Phospholipase, proteinase and haemolytic activities of *Candida albicans* isolated from oral cavities of patients with type 2 diabetes mellitus

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The aim of this study was to biotype and characterize phospholipase, proteinase and haemolytic activities of oral *Candida albicans* isolates from 210 Chinese patients with type 2 diabetes mellitus (DM) and 210 age- and sex-matched healthy controls. Seventy-six and 50 *C. albicans* isolates were obtained from type 2 DM patients and controls, respectively, using the oral rinse technique. The isolates were characterized with a biotyping system based on enzyme profiles, carbohydrate assimilation patterns and boric acid resistance of the yeasts, and the isolates were further tested for *in vitro* phospholipase, proteinase and haemolytic activities. The major biotypes of *C. albicans* isolates from the type 2 DM and control groups were A1R (42.1 %) and J1R (36.0 %), respectively. Significantly higher proteinase and haemolytic activities were found in the isolates from the type 2 DM group ($P<0.05$). Proteinase activity was higher in isolates from patients with ≥ 10 years of DM history than those with <10 years ($P<0.05$). Haemolytic activity was significantly higher in isolates from female DM patients than in those from male counterparts ($P<0.05$). These data provide evidence of increased extracellular enzyme activity in *Candida* isolates taken from DM patients.

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INTRODUCTION

Oral carriage rates of *Candida albicans* have been reported to be increased in patients with diabetes mellitus (DM) (Belazi *et al.*, 2005). Extracellular hydrolytic enzymes seem to play an important role in candidal overgrowth (Schaller *et al.*, 2005), as these enzymes facilitate adherence and tissue penetration, and hence invasion of the host. Among the most important hydrolytic enzymes produced by *C. albicans* are phospholipases and secreted aspartyl proteinases (Saps). Furthermore, the ability of *C. albicans* to acquire elemental iron through haemolysin production is pivotal in its survival and ability to establish infections within humans (Weinberg, 1978).

Seven phospholipase genes (*PLA*, *PLB1*, *PLB2*, *PLC1*, *PLC2*, *PLC3* and *PLD1*) have been identified, but only four of them (*PLB1*, *PLB2*, *PLC1* and *PLD1*) have been well characterized (Samaranayake *et al.*, 2006). Their roles in pathogenesis have not been fully elucidated.

Saps are encoded by 10 *SAP* genes that seem to play different roles in *C. albicans* infection. *SAP1* to *SAP6* take

part in adherence, tissue damage and evasion of host immune responses. The roles of *SAP7* to *SAP10* remain elusive, but there is evidence that *Sap9* and *Sap10* are not secreted from the cell, and instead are regulatory proteinases that play a role in maintaining cell surface integrity (Naglik *et al.*, 2003).

Haemolysin is another putative virulence factor thought to contribute to candidal pathogenesis. In particular, the secretion of haemolysin, followed by iron acquisition, facilitates hyphal invasion in disseminated candidiasis (Odds, 1998).

Few studies have investigated the secretion of both phospholipases and Saps together in *C. albicans* isolated from DM patients. Furthermore, to our knowledge, the secretion of haemolysin from *C. albicans* isolated from type 2 DM patients has not been studied before. Therefore, the aims of the present study were (1) to study the biotypes of *C. albicans* isolated from type 2 DM patients and controls; (2) to determine phospholipase, Sap and haemolysin activities of *C. albicans* isolated from type 2 DM patients; (3) to investigate any correlations between the enzyme profiles of *C. albicans* isolates and patients' demographic data, smoking habits, denture wearing and DM status; and

Abbreviations: DM, diabetes mellitus; FPG, fasting plasma glucose; Sap, secreted aspartyl proteinase.

(4) to correlate the *C. albicans* biotypes with their enzyme profiles.

METHODS

Subjects. Two hundred and ten Chinese patients aged 41 years or older with type 2 DM were consecutively recruited from the outpatient clinic of the Integrated Diabetes Mellitus Research and Training Centre, Tung Wah Eastern Hospital, Hong Kong SAR, China. Two hundred and ten age- and sex- matched controls without DM, that is, with a fasting plasma glucose (FPG) concentration of <7.0 mM (Alberti & Zimmet, 1998), were randomly recruited from the general outpatient clinic of the same hospital during the same period. The controls did not have any systemic disease except essential hypertension.

