on the basis of PCR amplification products: genotype A, 133 bp; genotype B, 512 bp; genotype C, 133 and 512 bp. The control strains used for genotyping were C. albicans ATCC 90028 (genotype A), C. albicans ATCC 90029 (genotype B) and a known tested laboratory strain for genotype C.

Statistical analysis. Statistical analyses were performed using SPSS software version 15.0. Student’s t-test was used to check the difference in mean virulence factor production between isolates from HIV-positive and -negative individuals. A χ2 test was used to test the association between genotypes and virulence factor activity. The correlation between virulence factors was assessed by a correlation coefficient. A value of P<0.05 was considered statistically significant.

RESULTS

Of the 210 HIV-infected individuals, 60 had lesions of oral candidiasis (symptomatic) and Candida species were isolated from all of them. Of the remaining 150 asymptomatic individuals, 88 (58.7%) were positive for oral carriage of Candida species. Of the 125 HIV-uninfected individuals, 28 (22.4%) showed candidial colonization. A total of 136 C. albicans isolates were detected, 115 from HIV-infected individuals (39 from symptomatic and 76 from asymptomatic subjects) and 21 from HIV-uninfected individuals. The distribution of Candida species and their antifungal susceptibility have been described previously (Mane et al., 2010).

Table 1 shows the production and quantitative expression of the various virulence factors.

All isolates showed ABEC. The number of yeast cells adhering per 100 BECs ranged from 28 to 989 and from 28 to 356 for isolates from HIV-infected and -uninfected individuals, respectively. The mean ABEC value was significantly higher in isolates from HIV-infected individuals (377.84 ± 227.25) compared with those from uninfected subjects (133.48 ± 90.62; P<0.001). No significant difference was observed in the mean ABEC in isolates from HIV-infected individuals causing candidiasis (484 ± 206.87) or colonization (423.18 ± 218.87; P=0.082) (Table 1).

Proteinase production was seen in 87.8 and 66.7% of isolates from HIV-infected and -uninfected individuals, respectively. Prz values ranged from 0.53 to 0.94 for the isolates from the HIV-infected group and from 0.75 to 0.93 for isolates from the HIV-uninfected group. The mean Prz value was significantly higher in isolates from HIV-infected individuals (0.728 ± 0.125) compared with those from uninfected subjects (0.826 ± 0.059; P=0.007). No significant difference was observed in the mean Prz value of isolates from HIV-infected individuals causing candidiasis (0.698 ± 0.133) or colonization (0.730 ± 0.098; P=0.062) (Table 1).

Phospholipase production was seen in 56.5 and 38.1% of isolates from HIV-infected and -uninfected individuals, respectively. Pz values ranged from 0.42 to 0.91 for isolates from the HIV-infected group and from 0.68 to 0.94 for isolates from the HIV-uninfected group. The mean Pz value was significantly higher in isolates from HIV-infected individuals (0.701 ± 0.124) compared with those from uninfected subjects (0.851 ± 0.104; P=0.002). No significant difference was observed in the mean Pz value of isolates from HIV-infected individuals causing candidiasis (0.715 ± 0.108) or colonization (0.694 ± 0.131; P=0.530) (Table 1).

Haemolytic activity was seen in all isolates from HIV-infected and -uninfected individuals. H2 values ranged from 0.46 to 0.86 for isolates from the HIV-infected group and from 0.55 to 0.81 for isolates from the HIV-uninfected group. The mean H2 value was significantly higher in isolates from HIV-infected individuals (0.622 ± 0.096) compared with those from uninfected individuals (0.676 ± 0.056; P=0.006). No significant difference was observed in the mean H2 value of isolates from HIV-infected individuals causing candidiasis (0.603 ± 0.089) or colonization (0.632 ± 0.099; P=0.114) (Table 1).

Phenotypic switching was seen in 35.7 and 28.6% of isolates from HIV-infected and -uninfected individuals, respectively. The predominant switched phenotype was the
myceliated type. Of the 41 isolates from HIV-infected individuals that were positive for phenotypic switching, 35 showed the myceliated form, four showed the heavily myceliated form and two showed irregular forms. All six isolates from HIV-uninfected individuals that were positive for phenotypic switching showed the myceliated form. There was no significant difference in the phenotype-switching activity of isolates from HIV-infected and -uninfected individuals or of isolates from HIV-uninfected individuals causing candidiasis or colonizers (Table 1).

Table 2 shows the genotypic distribution in *C. albicans* isolates. Genotypic analysis indicated that 71.3% isolates belonged to genotype A, 21.3% to genotype C and 7.4% to genotype B. Genotype B was not detected in isolates from HIV-negative individuals. No significant difference in genotype distribution was noted in isolates from HIV-infected and -uninfected individuals.

No significant association between genotype and any virulence factor was noted. No statistically significant correlation was noted between the virulence factors assayed.

DISCUSSION

In the present study, we evaluated the expression of five important virulence factors and the genotype distribution in *C. albicans* isolates from HIV-infected and -uninfected individuals. Adherence of the fungus to host cells is the initial step in the establishment of colonization or disease. Our results demonstrated that *C. albicans* isolates from HIV-infected individuals showed a significantly greater ability for ABEC compared with isolates from HIV-uninfected individuals, a finding that is in accordance with previous studies (Sweet *et al.*, 1995; Jain *et al.*, 2010). The increased adherence in HIV infection could be attributed to reduced salivary flow rate and alterations in the composition of saliva and mucosal tissues. In contrast, other investigators have shown a comparable degree of adhesion between HIV-positive and -negative isolates (Tsang & Samaranayake, 1999) and an even higher degree of adhesion by HIV-negative isolates (Pereiro *et al.*, 1997).

