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Genetic and phenotypic evaluation of *Candida albicans* strains isolated from subgingival biofilm of diabetic patients with chronic periodontitis

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Candida spp. are commensal microorganisms that are part of the microflora of different sites within the oral cavity. In healthy subjects, who have an unaltered immunological status, these yeasts do not cause disease. However, in immunosuppressed individuals whose condition may have been caused by diabetes mellitus, *Candida* spp. can express different virulence factors and may consequently become pathogenic. Studies have detected the presence of *Candida* spp. in periodontal sites of patients with chronic periodontitis, especially those that are immunologically compromised. However, the role of these microorganisms in the pathogenesis of periodontal disease is still unknown. The objectives of this study were: (1) to isolate and identify *Candida albicans* strains from subgingival sites of diabetic patients with chronic periodontitis; (2) to evaluate the following virulence factors; colony morphology, proteinase, phospholipase and hemolysin activities and cell surface hydrophobicity (CSH) under different atmospheric conditions; and (3) to determine the genetic patterns of these *C. albicans* isolates. Microbial samples were collected from subgingival sites and seeded on CHROMagar for subsequent identification of *C. albicans* by polymerase chain reaction (PCR). For the phenotypic tests, all strains of *C. albicans* were grown under reduced oxygen (RO) and anaerobiosis (ANA) conditions. Genotypes were defined by the identification through PCR of the transposable introns in the 25S rDNA. The results obtained relative to virulence factors were analyzed according to the atmospheric condition or genetic group, using Chi-square and Wilcoxon non-parametric tests. In this study, 128 strains were identified as *C. albicans* and of these, 51.6% were genotype B, 48.4% were genotype A and Genotype C was not found. Most of the strains were alpha-hemolytic in both atmospheric conditions, without a statistical difference. However, when comparing the genotypes, 46.1% of the genotype A strains were beta-hemolytic. In relation to colony morphology, 100% of the strains under ANA showed rough colonies, which were especially prevalent in genotype A isolates. In contrast, most of the colonies were smooth under RO. *C. albicans* strains did not produce proteinase and phospholipase activity in the total absence of oxygen. In RO, most strains had high proteinase activity and were positive by phospholipase tests ($P < 0.05$). Hydrophobicity was higher in anaerobiosis and was noted mainly for genotype A isolates. In conclusion, environmental oxygen concentration influenced the

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virulence factors of *C. albicans* strains isolated from subgingival sites of diabetic and periodontal patients. In addition, genotype A seems to be more virulent based on the phenotypic tests evaluated in this study.

Keywords periodontal disease, diabetes mellitus, *Candida* spp., virulence factors

Introduction

Candida species are microorganisms that live commensally in the oral cavity of healthy individuals, but their incidence and virulence seems to be increased in those that are immunocompromised [1,2]. These species can become pathogenic in response to physiological changes in the host, causing oral candidiasis or invasive systemic infections [3]. Mucosal surfaces are their primary oral reservoirs, but they can also be found in dental plaque, endodontic infections, peri-implantitis lesions and the subgingival biofilm of periodontal pockets of periodontitis, especially in HIV-positive and diabetic patients [4–9]. The higher prevalence of *Candida* spp. in diabetic patients has been associated with the type and duration of the disease and the degree of glycemic control [10,11].

Candida ssp. may produce some virulence factors that facilitate their proliferation and, consequently, adherence to the epithelium and invasion of the connective tissue. These yeasts produce essential exoenzymes that improve their capacity to establish colonizing and/or infections [12,13]. The exoenzymes, such as secreted aspartyl proteinase (SAPs) and phospholipases, degrade immunoglobulins and proteins from the extracellular matrix, inhibit phagocitosis by polymorphonuclear neutrophils and induce inflammatory reactions [6,14]. Another virulence factor is related to iron acquisition by *Candida* spp. Iron is an essential nutrient for many microorganisms and its uptake may play a special role in promoting infections [15]. The secretion of hemolysin, followed by iron acquisition, facilitates hyphal invasion and the development of disseminated candidiasis. Increase in blood glucose concentrations may contribute, directly or indirectly, to increased hemolysin activity among *C. albicans* isolates in diabetic patients [16].

Adherence to host tissue is the first step in the pathogenic process. The mechanisms of adherence to different cellular types or surfaces by *Candida* ssp. are complex and still not completely understood. It would appear that this process is achieved by a combination of specific (receptors) and non-specific mechanisms (electrostatic forces, aggregation and cell surface hydrophobicity [CSH]) [17]. CSH has a central role in the pathogenesis of *C. albicans*. Hydrophobic cells exhibit greater adherence to epithelial and endothelial cells and extracellular matrix proteins and seem to be more resistant to phagocytosis [18]. *Candida albicans* is unique among *Candida* species, in that the CSH status varies in response to different environmental conditions and growth phases [19].

Another property of *Candida* spp. is their ability to grow either aerobically or anaerobically [20] as they have developed adaptive mechanisms to survive in both situations. Oxygen can generate reactive products during an infection and induce an oxidative stress response. Treatment of *C. albicans* with low concentrations of superoxide generating agents, such as hydrogen peroxide, induces a redox potential with the activation of antioxidant enzymes which protects cells from the lethal effects of a subsequent challenge with higher concentrations of these oxidants [21]. Another situation is an anaerobic environment, such as root canal systems and periodontal pockets, which lead to polymicrobial infections. Rosa *et al*. [22] demonstrated an increment in SAP secretion when *C. albicans* strains recovered from periodontal pockets and other intra-oral sites were grown under anaerobic conditions, suggesting that the oxygen concentration in the atmosphere surrounding these cells can exert an influence on the virulence attributes of this yeast.

