Oral Microbiology Immunology 2006: 21: 183–189
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

In vitro evaluation of virulence attributes of *Candida* spp. isolated from patients affected by diabetes mellitus

M. Manfredi[1,2], M. J. McCullough[1,3], Z. M. Al-Karaawi[1], P. Vescovi[2], S. R. Porter[1]

[1]Oral Medicine, Division of Infection and Immunity, Eastman Dental Institute, UCL, University of London, London, UK, [2]Sezione di Odontostomatologia, Dipartimento di Scienze Otorino-Odonto-Oftalmologiche e Cervico Facciali, Università di Parma, Italy, [3]School of Dental Science, University of Melbourne, Victoria, Australia

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. © Blackwell Munksgaard, 2006.

Background: Diabetes mellitus is a common disease found worldwide and it has been previously suggested that oral candidal infections may be more frequent or severe in patients with this disease. Systemic and local factors may influence the balance between the host and yeasts, and favour the transformation of *Candida* isolates from commensal to pathogenic microorganisms. *Candida* species have developed specific virulence mechanisms that confer the ability to colonise host surfaces, to invade deeper host tissue, or to evade host defences. Few studies have investigated the expression of the virulence attributes of oral *Candida* isolates in patients with diabetes mellitus.
Material and methods: The *in vitro* extracellular proteinase production and the *in vitro* ability to adhere to fibronectin of 229 *Candida* isolates of two geographic different groups of patients with diabetes mellitus and of healthy subjects were assessed.
Results: *Candida* isolates of patients with diabetes mellitus expressed a higher ability to adhere than those of healthy subjects. Higher levels of adhesion were also recorded in patients with a lower oral *Candida* colonisation. No differences were observed in the *in vitro* expression of extracellular proteinase of *Candida* isolates of patients with diabetes mellitus and those of non-diabetic subjects. Isolates of patients with type 2 diabetes mellitus expressed greater levels of proteinase than isolates of type 1 diabetes mellitus.
Conclusions: Diabetes mellitus could be considered as an additional variable that may influence not only oral *Candida* carriage but also the ability of isolates to enhance the expression of virulence attributes.

Key words: *Candida* adhesion; *Candida* extracellular proteinases; *Candida* spp.; oral *Candida* isolates; virulence attributes

M. Manfredi, Sezione di Odontostomatologia, Dipartimento di Scienze Otorino-Odonto-Oftalmologiche e Cervico Facciali, Università di Parma, Parma, Italy
Accepted for publication November 5, 2005

Candida species, like all pathogenic microorganisms, have developed specific virulence mechanisms that confer the ability to colonise host surfaces, to invade deeper host tissue, or to evade host defences (25). Adherence to host tissue is the first step in the pathogenic process: once the first contact between host tissues and *Candida* has occurred, enzymes facilitate adherence by damaging or degrading cell membranes and extracellular proteins (8). The mechanisms of *Candida* species adherence to many cell types or surfaces are complex and still not elucidated. Adherence is achieved by a combination of specific (ligand–receptors interactions) and nonspecific mechanisms (electrostatic forces, aggregation, and cell surface hydrophobicity) (8). Specific adherence is mediated by a number of target proteins located as in the epithelial cell surface or in the subepithelial extracellular matrix. Of these, fibronectins are high molecular weight adhesive glycoproteins involved in cell adhesion and cell migration and located in the extracellular matrix *interstitium* (5, 8). Other microorganisms apart from *Candida*, such as *Staphylococcus aureus* and *Streptococcus pyogenes*, have also been shown to adhere to extracellular matrix protein fibronectins *in vitro* (48). Furthermore, molecules (transmembrane integrins) present on the surface of *Candi-*

da albicans are thought to mediate adherence to extracellular matrix molecules (20). These 'adhesins' function by recognising ligands that contain amino-acid sequences such as the Arg-Gly-Asp (RGD) sequence (53). *C. albicans* binds RGD-containing proteins, such as fibronectins, laminin and collagen types I and IV, through integrin-related structures (26).

The complex pathogenesis of *Candida* species is aided by the production of a range of extracellular enzymes that facilitate adherence and/or tissue penetration. The three most significant hydrolytic enzymes produced by *Candida* species, and most notably by *C. albicans*, are secreted aspartic proteinase (Sap), phospholipases and lipases (25). Many *Candida* species, including *C. albicans*, *Candida dubliniensis* (18), *Candida tropicalis* (33, 52, 63), and *Candida parapsilosis* (12, 33), are known to possess SAP genes and produce active extracellular proteinases *in vitro*. However, less pathogenic or non-pathogenic *Candida* species do not produce significant levels of proteinases, which has led to the conclusion that fungal pathogenicity directly correlates with the extent of Sap production. Extracellular matrix and host surface proteins such as laminin, fibronectin, and mucin are efficiently degraded by Saps, in particular by Sap2, the most effected Sap *in vitro*, aiding yeast cell adhesion to buccal epithelial cells. Sap2 has also been shown to hydrolyse secretory IgA, normally resistant to most bacterial proteinases (34). Sap2 activity is optimal at low pH (2.5–5.5) (34), but it is stable at neutral pH and is active under these conditions (4, 55). Sap4 and Sap6 are known to be significantly active at physiological pH (25). This stability of Saps at different pH ranges (2.0–7.0) may assist *Candida* species, and in particular *C. albicans*, to colonise and/or infect hosts in a neutral pH environment, such as the oral cavity.

