# RELATIONSHIP BETWEEN ORAL *CANDIDA* COLONISATION AND DIABETIC CONTROL

***J Fourie***

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# RELATIONSHIP BETWEEN ORAL *CANDIDA* COLONISATION AND DIABETIC CONTROL


by

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MChD (Oral Medicine and Periodontics)


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## DECLARATION

I, Jeanine Fourie, hereby declare that this dissertation is my own work. It is submitted in partial fulfilment of the requirements for the degree of Master of Dentistry in the field of Oral Medicine and Periodontics. It has not been submitted before for any degree or examination at this or any other University.

**J Fourie**

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# CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

## 1.1 INTRODUCTION

*Candida:* Latin: Candidus glowing white.

*Candida* belongs to a genus of yeastlike imperfect fungi of the family Cryptococcaceae that produce yeast cells, mycelia, pseudomycelia and blastospores. Some species are part of the normal flora of the skin and mucous membranes (commensal organisms) but can also cause infections.[1]

Candidiasis: "Infection with a fungus of the genus *Candida,* most commonly *C. albicans.* It is usually a superficial infection of the skin or mucous membranes, although sometimes it manifests as a systemic infection or as endocarditis; any form can become more severe in immunocompromised patients".[1]

Commensally carried oral *Candida* may be more prevalent in diabetic patients than in healthy controls.[2-4]

Oral *Candidiasis* is a common opportunistic infection.[5] Diabetic patients may be more susceptible to oral *Candida* infection owing to impaired host defence,[6] increased salivary glucose[7] and decreased salivary flow.[8]

Blood glucose levels have been thought to be the determining factor in oral *Candida* colonisation But an association between blood glucose levels and oral *Candida* colonisation has not been conclusively demonstrated.[2,3,9,10]

Another theory proposes that saliva with an increased glucose concentration may create ideal growth conditions for *Candida*.[11,12,13]

Although the saliva of diabetic patients may have a higher than normal glucose concentration, there does not seem to be a direct correlation between blood glucose and salivary glucose levels.[14]

This study will attempt to correlate the relationship between blood glucose and oral *Candida* presence in a diabetic population.

## 1.2 LITERATURE REVIEW

### *1.2.1 Candida*

*Candidiasis* is the most common oral fungal infection.[5] The usual pathogen is *Candida albicans,* but other strains of *Candida* are also commonly found, such as *Candida glabrata, Candida tropicalis, Candida parapsilosis, Candida krusei, Candida guilliermondii, Candida lusitaniae and Candida kefyr*.[15]

Yeasts which belong to the genus *Candida* are unicellular dimorphic fungi which can transform from blastospores to hyphae and/or pseudohyphae depending on environmental conditions.[16] Known predisposing factors for *Candida* infection include the wearing of a dental prosthesis, HIV infection, the use of broad spectrum antibiotics and glucocorticosteroids, as well as endocrine disorders such as diabetes mellitus (DM).[17-19]

*Candida albicans* can be isolated from the oral cavity in 40% to 65% of healthy persons.[20] Other strains of *Candida* are associated with certain systemic diseases[20] and with local conditions of the oral cavity.[21] *Candida* strains isolated from the mouths of patients with DM include: *Candida albicans* (89%), *Candida krusei* (2.8%), *Candida glabrata* (2.8%), *Candida tropicalis* (6.2%), *Candida stellatoidea* (2.8%), *Candida parapsilosis* (0.5%)[22] and *Candida dubliniensis*.[23,24]

#### *1.2.1.1* *Candida* infection

In healthy persons, oral *Candida* occurs commensally, primarily on the dorsum of the tongue in the form of blastospores.[18] These persons are known as carriers. The *Candida* counts from carriers and from those with infection show overlap and therefore isolation of *Candida* is not regarded as definite evidence of infection but should be considered relative to the clinical findings.[25]

Oral candidiasis may present clinically as either a primary or a secondary infection. In secondary oral candidiasis the oral presentation is the manifestation of systemic *Candida* infection, while the primary form is confined to the oral and peri-oral tissues.[26] Primary oral candidiasis may present clinically as pseudomembranous-, erythematous- or hyperplastic candidiasis, and also as denture stomatitis, angular cheilitis or median rhomboid glossitis.[26]

### 1.2.2 Diabetes mellitus

Diabetes: Greek: Diabétés a siphon, from *dia* through + *baineth* to go.[1]

Diabetes mellitus (DM) is an endocrine-mediated disease characterised by elevated blood glucose concentration. Type I DM, formerly known as insulin dependent DM (IDDM), is caused by the autoimmune mediated destruction of the insulin producing pancreatic beta cells resulting in insufficient insulin levels.[27]

Type II DM, formerly known as non-insulin dependent DM (NIDDM), is caused by the relative unresponsiveness or resistance of cells to insulin.[27]

Patients are diagnosed with DM when:

- random blood glucose concentration is greater than 11.1 mmol/L (200mg/dL);
- fasting blood glucose concentration is greater than 7 mmol/L (126mg/dL) and
- the glucose tolerance test results in blood glucose levels greater than 11.1 mmol/L (200 mg.dL) 2 hours following a 75 g glucose load.[27]