Pizzo *et al*. [23] suggested that heterogenotypes within subgingival *C. albicans* isolates is not just the result of the spreading of *Candida* spp. from saliva or biofilm, but also the adaption of new strains to subgingival pockets that can develop different virulence properties. This study evaluated some virulence factors of *C. albicans* isolated from subgingival biofilm of patients with chronic periodontitis who have insulin-dependent type 2 diabetes mellitus and investigated the relationship of the disease with different atmospheric conditions and genetic diversity of the etiologic agent.

Material and methods

This research was approved by the ethical committee for research of the Piracicaba Dental School, State University of Campinas, São Paulo, Brazil.

Inclusion and exclusion criteria

Residents of Piracicaba, São Paulo, Brazil, ranging in age from 31–68 years who had chronic periodontitis (CP) and medical diagnoses of type 2 diabetes mellitus were included in the study. Glycemic control with insulin supplementation

was confirmed by an endocrinologist. Exclusion criteria included use of antibiotics and periodontal treatment during the previous six months, pregnancy, smoking, systemic disease, immunodepression, clinical manifestation of oral candidiasis, use of partial and/or total prosthesis, use of orthodontic appliances or any medication that could interfere with the periodontium or the response to periodontal therapy.

Patient selection

Eleven patients with a clinical diagnosis of generalized chronic periodontitis, as defined by probing depths (PD) ≥5 mm in ≥10 teeth, radiographic bone loss ranging from 30–50%, and ≥20 teeth were included in the study. After clinical analysis, the supragingival biofilm was removed with sterile gauze, with samples taken from the sites with the deepest PD ≥5 mm in each subject using a sterile periodontal curette. Pooled biofilms from each site were transferred to separate Eppendorf microtubes containing 1 ml of reduced transport fluid (RTF). Immediately after collection, the samples from each site were diluted and plated onto a Sabouraud dextrose agar (SDA) with chloramphenicol and chromogenic medium (CHROMagar Candida®, Biomerieux, Paris, France) and incubated at 37°C for 48 h in a reduced oxygen atmosphere (10% CO_2 and 90% air). The green colonies growing on the CHROMagar plates were randomly selected and cultures stored in glycerol stock at −20°C for later identification by PCR.

PCR (polymerase chain reaction)

DNA from the *Candida* isolates was extracted using a protocol described by Nascimento *et al*. [24] and quantified in a spectrophotometer at 260 nm (Genesys 10UV, Rochester, NY,USA) to obtain a standard concentration of 100 ng/ml and stored at −20°C for subsequent PCR reactions. DNA samples were identified by PCR using specific primers (Forward: 5′-ACT GCT CAA ACC ATC TCT GG-3′ and Reverse: 5′-CAC AAG GCA AAT GAA GGA AT-3 with fragment size of 472 bp) for *Candida albicans* [25]. Purified DNA from a reference *C. albicans* (ATCC 90028) strain was used as a positive control, with the described primer designed specifically for *C. albicans*. The molecular mass ladder (100 bp DNA ladder, Gibco, Grand Island, NY, USA) was included for running in agarose gel. PCR amplification was performed with a GeneAmp PCR system 2400 (Perkin-Elmer-Applied Biosystems) under the following thermal conditions: 72°C for 5 min, 38 cycles of 95°C for 30 s, 55°C for 45s and 72°C for 30 s and extension at 72°C for 5 min. The PCR products were separated by electrophoresis in 2% agarose gels and Tris-borate-EDTA running buffer (pH.8.0). The DNA was stained with 0.5 μg ethidium bromide/ml and visualized under UV illumination (Pharmacia LKB-MacroVue, San Gabriel, CA, USA).

Colony morphology

Colony morphology was evaluated for all *C. albicans* isolates using a blood plate assay. Media were prepared by adding 7 ml fresh sheep blood to 100 ml SDA (Merck) supplemented with glucose at a final concentration of 3% (w/v). The final pH of the medium was approximately 5.6. Test isolates were grown on this medium for 24 h, and a single drop (10 μl) containing 10^8 CFU/ml of each inoculum in sterile saline (absorbance 0.5 at 600 nm) was inoculated onto the agar blood plates. These were incubated at 37°C in a reduced oxygen atmosphere (10% CO_2 and 90% air – Water-Jacked CO_2 Incubators – Cole Parmer Instruments, EUA) or anaerobiosis (10% CO_2, 10% H_2 and 80% N_2 – Anaerobic Workstation, Don Whitley Scientific, Shipley, UK) for 48 and 72 h, respectively. After this, the colony morphology was assessed as either smooth or rough using a stereoscopic microscope.