Data collection. Blood samples were taken from all diabetic patients for FPG and glycosylated haemoglobin (HbA_{1c}) measurements on the date of study recruitment as part of the patients' regular medical screening or during the baseline DM examination for new cases. A self-administered questionnaire was used to collect demographic data and information on education level, smoking habits and presence of any medical complications. All participants gave written informed consent. Approval for the study was obtained from the Ethics Committee of the Faculty of Dentistry, The University of Hong Kong, Hong Kong SAR, China.

Oral rinse sampling. A modified protocol for concentrated rinse culture was used to evaluate the overall oral yeast carriage (Tsang & Samaranyake, 2000). The patient was given 10 ml 0.1 M PBS, pH 7.3, in a sterile universal container and instructed to rinse the mouth for 60 s. Denture-wearing patients did not remove their prostheses. After the oral rinse was expectorated into the container, the sample was transferred immediately to the laboratory, where it was centrifuged at 1700 g for 10 min. The supernatant was discarded and the pellet resuspended in 2 ml PBS on a vortex mixer for 30 s. A spiral plater (model DU; Spiral Systems) was used to dispense 50 µl of the suspension onto various media, as described below.

Culture. The concentrated oral rinse was spiral-plated with an Archimedean spiral onto a Sabouraud glucose agar (SGA) plate (Gibco) and incubated for 48 h at 37 °C. Well-separated yeast colonies were subcultured onto SGA plates to obtain pure yeast cultures that were then harvested, suspended in water in sterile vials and stored at -20 °C. The yeast isolates were identified by the germ tube test, growth at 45 °C, chlamydospore formation and API 20C AUX (bioMérieux) assimilation tests. Their phenotypes were further defined by culture on CHROMagar *Candida* plates. Their identities were reconfirmed with the API AB Plus (bioMérieux) assay to exclude *Candida dubliniensis*. The yeasts were then stored in vials with multiple glass beads (Microbank) at -70 °C, subcultured monthly on SGA and maintained at 4 °C during the experimental period, unless otherwise stated. The purity of the cultures was confirmed periodically by Gram-staining and the germ tube test.

***C. albicans* biotyping.** All yeast isolates were biotyped with two commercially available biotyping kit systems, API ZYM and API 20C AUX (bioMérieux) (Leung *et al.*, 2000), and a boric acid-resistance test (Williamson *et al.*, 1987; Tsang *et al.*, 1995). In brief, the API ZYM system evaluated the enzyme activity of the isolates by means of a battery of 19 enzyme substrates contained in miniaturized plastic cupules of a multi-cupule tray. Colour reactions in each cupule were measured after inoculation of a standard quantity of *C. albicans* suspension, incubation for 4 h at 37 °C and addition of reagents according to the manufacturer's instructions. The API 20C AUX

system measured the ability of *C. albicans* isolates to assimilate 19 different carbohydrates as sole sources of carbon. The results were obtained from a comparison of the extent of opacity in test and control cupules. The boric acid-resistance test assessed the sensitivity of the isolates to 1.8 mg boric acid ml⁻¹ incorporated into the agar medium.

Determination of phospholipase activity. *C. albicans* isolates were screened for extracellular phospholipase activity by measuring the size of the zone of precipitation after growth on egg yolk agar (Samaranyake *et al.*, 1984). The egg yolk medium consisted of 13.0 g SGA (Oxoid), 11.7 g NaCl, 0.11 g CaCl₂ and 10 % sterile egg yolk (Oxoid) (all in 184 ml distilled water). First, the components without the egg yolk were mixed and sterilized, then the egg yolk was centrifuged at 500 g for 10 min at room temperature and 20 ml of the supernatant was added to the sterilized medium. Standard inocula of the test and control *Candida* isolates [5 µl, with 10⁸ yeast cells (ml saline)⁻¹] were deposited onto the egg yolk agar medium and left to dry at room temperature. A further 5 µl of saline, but without yeast cells, was overlaid onto the plate and left to dry at room temperature. Each culture was then incubated at 37 °C for 48 h, after which the diameter of the precipitation zone around the colony (an indicator of phospholipase activity) was determined.