Different classes of phospholipases are known and classified according to their mode of action (25). In *C. albicans*, phospholipases A, B, C and lysophospholipases may damage host-cell membranes and facilitate tissue invasion. Recently, non-*albicans* species have also been shown to secrete phospholipases, at lower levels (17). Furthermore, a correlation between phospholipase activity *in vitro* and virulence has been demonstrated (17, 25).

Few studies have investigated the expression of these two virulence attributes of oral *Candida* isolates in patients with diabetes mellitus. Diabetes mellitus is a common disease found worldwide and it has been previously suggested that oral candidal infections may be more frequent or severe in patients with this disease (2, 13, 57). However, it has been recently reported that only a small number of patients affected by diabetes mellitus develop oral candidosis (28). Systemic (e.g. the degree of glycaemic control) or local factors (e.g. presence of dentures) may influence the balance between the host and yeasts, and favour the transformation of *Candida* isolates from commensal to pathogenic microorganisms. It would be interesting to investigate whether there are virulent attributes, such as adhesion or proteinase expression, whose activity could be enhanced in isolates from diabetic patients who carry *Candida* species in their oral cavity.

The aim of this study was to evaluate *in vitro* extracellular proteinase production and the ability of *Candida* isolates to adhere *in vitro* to fibronectin. A comparison of these traits was made for isolates from two geographically different groups of patients affected by diabetes mellitus and from a healthy control group of non-diabetic subjects.

Material and methods

Patients affected by diabetes mellitus and healthy subjects

A total of 142 patients attending an outpatient Diabetology Clinic of The Middlesex Hospital, University College London Hospitals (UCLH), London, and 107 patients attending an outpatient Diabetology Clinic at the Parma Hospital, Parma, Italy, were enrolled during their routine diabetic review appointment. The medical history of each patient was recorded at the time of examination. This included tobacco smoking habits, type of diabetes mellitus (type 1 or type 2) and duration of diabetes (time since diagnosis < 10 years or > 10 years). The presence of most common long-term complications of diabetes (retinopathy, nephropathy and peripheral neuropathy) was similarly recorded. Glycaemic control, assessed by HbA_{1c} (glycosylated haemoglobin), was used as a measure of recent diabetic control. Patients were classified into three mutually exclusive groups depending upon the level of haemoglobin glycosylation at the time of oral examination (group 1: $< 7.5\%$; group 2: $> 7.5\%$, $< 8.5\%$; group 3: $> 8.5\%$).

In all, 130 healthy, non-diabetic patients were enrolled in London, UK. Patients were attending a General Dental Clinic ($n = 41$), a Special Needs Clinic ($n = 42$), a Prosthodontic Clinic ($n = 31$) or an Oral Medicine Clinic ($n = 16$). The medical history of each patient was recorded including current medication and tobacco smoking habits.

To evaluate presence or absence of mucosal abnormalities or oral conditions that could be associated with diabetes mellitus (e.g. oral candidal infection, xerostomia, sialosis, oral lichen planus, etc.) or that could influence their oral candidal colonisation, all patients received a detailed oral examination that included the presence or absence of dentures (either partial or full) and the presence of any mucosal abnormalities. Any other dental/prosthetic as well as periodontal evaluation was not specifically performed. The oral rinse technique (28, 56), was used to assess the growth and the degree of yeast colonisation for each patient. In particular, mouth swills using 10 ml of sterile distilled water collected from each patient in a 50 ml sterile tube. Each mouth rinse was vortexed for 30 s until homogenised and 100 µl was spread evenly on Sabouraud's dextrose agar (Sigma-Aldrich, Dorset, UK) plates containing chloramphenicol (1 mg/l, Sigma-Aldrich). Furthermore, two 10-fold dilutions of the vortexed mouthwash, diluted in sterile distilled water, were plated onto Sabouraud's dextrose agar/chloramphenicol agar plates for enumeration of the degree of colonisation. Each plate was incubated at 37°C for 48 h. Growth of *Candida* spp. was assessed by enumeration of colonies and expressed as colony forming units (cfu) per ml of mouth rinse. Non-identical, single colonies were isolated and cultured (re-incubated at 37°C for 48 h) to confirm their identity.

Candida isolates

A total of 229 oral yeasts (177 *C. albicans* and 52 non-*albicans* spp.) from adult diabetic patients (both type 1 and type 2) from Parma (DPR group, 71 strains), London, (DL group, 83 strains) and from non-diabetic subjects (ND group, 75 strains) were isolated. These isolates were then identified by conventional and molecular methods (1, 28). Oral yeasts isolated from each group of patients were evaluated in this study using adhesion and proteinase assays to assess phenotypic attributes postulated to be involved in *Candida* virulence.

Adhesion assay

The ability of individual isolates to adhere was assessed in an *in vitro* assay. A single

colony from a 48-h, 37°C Sabouraud's dextrose agar culture was inoculated into 5 ml of Yeast, Peptone and Dextrose (YPD) broth. This broth consisted of 20 g of dextrose powder (Sigma-Aldrich), 20 g of peptone powder (Sigma-Aldrich) and 10 g of yeast (Sigma-Aldrich) in 1 litre of sterile water. This inoculum was incubated at 37°C for 18 h, after which 45 ml of YPD was added and incubated at 37°C for a further 6 h. The cells were then centrifuged at 3600 *g* for 3 min and washed three times in Tris-EDTA (tris ethylene-diamine-tetra-acetate) buffer pH 8 (1×: 10 mM Tris HCl pH 8, 1 mM EDTA pH 8; Sigma-Aldrich) to remove traces of YPD. The cell pellet was then re-suspended into 5 ml of TE buffer pH 8. The number of yeast present was counted with a haemocytometer and adjusted to 10^8 cells/ml.