Proteinase and phospholipase activity determination by the agar plate method

All *C. albicans* isolates were tested in triplicate, during three independent experiments, to verify the enzymatic activity of proteinases (SAPs) and phospholipases [12,26]. The test medium for proteinases was a BSA (bovine serum albumin) agar medium containing 2 g BSA, 145 g YNB (Yeast Nitrogen Base) Difco Laboratories, Detroit), 20 g glucose and 20 g agar per liter of distilled water. The test medium for phospholipases consisted of 10 g peptone, 57.3 g sodium chloride, 0.55 g calcium chloride, 30 g glucose, 20 g agar, and 100 ml 50% sterile egg yolk (egg yolk enrichment) per liter of distilled water. Test isolates were grown on SDA for 24 h, and a single drop (10 μl) of each containing 10^8 CFU/ml of sterile saline (absorbance 0.5 at 600 nm) was inoculated onto the test media. The plates were incubated at 37°C in a reduced oxygen atmosphere or anaerobiosis for 48 and 72 h to examine the proteinases and phospholipases, respectively. The enzymatic activity was determined by the formation of a halo around the yeast colony, and measured in terms of the ratio of the diameter of the colony to the total diameter of the colony plus the zone of precipitation (Pz), according to the

method described by Price *et al*. [26]. According to this system, Pz = 1.0 indicated that the test strain was negative for proteinase/phospholipase, while a value of Pz ≤ 0.63 signified that the test strain was releasing large amounts of proteinases/phospholipases (strongly positive). Values of Pz of between 0.64 and 0.99 signified that the test strain was releasing small amounts of proteinases/phospholipases (positive).

Determination of hemolysin activity

Hemolysin activity was evaluated for all *C. albicans* isolates using a blood plate assay [16]. Media were prepared by adding 7 ml fresh sheep blood to 100 ml SDA (Merck) supplemented with glucose at a final concentration of 3% (w/v). The final pH of the medium was approximately 5.6. Test isolates were grown on SDA for 24 h, and a single drop of each (10 µl) containing 10^8 CFU/ml of sterile saline (absorbance 0.5 at 600 nm) was inoculated onto the blood agar plates which were then incubated at 37°C in a reduced oxygen atmosphere or anaerobiosis for 48 and 72 h, respectively. A further 10 µl of saline, but without yeast cells, was overlaid onto the same plate. A reference strain of *C. albicans* (ATCC 90028) was used as a positive control. In addition, one standard strain of *Staphylococcus aureus* (ATCC 10832) and one strain of *Streptococcus mutans* (UA159), which induce beta and alpha hemolysis, respectively, were used as positive controls. Gama-hemolytic strains (γ-hemolytic) produce no halo, alpha-hemolytic (α-hemolytic) strains produced a green halo and beta-hemolytic (β-hemolytic) strains produced a yellow halo. The assays were conducted in triplicate during three independent experiments for each of the tested yeast isolates.

Cellular Superficial Hydrophobicity assay (CSH)

Hydrophobicity assay (CSH) was evaluated according to Rodrigues *et al*. [27] for all *C. albicans* isolates, during two independent experiments. In these studies, identical glass tubes (10 ml, O.D × L – 13 × 100 mm) with screw caps were used to avoid methodologic caused differences in the results. Fifty ml of Sabouraud dextrose broth (SDB; Difco Laboratories Detroit, MI, USA) was inoculated with yeast cells and incubated overnight at 37°C in 10% reduced oxygen or anaerobiotic conditions. The yeast cells were then harvested and washed twice in phosphate buffer, pH 8.0. A yeast suspension was prepared in the same buffer to achieve an optical density (A0) of 0.4–0.6 (at 600 nm) and 150 µl of hexadecane was added to 3 ml of this yeast suspension. After 10 min of incubation at 30°C, the tubes were vortexed twice for 30 sec. After allowing phase separation for 30 min, the optical density of the lower aqueous phase (A1) was measured and compared with the optical density obtained prior to the mixing procedure (A0). The percentage of cells in the hexadecane layer (adhered cells) was used to estimate the hydrophobicity, using the following formula: % H = A0 – A1/A0 × 100%.

Genotyping

Ribosomal sequences are extensively used for genotyping of many fungal pathogens. The method developed by McCullough *et al*. [28] uses a pair of primers designed to span the region that includes the site of the 1 intron transposable group of the 25S rRNA gene (rDNA), to classify *C. albicans* strains into three genotypes, according to the size of the PCR products, i.e., genotype A (approximately 450 bp), genotype B (approximately 840 bp), and genotype C (two products of approximately 450 and 840 bp). DNA of the yeasts previously extracted and spectrophotometrically quantified (100 ng/ml) was submitted to PCR reactions. PCR was performed using the primers CA-INT-L (5′-ATA AAG GGA AGT CGG CAA ATA GAT CCG TAA-3′) and CA – INT- R (5′-CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT-3′). The products were analyzed by electrophoresis through the use of 3% (wt/vol) agarose gel. Bands were visualized by UV transllumination after ethidium bromide staining.

Statistical analysis

The results obtained for the tested virulence factors (enzymatic activities and CSH) were analyzed according to the atmospheric condition or genetic group, using Chi-square and Wilcoxon non-parametric tests.

Results

Identification of C. albicans by PCR

In this current study, 172 *Candida* spp. strains were obtained on CHROMagar from samples collected from the subgingival biofilm of patients with chronic periodontitis and controlled insulin-dependent type 2 diabetes mellitus. From these isolates, 128 were identified as *C. albicans* by PCR from 63.63% of the periodontal sites of 11 patients.

Virulence factor × atmospheric conditions

Colony morphology. The colony morphology of 100% of the isolates under anaerobiosis were rough, whereas in reduced oxygen, most (71.9%) were smooth (Table 1), indicating a statistical difference between the atmospheric conditions.