Extracellular hydrolases such as proteinases and phospholipases are major facilitators of host tissue invasion and of the disease process that ensues. A total of 89.7% of *C. albicans* isolates from HIV-positive individuals were proteinase producers, a finding that compares favourably with...
previous studies (Ollert et al., 1995; Koga-Ito et al., 2006; Menezes et al., 2006; Costa et al., 2010; Sacristán et al., 2011). Similar to the results of Ollert et al. (1995) and Wu et al. (1996), we found increased expression of proteinase activity in isolates from HIV-infected individuals compared with HIV-uninfected subjects. The proteinases of \textit{C. albicans} require a low pH for optimal activity. Interestingly, xerostomia, changes in salivary composition and the lower pH found in HIV-infected individuals favour the activity of proteinases (Wu et al., 1996).

Extracellular phospholipases are also considered a key attribute that aid invasion of the host mucosal epithelia. They are thought to contribute to virulence by lysing host cells or altering their surface characteristics so that adherence and penetration are facilitated. The phospholipase production in isolates from HIV-positive individuals was in agreement with other reports in the literature (Menezes et al., 2006; Gokce et al., 2007). Earlier studies comparing the phospholipase activity in isolates from diabetic and non-diabetic individuals and from subjects with and without denture stomatitis reported no significant differences between the test and control groups (Koga-Ito et al., 2006; Tsang et al., 2007). However, we found increased expression of phospholipase activity in isolates from HIV-infected individuals compared with uninfected subjects.

The ability of \textit{Candida} to acquire elemental iron through haemolysin production is pivotal to its survival and ability to establish infections in humans, in particular in disseminated candidiasis (Tsang et al., 2007). Studies demonstrating the important role of haemolysins in \textit{Candida} infections in different disease conditions were published recently (Tsang et al., 2007; Yenişekerli et al., 2010; Sacristán et al., 2011). In our study, all \textit{Candida} isolates exhibited haemolytic activity. The factors leading to increased expression of phospholipase and haemolysins in HIV-positive individuals are as yet unknown.

\textit{C. albicans} strains are capable of switching spontaneously, reversibly and at high frequencies between a number of general phenotypes distinguishable by colony morphology. Switching has been demonstrated to regulate a number of characteristics in pathogenesis (Vargas et al., 2000). However, data on phenotypic switching in clinical \textit{C. albicans} isolates are sparse. In the present study, isolates from HIV-positive individuals showed greater switching compared with isolates from healthy individuals, although the difference was not statistically significant. Vargas et al. (2000) demonstrated elevated phenotypic switching in \textit{C. albicans} isolates from HIV-positive individuals, although this high-frequency phenotypic switching was observed just prior to the first episode of oral thrush. Further investigations are warranted to clarify the role of phenotypic switching in the pathogenesis of candidiasis.

Our results confirm earlier genotypic and phenotypic findings that support the concept of preferential \textit{C. albicans} strain selection with overall higher virulence activities as a consequence of the HIV-positive phenotype of the host (Miyasaki et al., 1992; Schmid et al., 1992; Ollert et al., 1995; Sweet et al., 1995; Jain et al., 2010). We did not find any significant differences in virulence factor expression in isolates colonizing or infecting HIV-positive individuals, indicating that preferential selection in HIV infection occurs before the symptoms of immune deficiency are evident and prior to the sequelae of opportunistic mucosal infections and consequent treatment regimens (Sweet et al., 1995). The selection of more virulent strains may contribute to the predisposition of HIV-infected individuals to oral candidiasis; however, increased expression of virulence factors did not correlate with oral candidiasis, probably suggesting that, for oral candidiasis to occur, there has to be an interplay of host factors as well as different virulence factors of the organism.

Recent advances in technologies based on molecular biology have enabled an increase in information about the genetic diversity of \textit{C. albicans}. We used the current method for genotyping as it is easy to perform, gives clear results and can be used for analysis of a large number of isolates. The results showed that genotype A was predominant in isolates from HIV-infected as well as -uninfected individuals. A higher incidence of genotype A among \textit{Candida} genotypes has been reported previously (Sugita et al., 2002; Shinobu et al., 2007). However, we could not establish a relationship between the genotypes and the virulence factors, or the genotypes and clinical infection. Earlier studies have reported similar findings (Shinobu et al., 2007; Abaci, 2011).

In conclusion, we have reported here the increased quantitative expression of four virulence factors in \textit{C. albicans} isolates from HIV-positive individuals compared with isolates from HIV-negative individuals and showed that these data support the concept of preferential \textit{C. albicans} strain selection with overall higher virulence activities in HIV-infected individuals. The results emphasize the need for future studies correlating the expression of \textit{C. albicans} virulence factors with selected genetic linkages of the fungus, which could ultimately be helpful in dissecting the molecular cause of preferential strain selection, which in turn may open up new approaches for therapeutic interventions.

\textbf{REFERENCES}