In vitro adhesion was assessed using paramagnetic beads (Dynabeads® M-450 Tosylactivated, Dynal Biotech UK, Wirral, UK) coated with fibronectin (0.1%, Sigma-Aldrich). Non-specific sites were blocked with bovine serum albumin (Sigma-Aldrich) as outlined by the manufacturer (Dynal Biotech UK). To coat the paramagnetic beads (Dynal Biotech UK) with fibronectin they were initially vortexed in a 15 ml tube, which was then placed in the associated magnet for 2 min. The supernatant was discarded, the tube removed from the magnet and 1 ml Buffer A was added (Buffer A: 0.019 M NaH_2PO_4 and 0.081 M Na_2HPO_4, at pH 7.4). The washing procedure was repeated twice. Fibronectin 50 μg (Sigma-Aldrich) was added to the suspension and incubated at 37°C for 10 min on slow tilt. Buffer B 10 μl was added (80 ml of Buffer B consisted of 0.88 g of NaCl, 0.1 g of bovine serum albumin and a 10 times dilution of Buffer A in sterile distilled water) and the suspension was incubated at slow tilting for a further 24 h at 37°C. The mixture was put in the magnet for 3 min and the supernatant was removed carefully. Cold buffer B 1 ml was added and the suspension was put in the fridge for 5 min. This procedure was repeated twice and 1 ml of Buffer C (100 ml containing 2.42 g of Tris, Sigma-Aldrich, and 0.1 g of bovine serum albumin, pH 8.5) was added. The suspension was incubated at 37°C for 4–6 h. The mixture was put in the magnet for 3 min and supernatant removed carefully. Cold buffer B 1 ml was added and the suspension was put in the fridge for 5 min. Dynabeads coated primarily with fibronectin and secondarily with bovine serum albumin were then suspended in Buffer B and the concentration adjusted to 10^8 beads/ml (stock solution) and finally stored at 4°C until use. A final 100 times dilution (990 μl of Buffer B with 10 μl of beads stock solution) was prepared and used in the assay (10^6 beads/ml).

The Dynabeads coated with fibronectin were incubated with the yeast cells in a 200 ml final volume in TE buffer, pH 8 (Sigma-Aldrich). This was incubated in a ration of 10^6 beads/ml Dynabeads to 10^8 cells/ml yeast cells, shaking for 30 min at room temperature. The cells bound to the Dynabeads were collected using the supplied magnetic separator (Dynal MPC, Dynal Biotech UK) for 3 min. Unbound yeast cells were removed from the solution by washing three times with TE buffer. The adherent yeast cells were finally disassociated from the Dynabeads by re-suspending them in 0.1 N NaOH (150 μl). Free cells and Dynabeads were counted using a haemocytometer and results were expressed as number of *Candida* cells bound per fibronectin-coated Dynabead.

Extracellular proteinase production

The ability of the individual isolates to produce extracellular proteinases was assessed in an *in vitro* assay as previously described (27). A culture medium containing 1000 ml of sterile water, 2 g of bovine serum albumin (Sigma-Aldrich), 20 g of dextrose (Sigma-Aldrich), 1 g of KH_2PO_4 (Sigma-Aldrich), 0.5 g of $MgSO_4$ (Sigma-Aldrich) and a synthetic vitamin solution (20 μg of biotin, 200 μg nicotinic acid, 200 μg of riboflavin, 400 μg of thiamine and 400 μg of pyridoxal hydrochloride, Sigma-Aldrich) was prepared. A single colony from an 18-h, 37°C Sabouraud's dextrose agar culture of each isolate was inoculated into 1 ml of the above described medium and left shaking for 5 days at room temperature. The number of yeasts per ml was enumerated using a haemocytometer and then removed by centrifugation at 5000 *g* for 30 min. A 0.5-ml aliquot of the supernatant was incubated in 2 ml of 10 g/l bovine serum albumin solution (Sigma-Aldritch) in 0.05 M citric buffer (as protein substrate) (Sigma-Aldrich) pH 3.2, at 37°C for 30 min. The reaction was stopped by addition of 5 ml trichloracetic acid solution on ice (50 g/l, Sigma-Aldrich) for 15 min. In negative control samples, the incubation with bovine serum albumin and citric buffer was omitted. The absorbance of each sample at 280 nm was determined against a citrate-buffer blank and representative fractions containing ultraviolet-absorbing material were assayed for proteinase activity. Results were expressed as a ratio between spectrophotometer reading and numbers of cells/ml.

Statistical analysis

The results of the adhesion and proteinase assays in the present study did not pass the test of normality (Kolmogorov–Smirnov test for normality). Furthermore, the shape of the distribution of the data for both adhesion and proteinase of the groups studied was very different and anomalous values (outliers) were present. For these reasons, both parametric and some nonparametric tests (such as Mann–Whitney test) were not applicable. Thus, all the isolates were divided above and below the median for each of these respective tests (median for adhesion: 116.38; median for proteinase: 0.153442), and isolates whose values were either above or below these values were classified as having either high or low adhesiveness, or high and low extracellular proteinase activity, respectively.

The differences among or between groups were evaluated using Fisher's Exact Test or the Chi-squared Test; values were deemed significant when the probability (P) was less than or equal to 0.05.