Proteinase and phospholipase activities. All *C. albicans* isolates showed proteinase/phospholipase activity under

Table 1 Frequency of virulence factors of *Candida albicans*, according to atmospheric condition.

Virulence factor	Results	Scores	RO (%)	ANA (%)
Colony morphology	Rough	1	21.9	100
	Intermediate	2	6.3	0
	Smooth	3	71.9	0
Proteinase activity	Negative (Pz = 1)	0	2.4	100
	Positive (Pz = 1–0.63)	1	3.1	0
	Strongly positive (Pz < 0.63)	2	94.5	0
Phospholipase activity	Negative (Pz = 1)	0	8.7	100
	Positive (Pz = 1–0.63)	1	58.3	0
	Strongly positive (Pz < 0.63)	2	33.1	0
Hemolysin activity	Gama-hemolysis	0	1.6	1.6
	Alpha-hemolysis	1	64.1	64.1
	Beta-hemolysis	2	34.4	34.4
Hydrophobicity	Low (0-30%)	1	34.4	16.7
	Moderate (31–59%)	2	43.8	31.7
	High (above 60%)	3	21.9	51.6

RO, reduced oxygen; ANA, anaerobiosis; Pz, zone of precipitation.

reduced oxygen conditions but not under the anaerobiotic environment (Table 1). Pz values for the proteinase tests ranged from 0.29–1.0 for the isolates incubated in reduced O_2. In this atmospheric condition, only 2.4% of the strains did not produce these enzymes, 3.1% were positive and the vast majority of the strains (94.5%) were strongly positive for the production of proteinase enzyme. *C. albicans* showed different activities of phospholipases with Pz ranging from 0.41–1.0 in the reduced oxygen atmosphere, with 8.6% of the strains having no phospholipase activity (negative), 58.3% having low activity (positive) and 33.1% high activity (strongly positive). These enzymatic activities were statistically different when comparing the atmospheric condition. Figs 1A and 1B illustrate the proteinase and phospholipase activities of some *C. albicans* strains.

Hemolysin activity. The results obtained under both atmosphere conditions were 1.6% γ-hemolytic, 64.40% α-hemolytic, and 34.30% β-hemolytic demonstrating that atmospheric oxygen did not influence the hemolysin activity of the *C. albicans* strains (Table 1). Fig. 2 illustrates β-hemolytic activity of some *C. albicans* strains.

Cellular Superficial Hydrophobicity Assay (*CSH*). Hydrophobicity was classified as low (0–30%), moderate

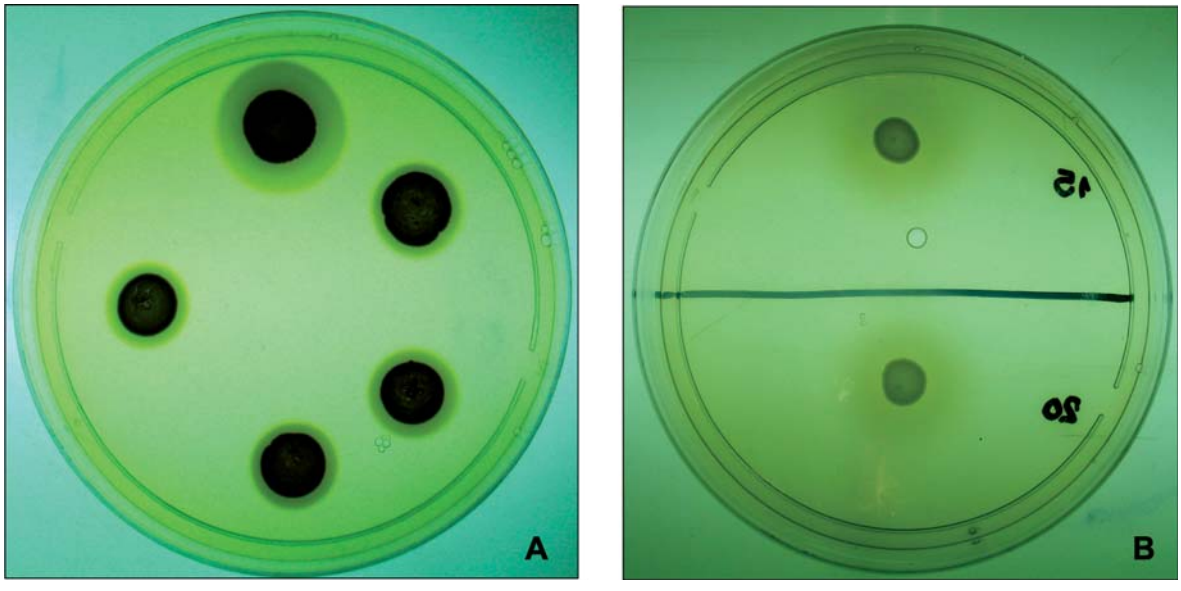

Fig. 1 (A) Proteinase activity of five *Candida albicans* strains and (B) Phospholipase activity of two *Candida albicans* strains. The enzymatic activity was determined by the formation of a halo around the yeast colony.