The plates were read with the aid of a computerized image analysis system (Quantimet 500 Qwin; Leica), which measured the diameter of the colonies relative to the precipitation zones on a magnified scale. Phospholipase activity (P_z value) was expressed as the ratio of the diameter of the colony to the diameter of the colony plus the precipitation zone (in mm) (Price *et al.*, 1982). The assay was conducted in duplicate on three separate occasions for each yeast isolate tested. Reference strains of *C. albicans* (ATCC 10231 and ATCC 24433) served as positive controls. A strain of *Candida glabrata* from our laboratory stock collection served as a negative control.

Determination of proteinase activity. Extracellular proteinase activity of *C. albicans* strains was analysed in terms of BSA degradation according to the technique described by Staib (1965). In brief, an 18 h yeast suspension of 1 × 10⁶ cells ml⁻¹ was prepared, and 10 µl suspension was inoculated onto a 1 % BSA plate. The plate was incubated for 5 days at 37 °C, flooded with 1.25 % naphthalene black solution for 15 min and washed with 90 % (v/v) methanol/water destaining solution, followed by 36 h of decolourization with several changes of destaining solution.

Results were examined and quantified by a computerized image analysis system (Quantimet 500 Qwin; Leica). The ratio of the diameter of the colony to that of the clear zone of proteolysis (in mm) was used as an index (Pr_z value) to represent the extent of proteinase activity by the different *C. albicans* isolates. The assay was conducted in duplicate on three separate occasions for each yeast isolate tested. Reference strains of *C. albicans* (ATCC 10231 and ATCC 10261) served as positive controls. *Candida parapsilosis* ATCC 22019 served as a negative control.

Determination of haemolysin activity. Haemolysin activity was evaluated with a blood plate assay (Manns *et al.*, 1994; Luo *et al.*, 2001). Media were prepared by adding 7 ml fresh sheep blood (Dixon) to 100 ml SGA (Merck) supplemented with glucose at a final concentration of 3 % (w/v). The final pH (mean ± SD) of the medium was 5.6 ± 0.2. A standard inoculum of both the test and the control *Candida* isolates [10 µl, with 10⁸ yeast cells (ml saline)⁻¹] was deposited onto the medium. A further 10 µl of saline but without yeast cells was overlaid onto the same plate. The plate was then incubated at 37 °C in 5 % CO₂ for 48 h.

After incubation, plates were examined and colonies quantified by a computerized image analysis system (Quantimet 500 Qwin; Leica).

The ratio of the diameter of the colony to that of the translucent zone of haemolysis (in mm) was used as the haemolytic index (H_z value) to represent the extent of haemolysin activity by different *C. albicans* isolates. The assay was conducted in duplicate on three separate occasions for each yeast isolate tested. A reference strain of *C. albicans* (ATCC 90028) served as a positive control. In addition, one strain each of *Streptococcus pyogenes* (Lancefield group A) and *Streptococcus sanguis*, which induce beta and alpha haemolysis, were used as positive controls. *Candida parapsilosis* ATCC 22019 served as a negative control.

Data analysis. Statistical analyses were performed using the Student's *t* test, chi square test, Fisher's exact test and correlation coefficient. A *P* value of 0.05 was considered significant.

RESULTS

The mean $\pm$ SD age of the diabetic group was 64 ± 10 years; 54 % ($n=113$) were female. These results were similar for the control group. The two groups were also similar in terms of education (e.g. 51 % of controls and 60 % of DM patients had received no or only primary education) and smoking habits (e.g. 71 % of controls and 72 % of DM patients were non-smokers). Controls and DM patients were significantly different, however, in terms of hypertension (43.8 vs 63.3 %), denture wearing (34.8 vs 47.1 %) and *C. albicans* isolation (23.8 vs 36.2 %) (all *P* values <0.05).