Results

Adhesion and proteinase activity of oral *Candida* isolates of patients with and without diabetes mellitus

Analysis of adherence and proteinase production of the 154 *Candida* isolates from diabetic patients demonstrated that there were no significant associations with age, gender, smoking habits or denture status of the patients. However, more *Candida* isolates from diabetic patients with lower levels of yeast colonisation (< 100 cfu/ml) expressed higher levels of adhesion ($P = 0.0001$) when compared with isolates from diabetic patients with a higher oral candidal load (> 100 cfu/m).

No statistical differences were found between *Candida* spp. or genotypic distribution of *C. albicans* and adhesion or proteinase expression ($P > 0.05$).

Furthermore, although the type of diabetes, duration of disease, long-term diabetic complications and haemoglobin glycosylation levels did not influence the adhesion ($P > 0.05$) of the *Candida* spp. isolated from the diabetic patients, significantly higher levels of proteinase ($P = 0.02$) were observed in type 2 diabetes mellitus patients when compared with type 1 diabetes mellitus patients.

In vitro adherence ability and proteinase production by the 75 *Candida* isolates of non-diabetic subjects were not significantly associated with patient age, gender, smoking habits or denture status, oral candidal load (cfu/ml), species of *Candida* or genotypic distribution of *C. albicans* ($P > 0.05$).

The comparison of adhesion levels of *Candida* isolates of the diabetic patient population with those of the oral yeasts of the non-diabetic subjects showed that there were significantly high levels of adhesion in diabetic patient groups ($P < 0.0001$) (Table 1). More specifically, for all the variables evaluated the *in vitro* ability to adhere was found to be significantly higher for *Candida* isolates from patients with diabetes mellitus than for isolates from non-diabetic controls.

No significant differences in the proteinase production were observed between patients with diabetes mellitus and non-diabetic subjects ($P = 0.48$). However, higher levels of proteinase were found in isolates from non-diabetic control subjects with a lower degree of colonisation (< 100 cfu/ml) than in isolates from diabetic patients ($P = 0.04$) (Table 1).

Table 1. Comparison of adhesion and proteinase activity of *Candida* isolates of patients with diabetes mellitus and *Candida* isolates of non-diabetic subjects

Patients	Adhesion above median	Adhesion below median	*P*-value	Proteinase above median	Proteinase below median	*P*-value
Male						
D	45	34	0.003	41	38	0.42
ND	9	28		16	21	
Female						
D	51	24	< 0.0001	33	42	0.07
ND	10	28		24	14	
< 60 years*						
D	41	25	0.0001	33	33	0.44
ND	11	34		26	19	
> 60 years*						
D	55	33	0.02	41	47	1.00
ND	6	13		9	10	
Tobacco users						
D	20	10	0.001	15	15	0.55
ND	3	15		11	7	
Non-tobacco users						
D	76	48	< 0.0001	59	65	0.74
ND	16	41		29	28	
< 100 cfu/ml						
D	67	28	< 0.0001	40	55	0.03
ND	6	18		16	8	
> 100 cfu/ml						
D	29	30	0.01	34	25	0.33
ND	13	38		24	27	
Dentate						
D	46	30	0.001	39	37	0.70
ND	14	32		26	20	
Dentures						
D	50	28	< 0.0001	35	43	0.82
ND	5	24		14	15	
Candida albicans						
D	73	48	< 0.0001	60	61	0.41
ND	14	42		32	24	
Non-albicans						
D	23	10	0.03	14	19	1.00
ND	5	14		8	11	
Candida albicans A						
D	58	41	< 0.0001	52	47	0.37
ND	10	36		28	18	
Candida albicans B						
D	12	5	0.08	7	10	1.00
ND	2	6		3	5	
Candida albicans C						
D	3	2	1.00	1	4	1.00
ND	1	1		1	1	

Statistical analysis was performed by subclassifying the values above and below the median of each assay (adhesion: 116.38 and proteinase: 0.153442), then Fisher's exact or Chi-squared tests were used to analyse data obtained.
D, diabetic patients; ND, non-diabetic control subjects.
*Twelve non-diabetic patients did not give their date of birth.

Adherence and proteinase activity of all *Candida* isolates

Analysis of all 229 *Candida* isolates from patients with diabetes mellitus and healthy subjects indicated that the adherence of isolates from individuals with levels of oral yeast colonisation of fewer than < 100 cfu/ml were significantly higher ($P = 0.0006$) than other individuals. No other differences ($P > 0.05$) were observed in the adherence or proteinase expression of isolates from any of the 229 *Candida* carriers evaluated in this study.

Influence of geographical locale of patients with diabetes mellitus

There was a tendency ($P = 0.06$) for oral isolates from patients with diabetes mellitus from Italy to have greater adherence in comparison with patients with diabetes mellitus from UK. A tendency ($P = 0.053$) for *Candida* isolates from UK patients with diabetes mellitus to produce higher levels of extracellular proteinase than isolates of patients from Italy was also observed. Adherence of *Candida* isolates of patients with diabetes mellitus from Italy wearing dentures was significantly greater ($P = 0.03$) than those of isolates of patients with diabetes mellitus from UK.

Higher levels of proteinase activity were found in *C. albicans* isolates from UK patients with diabetes mellitus ($P = 0.04$), particularly from *C. albicans* genotype A ($P = 0.04$), compared to the oral isolates of patients with diabetes mellitus from Italy.

Analyses of proteinase activity of isolates from tobacco users showed that *Candida* isolates from UK diabetic patients expressed higher levels ($P = 0.02$) than those from patients with diabetes mellitus from Italy.