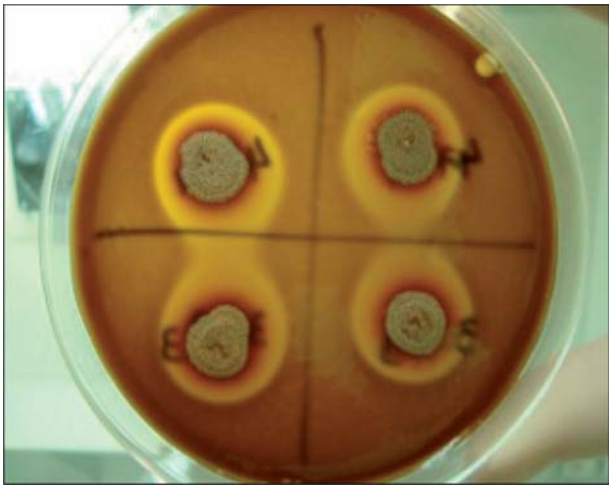

Fig. 2 Hemolytic activity of four *Candida albicans* strains. All of the isolates were β-hemolytic strains.

(31–59%) and high (above 60%). In the reduced oxygen atmosphere, the results obtained indicated that 34.40% of isolates showed low hydrophobicity, 43.70% moderate and 21.90% had high hydrophobicity. Under anaerobiosis, the results were 13.47% of strains having low hydrophobicity, 34.56% moderate and 51.97% high hydrophobicity (Table 1). Yeast cells grown under anaerobic conditions were more hydrophobic than when grown in the reduced oxygen condition, with a statistical difference between the atmospheric conditions ($P < 0.05$, Chi-square test).

Genotyping

A total of 128 strains isolated from patients with chronic periodontitis and diabetics were submitted to molecular typing by the method described above which showed that 51.6% were genotype B and 48.4% were genotype A. Genotype C was not found in this study.

Virulence factors × genotyping

Genotype A. In reduced oxygen atmospheric condition, 95.2% and 51.6% were strongly positive for proteinase and phospholipase activities, respectively. Under both atmospheric conditions, 51.6% of *C. albicans* strains were α-hemolytic. Smooth morphology was observed in 59.7% of the strains and 61.3% had high hydrophobicity under anaerobiosis and 37.1% under reduced oxygen (Table 2). A statistical difference was observed for all virulence factors, except hemolysis ($P < 0.05$, Chi-square test).

Genotype B. In the reduced oxygen atmospheric condition, 95.5% and 15.6% were strongly positive for proteinase and phospholipase activities, respectively, with 81.8% positive for phospholipase activity. Under both atmospheric conditions, 75.8% were α-hemolytic. Smooth colony morphologies were observed in 83.3% of the strains and 41.5% had high hydrophobicity under anaerobiosis and only 7.6% in the reduced oxygen atmosphere (Table 2). A statistical difference was observed for all virulence factors, except for hemolysis and CSH ($P < 0.05$, Chi-square).

When genotypes A and B were compared, there was a statistical difference between them for phospholipase activity under reduced oxygen and CSH under anaerobiosis.

Discussion

Candida is present as a commensal 'organism' in the oral cavities of up to 40% of healthy individuals. The number of these organisms in the saliva of carriers increases in pregnancy, with tobacco smoking, and when dentures are worn [29]. The prevalence of *Candida* species in the oral cavity of immunosuppressed individuals has been found to be higher when compared to the healthy population. Peterson *et al*. [30] observed that the occurrence of oral yeasts from saliva of hospitalized patients was 55%. In patients with advanced cancer, this number ranged between 47% and 87% of the population. In diabetic patients, the presence of *Candida* spp. in the oral mucosa reached up to 80% [31]. Few studies have demonstrated the incidence of *Candida* spp. in periodontal pockets. Barros *et al*. [32] found that *C. albicans* was present in 39.6% of periodontal sites in healthy patients. In this study, *C. albicans* strains were identified in 63.63% of periodontal sites of patients with diabetes mellitus and chronic periodontitis. The prevalence of *Candida* spp. in subgingival sites was 42.3% in HIV-positive children and 7.1% in control individuals [8].

Virulence factors of *Candida* species may also be involved in the pathogenesis of several oral diseases. However, few studies have investigated their role in host colonization and the development of infection [33]. Enzymatic secretion from oral isolates of *C. albicans*, especially phospholipases (PLs) and secreted aspartyl proteinases (SAPs), are determinant of not only commensal colonization, but also for the pathogenic potential of these yeasts [11,34]. Studies have reported that 30–100% of the oral isolates of *C. albicans* produce phospholipases, with variable degrees of enzymatic activity [31,35]. This enzyme was detected in 91.4% of *C. albicans* isolates in the current study when they were incubated in a reduced oxygen atmosphere. Enzymatic activity may depend on the site where the pathogen was recovered, as for example, phospholipase activity has been found in 100, 55, 50 and 30% of the *Candida* species isolated from chronic periodontitis, blood, wound infections and urine, respectively [25,32]. Phospholipase expression has been shown to be affected by growth conditions [36]. It has also been hypothesized that the presence of a high concentration of salivary glucose, combined with a reduced

Table 2 Frequency of virulence factors of *Candida albicans,* according to genotyping.