The mean age of DM onset was 56 years, and the duration of diabetes was 6.8 and 8.9 years in male and female diabetic patients, respectively. The mean HbA_{1c} was 7.8 ± 1.3 % for male diabetic patients and 8.0 ± 1.3 % for female patients. The mean FPG was 8.3 ± 2.4 mM in male patients and 8.6 ± 2.9 mM in female patients.

The oral carriage of *C. albicans* in the test group ($n=76$; 36.2 %) was significantly greater than that in the control group ($n=50$; 23.8 %) (chi square test, $P<0.05$). Other *Candida* species isolated included *Candida tropicalis* (five

isolates from DM and two from control groups), *C. glabrata* (five isolates from DM and three from control groups), *C. parapsilosis* (three isolates from DM and two from control groups), *Candida guilliermondii* (one isolate from the control group) and *Candida famata* (one isolate from the DM group). Fourteen diabetic patients harboured two *Candida* species simultaneously, while eight non-diabetic patients harboured two *Candida* species.

Biotype

The biotype profiles of the *C. albicans* are shown in Fig. 1. Altogether, 11 biotypes were detected (six from the test group and eight from the control group). Three biotypes were detected in both groups (A1S, J1R and J1S). The major biotype detected in the test group was A1R ($n=32$; 42.1 %), whereas J1R was the major biotype detected in the control group ($n=18$; 36 %). Significantly more *C. albicans* isolates of API ZYM biotype A than any other were detected in the test group (Fisher's exact test, $P<0.0001$), whereas significantly more isolates of API ZYM biotype J than any other were detected in the control group (Fisher's exact test, $P<0.0001$). No correlations could be found between biotype and age or sex in general, or between test-group biotypes and HbA_{1c} or FPG.

Of the 126 *C. albicans* isolates (test and control) tested, all were found to be positive for phospholipase, Sap and haemolysin activities.

Phospholipase

The P_z values of the *C. albicans* isolates ranged from 0.68 to 0.89 for the DM group and from 0.65 to 0.89 for the controls. No significant difference in phospholipase activity could be detected between the isolates from the test and control groups (Table 1). No significant association could

Fig. 1. Biotypes of *Candida albicans* isolates from diabetic (open bars) and non-diabetic (closed bars) subjects. The y axis shows the number of isolates in each biotype as a percentage of the total number of isolates.

Table 1. Phospholipase, proteinase and haemolysin activities of oral *C. albicans* isolates from diabetic and non-diabetic subjects

Results are expressed as mean $\pm$ SD. NS, Not significant.

Activity	Non-diabetic group, <i>n</i> =50	Diabetic group, <i>n</i> =76	Test	Statistic	<i>P</i> value
Phospholipase (P_z)	0.794 $\pm$ 0.060	0.776 $\pm$ 0.050	<i>t</i>	1.43	NS
Proteinase (Pr_z)	0.517 $\pm$ 0.156	0.425 $\pm$ 0.122	<i>t</i>	3.1	0.0027
Haemolysin (H_z)	0.764 $\pm$ 0.08	0.673 $\pm$ 0.06	<i>t</i>	4.71	<0.0001

be detected between P_z and biotype. Similarly, no significant association was observed between phospholipase activities of *C. albicans* isolates from type 2 DM patients and demographic data, smoking habit, denture-wearing status, HbA_{1c}, FPG or duration of diabetes.

Proteinase

The Pr_z values ranged from 0.23 to 0.79 for the DM group and from 0.30 to 0.87 for the controls. The mean proteinase activity of isolates from the test group (0.425 $\pm$ 0.122) was significantly higher than that of the control isolates (0.571 $\pm$ 0.156; Table 1). *C. albicans* isolated from type 2 DM patients with a diabetes history of ≥ 10 years had a lower mean Pr_z (0.428 $\pm$ 0.081), i.e. higher proteinase activity, than patients with a diabetes history of < 10 years (0.548 $\pm$ 0.168) (Student's *t* test, $P < 0.05$). There were no significant associations between Pr_z and biotype. No correlation could be found between Pr_z of isolates from type 2 DM patients and demographic data, smoking habit, denture-wearing status, HbA_{1c} or FPG.