Finally, higher levels of proteinase activity ($P = 0.02$) were observed in the oral isolates from UK diabetic patients with good metabolic control ($HbA1_c < 7.5\%$) when compared with isolates from Italian diabetic patients with the same level of glycaemic control.

Discussion

This study has investigated the expression of two important virulence attributes of *Candida* isolates of patients with

diabetes mellitus and healthy control subjects.

Few studies have investigated these two pathogenic factors of oral isolates of patients with diabetes mellitus and, in general, these have not examined individuals with a spectrum of diabetes mellitus. In addition, authors have not always examined more than one virulence attribute at time (10, 58).

In the present study, the comparison of adherence values obtained from the 154 isolates of patients with diabetes mellitus and the isolates from non-diabetic subjects detected significant differences in the expression of the *in vitro* adhesion between these two populations. In particular, the isolates from diabetic patients had a higher *in vitro* ability to adhere to fibronectin ($P < 0.0001$) than isolates from non-diabetic control subjects.

The assay used in this study to evaluate the *in vitro* ability to adhere of the *Candida* isolates was based on the binding of yeasts to fibronectin. This is one of the extracellular matrix glycoproteins and it has been recently recognised, thanks to the study of mutans deleted in the encoding gene, such as a *Candida* adhesin-receptor (Ala1p) (14–16). The main purpose of this study was to investigate variability in the ability of *Candida* isolates to adhere in general, rather than to any specific protein. Fibronectin was therefore chosen as a marker for adherence as the role of interactions between fibronectin and *Candida* spp. has already been established (9, 19, 36, 38, 40, 46, 47, 60–62), in the adhesion process. A number of different proteins (e.g. keratin, involucrin, salivary mucins) that may be involved in surface interaction could also have been used, but this type of assay would have been more suitable for evaluating the molecular adherence mechanism of *Candida* to epithelial surfaces, rather than variability of the yeast in adherence.

The enhanced adhesion of isolates of patients with diabetes mellitus may reflect the presence of a higher concentration of sucrose/glucose in the saliva of patients affected by diabetes compared to healthy non-diabetic subjects. It has been observed that yeast growth and adhesion may be enhanced by high blood and saliva glucose levels (35, 42), which can serve as nutrients for *Candida* cells. We therefore conclude that strains isolated from, for example, diabetes mellitus patients with higher glucose salivary levels may show a higher ability to adhere *in vitro*.

Although this study did not show that diabetic patients with lower levels of glycaemic control had higher levels of oral *Candida* carriage or a higher adhesion expression, the strains isolated from the oral cavity of patients suffering from diabetes mellitus may have increased their ability to adhere *in vitro*. It is likely that more than one factor promotes *Candida* carriage and enhances the isolates' adhesion activity in diabetic patients.

The analysis of proteinase production for *Candida* isolates from diabetic patients and isolates from non-diabetic subjects did not reveal any significant differences between the two groups ($P = 0.48$). However, a significant association was observed within the diabetic patient group. Interestingly, it has been shown that oral yeast isolates from patients with type 2 diabetes mellitus produced a significantly larger number of extracellular proteinases ($P = 0.02$) compared to patients with type 1 diabetes mellitus.

The higher levels of extracellular proteinase production by oral yeast isolated from type 2 diabetes mellitus patients may well reflect a variation in certain constituents in the saliva, such as higher salivary IgA levels, an increase in salivary glucose concentration and a variation in the type or concentration of certain salivary enzyme levels, e.g. matrix metalloproteinase (MMP-8) and gelatinase (MMP-9) (7). It has been reported (32) that some oral infections such as periodontitis in patients affected by type 2 diabetes mellitus may be related to elevated salivary MMP-8 levels.

Salivary lysozyme, which may be affected by diabetes mellitus (49), may also influence (reduce) (39, 45) extracellular proteinase activity and concentration, although this remains unproven.

Although many of the pathogenetic *Candida* spp. have been shown to possess SAP genes and produce active extracellular proteinases *in vitro*, it has also been reported that the most virulent species, such as *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis*, produce more proteinases *in vitro* than do less virulent species (34, 41).

Interestingly, no differences were observed in the proteinase production between *C. albicans* and non-*C. albicans* spp. in the present study. This may be due to the great difference in the number of species between the two groups. In addition, it was not possible to classify the isolates as less virulent or use more virulent ones to compare them, due to the relatively low number of non-*C. albicans* spp. collected and analysed in the present study.

However, it has been reported that even if *C. albicans* has been found to produce more Saps *in vitro*, the *Candida* spp. ability to exhibit different extracellular proteinase production may be due, at least in part, to the sensitivity of the assays used to determine proteolytic activity (34).

Recently, it has been reported that *C. albicans* genotype B showed a significant increase in proteinase and phospholipase activity when compared to genotypes A or C (50).

In this study, no differences in extracellular proteinase production and *C. albicans* genotype were observed when the isolates from all the carriers were evaluated together. However, *C. albicans* genotype A isolated from London diabetic patients expressed higher levels of extracellular proteinase than those from Italian diabetic patients.

Further research is necessary to make a connection between Saps and/or phospholipase activity and the *C. albicans* genotype.

Isolates from diabetes mellitus patients carrying a lower number of oral yeasts (fewer than 100 cfu/ml) expressed greater adhesion ($P = 0.011$) than isolates from patients who had a higher number of oral yeasts. This was also observed for isolates from all the *Candida* carriers evaluated in this study.