			Genotype A (%)		Genotype B (%)	
Virulence factor	Results	Scores	RO	ANA	RO	ANA
Colony morphology	Rough	1	35.5	100	9.1	100
	Intermediate	2	4.8	0	7.6	0
	Smooth	3	59.7	0	83.3	0
Proteinase activity	Negative (Pz = 1)	0	0	100	3.0	100
	Positive (Pz = 1–0.63)	1	4.8	0	1.5	0
	Strongly positive (Pz < 0.63)	2	95.2	0	95.5	0
Phospholipase activity	Negative (Pz = 1)	0	16.1	100	1.5	100
	Positive (Pz = 1–0.63)	1	32.3	0	83.1	0
	Strongly positive (Pz < 0.63)	2	51.6	0	15.4	0
Hemolysin activity	Gama-hemolysis	0	3.2	3.2	0	0
	Alpha-hemolysis	1	51.6	51.6	75.8	75.8
	Beta- hemolysis	2	45.2	45.2	24.2	24.2
Hydrophobicity	Low (0-30%)	1	22.6	11.7	45.5	16.9
	Moderate (31–59%)	2	40.3	25.0	47.0	41.5
	High (above 60%)	3	37.1	63.3	7.6	41.5

RO, reduced oxygen; ANA, anaerobiosis; Pz, zone of precipitation.

salivary secretion rate, enhances the growth of yeasts and their adherence to epithelial oral cells in type 2 DM.

Considering proteinase production, Koga-Ito *et al.* [33] showed that the activity of SAPs is significantly higher in denture wearers with signs of candidiasis than in denture wearers with a normal palatal mucosa. Another study has demonstrated that proteinase expression is not significantly higher in *Candida* isolates of patients with diabetes when compared to healthy patients, and that type 2 diabetes mellitus patients have higher proteinase levels than type 1 DM patients [17]. In the present investigation, proteinase expression was detected in 94.5% of the *C. albicans* isolates when incubated in a reduced oxygen atmosphere. These findings are in agreement with Tsang *et al*. [16], who found a high proteinase activity in type 2 diabetes mellitus patients. In this study, there was no activity of phospholipase or proteinase under anaerobic conditions. It is believed that these yeasts can grow under anaerobic conditions but under such conditions, fermentation is the dominant pathway for ATP production. The glycolysis pathway generates ATP, NADH and pyruvate molecules under both aerobic and anaerobic conditions. Pyruvate is not incorporated into the tricarboxylic acid (TCA) cycle under anaerobic conditions, but is metabolized to ethanol by alcohol dehydrogenase (ADH). This metabolism is controlled by oxidation of NADH to NAD, and this reaction is essential for maintaining the redox balance in the cytoplasm of fermenting cells. It is possible that *C. albicans* does not produce these enzymes under anaerobic conditions [37]. Rosa *et al*. [22] evaluated strains of *C. albicans* recovered from healthy patients with chronic periodontitis and observed an increase in the activity of SAPs under anaerobiosis when compared to aerobiosis. Unfortunately, the current results cannot be compared to those obtained in this previous study. Strains of *C. albicans* were isolated from healthy patients and in our investigation they were taken from diabetic patients. Although the atmospheric conditions seem to be similar, the current study incubated isolates in an anaerobic chamber as opposed to the use of anaerobic jars employed in the earlier study, indicating differences in methodology.

Investigations evaluating the activity of hemolysin in *C. albicans* are limited and as far as the authors can determine, there have been no studies of hemolysin activity of *C. albicans* isolates recovered from periodontal pockets of diabetic patients. Manns *et al*. [38] defined the conditions under which *C. albicans* can display hemolytic activity and found that hemolysis is not observed when glucose is not available in the culture medium. In this present study, hemolytic activity was detected in 98.5% of the *C. albicans* isolates when studied under both atmospheric conditions and that, 64.3% were alpha-hemolytic, 34.3% beta-hemolytic and 1.5% gamma-hemolytic. Luo *et al*. [39] evaluated 80 *Candida* isolates from clinical sources in different geographical locations and detected only alpha hemolysis in experiments with glucose free sheep blood agar. A high blood glucose concentration may contribute,

directly or indirectly, to increased hemolysin activity among *C. albicans* isolates in diabetic patients [16].

In this investigation, the cell surface hydrophobicity (CSH) was studied under reduced oxygen and anaerobiosis, with the results indicating that strains were more hydrophobic when grown under anaerobic conditions. The present results indicated that 51.97% of isolates were highly hydrophobic under anaerobic conditions and 21.90% under the reduced oxygen atmosphere. Hydrophobic interactions may be of importance in promoting tissue invasion by the mycelia phase of yeast cells. Germ tubes of *C. albicans* are able to adhere to fibronectin, fibrinogen, and complement via cell surface receptors [40]. CSH favors attachment of *C. albicans* to extracellular matrix components (ECM), which are intimately associated with host cell surfaces [41]. A recent investigation has suggested that an increase in CSH produces impairment of phagocytosis, increasing the resistance to blood clearance and, consequently, the virulence of *Candida* species [27]. Our study reports the first evaluation of the different genotypes of *C. albicans* from patients with diabetes mellitus and periodontal disease through the use of 25S rDNA PCR. The findings indicated that these patients are preferentially colonized with genotype B (51.6%), although published studies have shown a high incidence of genotype A in the healthy population [28,42]. However, there is some evidence to show an increase in the isolation of genotype B in immunocompromised patients, particularly in HIV patients [4,43]. The transposable intron region of the 25S rDNA gene was found to be associated with flucytosine susceptibility and genotype B is more susceptible to flucytosine [28]. Genotype B was prevalent but genotype A was the most virulent in our studies. We noted that 45.2% of the genotype A isolates were β-hemolytic and 61.3% had high hydrophobicity, under both atmospheric conditions. Only 24.2% of genotype B *C. albicans* strains were β-hemolytic under both conditions and 41.5% had high hydrophobicity under anaerobiosis but only 7.6% under reduced O_2. In another study, genotype A was associated with increased resistance to antifungals [28]. This PCR method offers high levels of reproducibility and discrimination, facilitating the delineation of *C. albicans* genotypes, while being easily performed in a laboratory [44].