Haemolysin

The H_z values ranged from 0.60 to 0.79 for the DM group and from 0.62 to 0.87 for controls. Higher haemolysin activities were detected in the test group isolates (0.673 $\pm$ 0.06) than in the control isolates (0.764 $\pm$ 0.08) (Table 1). *C. albicans* isolates from female type 2 DM patients had a significantly higher haemolysin activity (0.692 $\pm$ 0.050) than their male counterparts (0.749 $\pm$ 0.081) (Student's *t* test, $P < 0.05$). No correlation could be found between H_z of isolates from type 2 DM patients and demographic data, smoking habit, denture-wearing status, HbA_{1c}, FPG or duration of diabetes. No significant association could be detected between H_z and biotype.

Finally, no significant association was observed between phospholipase, proteinase and haemolysin activities of *C. albicans* isolates from DM or control groups.

DISCUSSION

In this study, *C. albicans* was isolated from 36.2 % of patients with DM. This finding agrees with those from previous studies in which *Candida* species were isolated from

between 18 and 80 % of DM patients (Soysa *et al.*, 2006). Some host factors, such as salivary flow rate, salivary pH, wearing of dentures, alcohol use and smoking habits, are associated with an increased oral carriage rate of *Candida* species (Kadir *et al.*, 2002). Virulence factors of *Candida* species may also be involved, although they have usually been studied in isolation and only a few researchers have investigated their role in host candidal infection/colonization (Kantarcioğlu & Yücel, 2002; Koga-Ito *et al.*, 2006).

Phospholipase activity was detected in 100 % of the *C. albicans* isolates in this study. Previous studies have reported phospholipase activity in 30 to 100 % of candidal isolates from various groups of patients and from various sites (Price *et al.*, 1982; Wu *et al.*, 1996). The proportion may depend on the site; for example, phospholipase activity has been found in 55, 50 and 30 % of the *Candida* species isolated from blood, wound infections and urine, respectively (Price *et al.*, 1982).

Phospholipase gene expression has been shown to be affected by growth conditions (Samaranayake *et al.*, 2006). It has also been hypothesized that the presence of a high concentration of salivary glucose combined with a reduced salivary secretion rate enhances the growth of yeasts and their adherence to epithelial oral cells of type 2 DM patients (Darwazeh *et al.*, 1991), perhaps by increasing phospholipase activity. In this study, there was no significant difference in phospholipase activity between the diabetic and non-diabetic groups, and no correlation between phospholipase activity and HbA_{1c} or FPG. However, we did not measure the salivary glucose concentration, which is known to be generally higher in diabetic patients (Darwazeh *et al.*, 1991). Whether salivary glucose concentration has any effect on phospholipase gene expression warrants further investigation.

Koga-Ito *et al.* (2006) showed that Sap activity 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 DM than in those of healthy patients, and that type 2 DM patients have higher proteinase levels than type 1 DM patients (Manfredi *et al.*, 2006).

The results of this study were similar to those of Koga-Ito's group: that is, there was no significant difference in oral *C.*

albicans phospholipase activities between DM patients and controls, but proteinase activities were significantly higher in isolates from the DM group. A number of constituents in the saliva may contribute to the higher levels of oral proteinase observed in type 2 DM patients (Manfredi *et al.*, 2006). Higher salivary levels of glucose, IgA, and other salivary enzymes such as matrix metalloproteinase (MMP-8), gelatinase (MMP-9) and lysozyme, may all influence salivary proteinase activity and concentration in a direct or indirect fashion (Rayfield *et al.*, 1982; Stevens *et al.*, 1990; Collin *et al.*, 2000). Our finding of increased *in vitro* proteinase activity in *C. albicans* isolates from DM patients, particularly those with a DM history of ≥ 10 years, suggests that proteinase secretion by *C. albicans* is another plausible explanation of the increased oral proteinase activities observed in DM patients.