The inverse relationship between the degree of colonisation and the ability of isolates to adhere is intriguing. The assay used to evaluate adhesiveness directly measured the isolates' ability to adhere to fibronectin. Therefore, it may be assumed that, as the number of yeasts present in the oral cavity increases, interaction between yeasts themselves becomes of greater importance than interaction between the yeast and the host, as was analysed in this study. Such a theory supports the concept of biofilm formation (3) and the increasing importance of interaction between microorganisms (51, 59). The results of previous study (28), where it was established that diabetes mellitus does not significantly increase the carriage or alter the character of oral yeast carriage, and thus the present evidence of virulence factors, could further support the notion that diabetes mellitus, at least when treated, does not greatly affect adherence (and resultant infections) or enhance the ability of *Candida* to modify its local environment.

Further research could be directed at examining agglutinin-like sequence genes (ALS), a family of at least nine genes that encode cell surface glycoproteins, mainly characterised in *C. albicans* and related

Candida spp. (6, 21–24), and vary in expression depending on environmental influence, to evaluate which of these genes are expressed during this *in vitro* assay.

The presence of oral prostheses may influence the capability of oral yeasts to adhere. In this study, particularly high levels of adhesion were observed in *Candida* isolates of Italian diabetic patients wearing dentures.

It has previously been reported that saliva of patients with controlled diabetes mellitus may be able to promote the growth of *C. albicans in vitro* and the present observation would confirm this. Certainly, the presence of oral prostheses such as dentures and removable (or fixed) orthodontic appliances can increase oral yeast carriage. The denture may function as a chronic reservoir of infection and dissemination (11, 29, 54), and surface irregularities would increase the likelihood of microorganisms remaining on the surface after the prosthesis has been cleaned.

It may be that diabetes, rather than the sole presence of a dental prosthesis, affects adherence, as the adherence of isolates from the control subgroup was not influenced by the presence of dentures. Furthermore, it has been reported (37) that, *in vivo,* microorganisms never attach directly to prostheses surfaces, as they are always covered with a salivary pellicle of various degrees of maturity. It may be that any enhanced interactions between yeasts and local factors such as a low salivary medium (due to the presence of dentures and hypoglycaemic agents) or poor metabolic control may increase the ability of *Candida* to adhere to oral mucosa, rather than any yeast-derived factor.

It is known that adhesion is strongly related to the presence of specific sugars (30, 43, 44), and that the adherence of *C. albicans* to acrylic surfaces increases when cultured in various carbon sources, including glucose, maltose and galactose (31). This increase has been attributed to enhanced formation of a fibrillar–floccular layer, demonstrated by ruthenium red or polycationic ferritin staining, which indicates that this layer may be rich in acidic polysaccharides (30).

This study has investigated the influence of diabetes mellitus upon the ability of oral *Candida* isolates to enhance the expression of two of the most important virulence attributes of *Candida* spp. adhesion and production of extracellular proteinase.

The assay used in this study detected that although there was a greater ability to adhere in *Candida* isolates of patients with diabetes mellitus than in healthy subjects, higher adhesion levels were shown in Italian patients with diabetes mellitus wearing dentures than the UK patients with diabetes mellitus and wearing dentures. In addition, higher levels of adhesion were also recorded in patients with a lower oral *Candida* colonisation (fewer than 100 cfu/ml).

In vitro extracellular proteinase expression was not significantly higher in *Candida* isolates of patients with diabetes mellitus in those of healthy subjects. However, higher extracellular proteinase levels were observed in isolates of type 2 diabetes mellitus patients when compared with type 1 diabetes mellitus patients.

As reported, it would be interesting to correlate the ALS and SAP genes expression obtained by reverse transcriptase-polymorphonuclear reaction for *Candida* isolates grown in the culture media previously used in the adhesion and extracellular proteinase production assays.