In conclusion, oxygen concentration in the environment influences the virulence factors of *C. albicans* strains isolated from subgingival sites of diabetic and periodontal patients. Genotype A seems may be more virulent, as per the phenotypic tests evaluated in this current study.

Declaration of interest: The author report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1 Arendorf TM, Walker DM. The prevalence and intra-oral distribution of *Candida albicans* in man. *Arch Oral Biol* 1980; **25**: 1–10.
2 Belazi M, Velegraki A, Fleva A, *et al*. Candidal overgrowth in diabetic patients: potential predisposing factors. *Mycoses* 2005; **48**: 192–196.
3 Jabra-Rizk MA, Falkler WA Jr, Merz WG, *et al*. Coaggregation of *Candida dubliniensis* with *Fusobacterium nucleatum.J Clin Microbiol* 1999; **37**: 1464–1468.
4 Velegraki A, Nicolatou O, Theodoridou M, Mostrou G, Legakis NJ. Paediatric AIDS-related linear gingival erythema: a form of erythematous candidiasis? *J Oral Pathol Med* 1999; **28**: 178–182.
5 Baumgartner JC, Watts CM, Xia T. Occurrence of *Candida albicans* in infections of endodontic origin. *J Endod* 2000; **26**: 695-698.
6 Reynaud AH, Nygaard-Østby B, Bøygard GK *et al*. Yeasts in periodontal pockets. *J Clin Periodontol* 2001; **28**: 860–864.
7 Yuan K, Chang CJ, Hsu PC *et al*. Detection of putative periodontal pathogens in non-insulin-dependent diabetes mellitus and non-diabetes mellitus by polymerase chain reaction. *J Periodontal Res* 2001; **36**:18–24.
8 Portela MB, Souza IP, Costa EM *et al*. Differential recovery of *Candida* species from subgingival sites in human immunodeficiency virus-positive and healthy children from Rio de Janeiro, Brazil. *J Clin Microbiol* 2004; **42**: 5925–5927.
9 Song X, Eribe ER, Sun J, Hansen BF, Olsen I. Genetic relatedness of oral yeasts within and between patients with marginal periodontitis and subjects with oral health. *J Periodontal Res* 2005; **40**: 446–452.
10 Darwazeh AM, MacFarlane TW, McCuish A, Lamey PJ. Mixed salivary glucose levels and candidal carriage in patients with diabetes mellitus. *J Oral Pathol Med* 1991; **20**: 280–283.
11 Willis AM, Coulter WA, Hayes JR, Bell P, Lamey PJ. Factors affecting the adhesion of *Candida albicans* to epithelial cells of insulin-using diabetes mellitus patients. *J Med Microbiol* 2000; **49**: 291–293.
12 Willis AM, Coulter WA, Fulton CR *et al*. The influence of antifungal drugs on virulence properties of *Candida albicans* in patients with diabetes mellitus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2001; **91**: 317–321.
13 Sardi JC, Duque C, Mariano FS, *et al*. *Candida* spp. in periodontal disease: a brief review. *J Oral Sci* 2010; **52**: 177–185.
14 Hube B, Naglik J. *Candida albicans* proteinases: resolving the mystery of a gene family. *Microbiology* 2001; **147**: 1997–2005.
15 Ramanan N, Wang Y. A high-affinity iron permease essential for *Candida albicans* virulence. *Science* 2000; **288**: 1062–1064.
16 Tsang CS, Chu FC, Leung WK *et al*. Phospholipase, proteinase and haemolytic activities of *Candida albicans* isolated from oral cavities of patients with type 2 diabetes mellitus. *J Med Microbiol* 2007; **56**: 1393–1398.
17 Manfredi M, McCullough MJ, Al-Karaawi ZM, Vescovi P, Porter SR. *In vitro* evaluation of virulence attributes of *Candida* spp. isolated from patients affected by diabetes mellitus. *Oral Microbiol Immunol* 2006; **21**: 183–189.
18 Hazen KC, Glee PM. Cell surface hydrophobicity and medically important fungi. *Curr Top Med Mycol* 1995; **6**: 1–31.
19 Hazen KC, Wu JG, Masuoka J. Comparison of the hydrophobic properties of *Candida albicans* and *Candida dubliniensis*. *Infect Immun* 2001; **69**: 779–786.
20 Dumitru R, Hornby JM, Nickerson KW. Defined anaerobic growth medium for studying *Candida albicans* basic biology and resistance to eight antifungal drugs. *Antimicrob Agents Chemother* 2004; **48**: 2350–2354.
21 Jamieson DJ, Stephen DW, Terrière EC. Analysis of the adaptive oxidative stress response of *Candida albicans.FEMS Microbiol Lett* 1996; **138**: 83–88.