Studies on the activity of haemolysin in *C. albicans* are limited and, to the best of our knowledge, there are no studies on the activity of haemolysin from oral *C. albicans* isolates of type 2 DM patients. We noted that haemolysin activity was significantly higher in oral *C. albicans* isolated from DM patients than in those isolated from controls.

Luo *et al.* (2004) studied *HLP* gene expression in *C. albicans*, but failed to obtain specific RT-PCR products. They surmise that there is either negative *HLP* gene expression in *C. albicans* or a relatively large amount of sequence variation in *HLP* genes between *C. glabrata* and *C. albicans*. Therefore, factors affecting haemolysin gene expression in *C. albicans* remain elusive.

Manns *et al.* (1994) defined the conditions under which *C. albicans* can display haemolytic activity, but found out that haemolysis is non-existent when no glucose is available in the culture medium. On the other hand, Luo *et al.* (2001) have tested 80 *Candida* isolates from clinical sources in different geographical locales and detected only alpha, and not any beta, haemolysis in experiments with glucose-free sheep blood agar. An increased blood glucose concentration may contribute, directly or indirectly, to increased haemolysin activity among *C. albicans* isolates in DM patients. In addition, an increased salivary glucose concentration in DM patients may also influence haemolysin production in *C. albicans*. Interestingly, *C. albicans* isolates from female DM patients showed significantly higher haemolytic activity than those from male counterparts, and female patients had higher FPG values than males. However, we were unable to detect any correlation between *C. albicans* H₂ and FPG. Further investigations are needed to clarify this issue.

A1R and J1R were the most common biotypes isolated from the test and control groups, respectively, in this study. Tsang *et al.* (1995) previously showed that A1R is the most common biotype isolated from HIV-infected patients in different geographical locales. Xu & Samaranayake (1995) studied the biotypes in health and disease in China, and found that A1R is only isolated in the diseased group, but that otherwise there are no significant differences. They

speculate that A1R is more virulent than other biotypes. Nevertheless, Samaranayake *et al.* (2003) found that J1R is the commonest biotype isolated from HIV-infected patients with or without oral candidiasis. This variation in biotypes regardless of their pathological source concurs with the view that *C. albicans* is an opportunist pathogen that initiates infection only when host defences are compromised (Samaranayake, 1990).

In this study, although significantly more biotype A *C. albicans* isolates were isolated from the DM group than from controls, no correlation between biotype and secretion of extracellular enzymes was noted. This finding implies that no direct relationship exists between biotype and expression of extracellular phospholipases, Saps and haemolysins. The reasons for the increased expression of Sap and haemolysin in oral *C. albicans* strains isolated from DM patients are as yet unknown. Possible factors, such as low pH and reduced salivary flow rate in DM patients, may further enhance strain selection in these patients.

Very few workers have investigated the activities of a number of extracellular enzymes in the same *Candida* strain. Kantarcioğlu & Yücel (2002) found that 56 of 60 strains of *C. albicans* and two of four strains of *Candida kefyr* produce both phospholipase and protease, whereas the remaining strains do not produce either enzyme. Shimizu *et al.* (1996) reported that only 73 % of *C. albicans* strains studied could produce hyaluronidase, chondroitin sulphatase, proteinase and phospholipase simultaneously; these strains were also highly virulent in mice. We noted that all *C. albicans* isolates from both DM and control groups expressed all three extracellular enzymes tested, and that Sap and haemolysin activities were higher in the DM group than in controls. This result suggests that the degree of extracellular enzyme activity may be important for successful oral colonization of *C. albicans*, at least in DM patients.

In conclusion, we report here for the first time that oral *C. albicans* isolates from type 2 DM patients possess significantly higher haemolysin activity than isolates from healthy, non-DM controls. Proteinase activity of *C. albicans* isolates was also significantly elevated in isolates of the DM group. Our results suggest that there are both qualitative and quantitative variations in the expression of the array of extracellular enzymes secreted by *C. albicans*, and that they appear to be modulated by a variety of host factors pertaining to the ecosystem within which the yeast resides. Further studies on the simultaneous expression of candidal extracellular enzymes are urgently needed to understand the natural history and host-pathogen relationships associated with mucosal candidal infections.

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