References

1. Al Karaawi ZM, Manfredi M, Waugh AC, McCullough MJ, Jorge J, Scully C, et al. Molecular characterization of *Candida* spp. isolated from the oral cavities of patients from diverse clinical settings. Oral Microbiol Immunol 2002: **17**: 44–49.
2. Aly FZ, Blackwell CC, MacKenzie DA, Weir DM, Clarke BF. Factors influencing oral carriage of yeasts among individuals with diabetes mellitus. Epidemiol Infect 1992: **109**: 507–518.
3. Baillie GS, Douglas LJ. Effect of growth rate on resistance of *Candida albicans* biofilms to antifungal agents. Antimicrob Agents Chemother 1998: **42**: 1900–1905.
4. Capobianco JO, Lerner CG, Goldman RC. Application of a fluorogenic substrate in the assay of proteolytic activity and in the discovery of a potent inhibitor of *Candida albicans* aspartic proteinase. Anal Biochem 1992: **204**: 96–102.
5. Castellani P, Siri A, Rosellini C, Infusini E, Borsi L, Zardi L. Transformed human cells release different fibronectin variants than do normal cells. J Cell Biol 1986: **103**: 1671–1677.
6. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. J Bacteriol 2001: **183**: 5385–5394.
7. Collin HL, Sorsa T, Meurman JH, Niskanen L, Salo T, Ronka H, et al. Salivary matrix metalloproteinase (MMP-8) levels and gelatinase (MMP-9) activities in patients with type 2 diabetes mellitus. J Periodontal Res 2000: **35**: 259–265.
8. Cotter G, Kavanagh K. Adherence mechanisms of *Candida albicans*. Br J Biomed Sci 2000: **57**: 241–249.
9. Cotter G, Weedle R, Kavanagh K. Monoclonal antibodies directed against extracellular matrix proteins reduce the adherence of *Candida albicans* to HEp-2 cells. Mycopathologia 1998: **141**: 137–142.
10. Darwazeh AM, Lamey PJ, Samaranayake LP, MacFarlane TW, Fisher BM, Macrury SM, et al. The relationship between colonisation, secretor status and *in-vitro* adhesion of *Candida albicans* to buccal epithelial cells from diabetics. J Med Microbiol 1990: **33**: 43–49.
11. Davenport JC. The oral distribution of *Candida* in denture stomatitis. Br Dent J 1970: **129**: 151–156.
12. de Viragh PA, Sanglard D, Togni G, Falchetto R, Monod M. Cloning and sequencing of two *Candida parapsilosis* genes encoding acid proteases. J Gen Microbiol 1993: **139**: 335–342.
13. Dorocka-Bobkowska B, Budtz-Jorgensen E, Wloch S. Non-insulin-dependent diabetes mellitus as a risk factor for denture stomatitis. J Oral Pathol Med 1996: **25**: 411–415.
14. Gaur NK, Klotz SA. Expression, cloning, and characterization of a *Candida albicans* gene, ALA1, that confers adherence properties upon *Saccharomyces cerevisiae* for extracellular matrix proteins. Infect Immun 1997: **65**: 5289–5294.
15. Gaur NK, Klotz SA, Henderson RL. Overexpression of the *Candida albicans* ALA1 gene in *Saccharomyces cerevisiae* results in aggregation following attachment of yeast cells to extracellular matrix proteins, adherence properties similar to those of *Candida albicans*. Infect Immun 1999: **67**: 6040–6047.
16. Gaur NK, Smith RL, Klotz SA. *Candida albicans* and *Saccharomyces cerevisiae* expressing ALA1/ALS5 adhere to accessible threonine, serine, or alanine patches. Cell Commun Adhes 2002: **9**: 45–57.
17. Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev 2000: **13**: 122–143.
18. Gilfillan GD, Sullivan DJ, Haynes K, Parkinson T, Coleman DC, Gow NA. *Candida dubliniensis:* phylogeny and putative virulence factors. Microbiology 1998: **144**: 829–838.
19. Gozalbo D, Gil-Navarro I, Azorin I, Renau-Piqueras J, Martinez JP, Gil ML. The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is also a fibronectin and laminin binding protein. Infect Immun 1998: **66**: 2052–2059.
20. Hostetter MK. Adhesins and ligands involved in the interaction of *Candida* spp. with epithelial and endothelial surfaces. Clin Microbiol Rev 1994: **7**: 29–42.
21. Hoyer LL. The ALS gene family of *Candida albicans*. Trends Microbiol 2001: **9**: 176–180.
22. Hoyer LL, Payne TL, Bell M, Myers AM, Scherer S. *Candida albicans* ALS3 and insights into the nature of the ALS gene family. Curr Genet 1998: **33**: 451–459.
23. Hoyer LL, Fundyga R, Hecht JE, Kapteyn JC, Klis FM, Arnold J. Characterization of agglutinin-like sequence genes from non-albicans *Candida* and phylogenetic analysis