22 Rosa EA, Rached RN, Ignácio SA, *et al*. Phenotypic evaluation of the effect of anaerobiosis on some virulence attributes of *Candida albicans*. *J Med Microbiol* 2008; **57**: 1277–1281.
23 Pizzo G, Barchiesi F, Falconi Di Francesco L, *et al*. Genotyping and antifungal susceptibility of human subgingival *Candida albicans* isolates. *Arch Oral Biol* 2002; **47**: 189–196.
24 Nascimento E, Martinez R, Lopes AR, *et al*. Detection and selection of microsatellites in the genome of *Paracoccidioides brasiliensis* as molecular markers for clinical and epidemiological studies. *J Clin Microbiol* 2004; **42**: 5007–5014.
25 Sardi JC, Duque C, Camargo GA, Hofling JF, Gonçalves RB. Periodontal conditions and prevalence of putative periodontopathogens and *Candida* spp. in insulin-dependent type 2 diabetic and non-diabetic patients with chronic periodontitis. A pilot study. *Arch Oral Biol* 2011; **56**: 1098–1105.
26 Price MF, Wilkinson ID, Gentry LO. Plate method for detection of phospholipase activity in *Candida albicans*.*Sabouraudia* 1982; **20**: 7–14.
27 Rodrigues AG, Mårdh PA, Pina-Vaz C, Martinez-de-Oliveira J, Fonseca AF. Germ tube formation changes surface hydrophobicity of *Candida* cells. *Infect Dis Obstet Gynecol* 1999; **7**: 222–226.
28 McCullough MJ, Clemons KV, Stevens DA. Molecular and phenotypic characterization of genotypic *Candida albicans* subgroups and comparison with *Candida dubliniensis* and *Candida stellatoidea*. *J Clin Microbiol* 1999; **37**: 417–421.
29 Ellepola ANB, Samaranayake LP. Oral candidal infections and antimycotics *CritRev Oral Biol Med* 2000; **11**: 172–198.
30 Peterson DE, Minah GE, Overholser CD, *et al*. Microbiology of acute periodontal infection in myelosuppressed cancer patients. *J Clin Oncol*. 1987; **5**: 1461–1468.
31 Soysa NS, Samaranayake LP, Ellepola AN. Diabetes mellitus as a contributory factor in oral candidosis. *Diabet Med* 2006; **23**: 455–459.
32 Barros LM, Boriollo MF, Alves AC, *et al*. Genetic diversity and exoenzyme activities of *Candida albicans* and *Candida dubliniensis* isolated from the oral cavity of Brazilian periodontal patients. *Arch Oral Biol* 2008; **53**: 1172–1178.

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33 Koga-Ito CY, Lyon JP, Vidotto V, de Resende MA. Virulence factors and antifungal susceptibility of *Candida albicans* isolates from oral candidosis patients and control individuals. *Mycopathologia* 2006; **161**: 219–223.
34 Sugita T, Kurosaka S, Yajitate M, Sato H, Nishikawa A. Extracellular proteinase and phospholipase activity of three genotypic strains of a human pathogenic yeast, *Candida albicans*. *Microbiol Immunol* 2002; **46**: 881–883.
35 Hannula J, Dogan B, Slots J, Okte E, Asikainen S. Subgingival strains of *Candida albicans* in relation to geographical origin and occurrence of periodontal pathogenic bacteria. *Oral Microbiol Immunol* 2001; **16**: 113–118.
36 Samaranayake YH, Dassanayake RS, Cheung BP, *et al*. Differential phospholipase gene expression by *Candida albicans* in artificial media and cultured human oral epithelium. *APMIS* 2006; **114**: 857–866.
37 Ogasawara A, Odahara K, Toume M, *et al*. Change in the respiration system of *Candida albicans* in the lag and log growth phase. *Biol Pharm Bull* 2006; **29**: 448–450.
38 Manns JM, Mosser DM, Buckley HR. Production of a hemolytic factor by *Candida albicans*. *Infect Immun* 1994; **62**: 5154–5156.
39 Luo G, Samaranayake LP, Yau JY. *Candida* species exhibit differential in vitro hemolytic activities. *J Clin Microbiol* 2001; **39**: 2971–2974.
40 Calderone RA, Braun PC. Adherence and receptor relationships of *Candida albicans*. *Microbiol Rev* 1991; **55**: 1–20.
41 Silva TM, Glee PM, Hazen KC. Influence of cell surface hydrophobicity on attachment of *Candida albicans* to extracellular matrix proteins. *J Med Vet Mycol* 1995; **33**: 117–122.
42 Tamura M, Watanabe K, Mikami Y, Yazawa K, Nishimura K. Molecular characterization of new clinical isolates of *Candida albicans* and *C. dubliniensis* in Japan: analysis reveals a new genotype of *C. albicans* with group I intron. *J Clin Microbiol* 2001; **39**: 4309–4315.
43 Vidotto V, Yumi Koga-Ito C *et al*. Correlation between germ tube production, phospholipase activity and serotype distribution in *Candida albicans*. *Rev Iberoam Micol* 1999; **16**: 208–210.
44 Kumar M, Mishra NK, Shukla PK. Sensitive and rapid polymerase chain reaction based diagnosis of mycotic keratitis through single stranded conformation polymorphism. *Am J Ophthalmol* 2005; **140**: 851–857.