of the ALS family. Genetics 2001: **157**: 1555–1567.
24. Hoyer LL, Scherer S, Shatzman AR, Livi GP. *Candida albicans* ALS1: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. Mol Microbiol 1995: **15**: 39–54.
25. Hube B, Naglik J. Extracellular hydrolases. In: Calderone RA, ed. Candida and candidiasis. Washington DC: American Society of Microbiology Association (ASM) Press, 2002: 107–122.
26. Klotz SA, Hein RC, Smith RL, Rouse JB. The fibronectin adhesin of *Candida albicans*. Infect Immun 2002: **62**: 4679–4681.
27. Macdonald F, Odds FC. Inducible proteinase of *Candida albicans* in diagnostic serology and in the pathogenesis of systemic candidosis. J Med Microbiol 1980: **13**: 423–435.
28. Manfredi M, McCullough MJ, Al Karaawi ZM, Hurel SJ, Porter SR. The isolation, identification and molecular analysis of *Candida* spp. isolated from the oral cavities of patients with diabetes mellitus. Oral Microbiol Immunol 2002: **17**: 181–185.
29. Maza JL, Elguezabal N, Prado C, Ellacuria J, Soler I, Ponton J. *Candida albicans* adherence to resin-composite restorative dental material: influence of whole human saliva. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002: **94**: 589–592.
30. McCourtie J, Douglas LJ. Relationship between cell surface composition of *Candida albicans* and adherence to acrylic after growth on different carbon sources. Infect Immun 1981: **32**: 1234–1241.
31. McCourtie J, Douglas LJ. Relationship between cell surface composition, adherence, and virulence of *Candida albicans*. Infect Immun 1984: **45**: 6–12.
32. McCullough MJ, Ross BC, Reade PC. Genetic differentiation of *Candida albicans* strains by mixed-linker polymerase chain reaction. J Med Vet Mycol 1995: **33**: 77–80.
33. Monod M, Togni G, Hube B, Sanglard D. Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. Mol Microbiol 1994: **13**: 357–368.
34. Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 2003: **67**: 400–428, table of contents.
35. Odds FC, Evans EG, Taylor MA, Wales JK. Prevalence of pathogenic yeasts and humoral antibodies to *Candida* in diabetic patients. J Clin Pathol 1978: **31**: 840–844.
36. Pendrak ML, Krutzsch HC, Roberts DD. Structural requirements for hemoglobin to induce fibronectin receptor expression in *Candida albicans*. Biochemistry 2000: **39**: 16110–16118.
37. Radford DR, Challacombe SJ, Walter JD. Denture plaque and adherence of *Candida albicans* to denture-base materials *in vivo* and *in vitro*. Crit Rev Oral Biol Med 1999: **10**: 99–116.
38. Rauceo JM, Gaur NK, Lee KG, Edwards JE, Klotz SA, Lipke PN. Global cell surface conformational shift mediated by a *Candida albicans* adhesin. Infect Immun 2004: **72**: 4948–4955.
39. Rayfield EJ, Ault MJ, Keusch GT, Brothers MJ, Nechemias C, Smith H. Infection and diabetes: the case for glucose control. Am J Med 1982: **72**: 439–450.
40. Rodier MH, el Moudni B, Kauffmann-Lacroix C, Daniault G, Jacquemin JL. A *Candida albicans* metallopeptidase degrades constitutive proteins of extracellular matrix. FEMS Microbiol Lett 1999: **177**: 205–210.
41. Ruchel R. Proteinase. In: Bennett, JE, Hay, RJ Peterson, PK, eds. New strategies in fungal disease. Edinburgh: Churchill Livingstone, 1992: 17–31.
42. Samaranayake LP, Hughes A, MacFarlane TW. The proteolytic potential of *Candida albicans* in human saliva supplemented with glucose. J Med Microbiol 1984: **17**: 13–22.
43. Samaranayake LP, MacFarlane TW. An *in-vitro* study of the adherence of *Candida albicans* to acrylic surfaces. Arch Oral Biol 1980: **25**: 603–609.
44. Samaranayake LP, McCourtie J, MacFarlane TW. Factors affecting the *in-vitro* adherence of *Candida albicans* to acrylic surfaces. Arch Oral Biol 1980: **25**: 611–615.
45. Scherer S, Stevens DA. Application of DNA typing methods to epidemiology and taxonomy of *Candida species*. J Clin Microbiol 1987: **25**: 675–679.
46. Senet JM. *Candida* adherence phenomena, from commensalism to pathogenicity. Int Microbiol 1998: **1**: 117–122.
47. Singleton DR, Masuoka J, Hazen KC. Cloning and analysis of a *Candida albicans* gene that affects cell surface hydrophobicity. J Bacteriol 2001: **183**: 3582–3588.
48. Skerl KG, Calderone RA, Segal E, Sreevalsan T, Scheld WM. *In vitro* binding of *Candida albicans* yeast cells to human fibronectin. 1984: **30**: 221–227.
49. Stevens DA, Odds FC, Scherer S. Application of DNA typing methods to *Candida albicans* epidemiology and correlations with phenotype. Rev Infect Dis 1990: **12**: 258–266.
50. 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.
51. Sullivan DJ, Coleman DC. *Candida dubliniensis*: an emerging opportunistic pathogen. Curr Top Med Mycol 1997: **8**: 15–25.
52. Togni G, Sanglard D, Falchetto R, Monod M. Isolation and nucleotide sequence of the extracellular acid protease gene (ACP) from the yeast *Candida tropicalis*. FEBS Lett 1991: **286**: 181–185.
53. Varner JA, Cheresh DA. Integrins and cancer. Curr Opin Cell Biol 1996: **8**: 724–730.
54. Verran J, Maryan CJ. Retention of *Candida albicans* on acrylic resin and silicone of different surface topography. J Prosthet Dent 1997: **77**: 535–539.
55. Wagner T, Borg Z van Ruchel R. pH-dependent denaturation of extracellular aspartic proteinases from *Candida* species. J Med Vet Mycol 1995: **33**: 275–278.
56. Williams DW, Lewis MA. Isolation and identification of *Candida* from the oral cavity. Oral Dis 2000: **6**: 3–11.
57. Willis AM, Coulter WA, Fulton CR, Hayes JR, Bell PM, Lamey PJ. Oral candidal carriage and infection in insulin-treated diabetic patients. Diabet Med 1999: **16**: 675–679.
58. 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.
59. Willis AM, Coulter WA, Sullivan DJ, Coleman DC, Hayes JR, Bell PM, et al. Isolation of *C. dubliniensis* from insulin-using diabetes mellitus patients. J Oral Pathol Med 2000: **29**: 86–90.
60. Yan S, Negre E, Cashel JA, Guo N, Lyman CA, Walsh TJ, et al. Specific induction of fibronectin binding activity by hemoglobin in *Candida albicans* grown in defined media. Infect Immun 1996: **64**: 2930–2935.
61. Yan S, Rodrigues RG, Cahn-Hidalgo D, Walsh TJ, Roberts DD. Hemoglobin induces binding of several extracellular matrix proteins to *Candida albicans*. Identification of a common receptor for fibronectin, fibrinogen, and laminin. J Biol Chem 1998: **273**: 5638–5644.
62. Yan S, Rodrigues RG, Roberts DD. Hemoglobin-induced binding of *Candida albicans* to the cell-binding domain of fibronectin is independent of the Arg-Gly-Asp sequence. Infect Immun 1998: **66**: 1904–1909.
63. Zaugg C, Borg-Von Zepelin M, Reichard U, Sanglard D, Monod M. Secreted aspartic proteinase family of *Candida tropicalis*. Infect Immun 2001: **69**: 405–412.