A record of the patient's long term glucose control over a 30 to 90 day period can be obtained from the glycated haemoglobin levels ($HbA_1$ and $HbA_{1c}$).[28]

Classic signs and symptoms of DM include polyuria, polydipsia, polyphagia, weakness and fatigue. Complications associated with DM include micro- and macrovascular disease (atherosclerosis), retinopathy, nephropathy, neuropathy and periodontal disease.[27] Chronic hyperglycaemia results in the non-enzymatic and irreversible glycation of body proteins, resulting in advanced glycation end-products (AGE); which are responsible for the complications of DM.[29] The AGE bind to specific receptors for AGE (RAGE) on monocytes, macrophages, and endothelial cells, altering intracellular signalling pathways.[30] AGE-RAGE binding lead to increased expression of pro-inflammatory cytokines and the production of oxygen free radicals by activated monocytes and macrophages, creating a chronic state of inflammation.[31]

Patients with DM are predisposed to infections by the combined effects of angiopathy, neuropathy and hyperglycaemia.[6] Hyperglycaemia may lead to impaired host defence mechanisms such as impaired wound healing, impaired granulocyte function, decreased cellular immunity, impaired complement function and decreased lymphokine response.[6] In the mouth, local conditions such as raised salivary glucose[7] and decreased salivary flow may further predispose to oral candidiasis.[8]

### 1.2.3 Association between diabetes mellitus and *Candida* colonisation of the oral cavity

The carriage and the density of growth of *Candida* species in the oral cavity is claimed to be increased in patients with DM.[32] However, the validity of this contention remains controversial since a number of studies could not demonstrate such an association.[14,33]

#### 1.2.3.1 Studies that demonstrated an increase in *Candida* colonisation of the oral mucosa in diabetic patients

Some studies have shown that DM is a significant risk factor for increasing the carriage rate and density of *Candida* colonisation.[2-4,10,33-38]

Either the *Candida* carriage rate,[2,3] the density of *Candida*, or both are increased in diabetic patients.[4] Diabetic patients are more likely to have *Candida* pseudohyphae in greater numbers in oral cytological smears,[35] as well as a greater diversity of *Candida* species.[38]

The wearing of dentures further predisposes diabetic patients to *Candida* colonisation as is evident from their increased prevalence of denture stomatitis.[10,33,36,37] One explanation given for this high prevalence of denture stomatitis in type II diabetic patients is the increased adherence of *Candida* to palatal epithelial cells.[34]

Other clinical manifestations of oral candidiasis are also more prevalent in diabetic patients than in healthy control subjects,[35] erythematous candidiasis being the most common clinical presentation in a type I DM population.[10]

Overall, it is apparent from the literature that *Candida* is more prevalent in diabetic than in non-diabetic subjects.

#### 1.2.3.2 Studies that did not demonstrate an increase in *Candida* colonisation of the oral mucosa in diabetic patients

Phelan and Levin, 1986, found that denture stomatitis was equally prevalent in patients with DM, in patients with elevated plasma glucose levels and in healthy control subjects.[39] Fisher et al., 1987, postulated that denture wearing, and not DM per se may be the only risk factor for *Candida* colonisation of the mouth in diabetic patients; and that this also determines the *Candida* colony forming units (CFU).[22] Dorocka-Bobkowska and co-workers, 1996, on the other hand demonstrated that even though denture stomatitis was observed more frequently in Type II DM patients, the frequency of *Candida* colonisation was not significantly increased relative to that in non-DM subjects with denture stomatitis.[34]

Peters, Bahn and Barens, 1966, could not find differences in the frequency of colonisation of the oral mucosa with *Candida* spp., between uncontrolled diabetic patients, controlled diabetic patients and healthy controls.[40]

In addition, no significant differences were found between long- and short-duration diabetic patients and healthy controls with regard to the mean number of CFU of *Candida.*[8]

### 1.2.4 Association between blood glucose levels and *Candida* colonisation of the oral mucosa

Many researchers have compared diabetic patients with healthy controls to illustrate differences between these groups, while others have grouped their subjects according to the type of DM and compared them with healthy controls.[3,4,9,35] Yet other researchers have attempted to draw a direct correlation between blood glucose levels and the density of *Candida* colonisation of the mouth of diabetic patients.[2,3,9,10,35,41]

Despite confirming the increased oral carriage of *Candida* in diabetic patients, a number of authors have failed to draw correlations between the increased oral carriage of *Candida* and certain diabetic parameters such as glycaemic (blood glucose) control,[2,3] diabetic therapy[4] or $HbA_{1c}$ values.[9]

However, Guggenheimer *et al.,* 2000, did correlate the presence of *Candida* pseudohyphae with elevated levels of $HbA_{1c}$[35] and Aly *et al.* 1992, correlated oral carriage of *Candida* with plasma glucose levels.[41]

### 1.2.5 Association between salivary glucose levels and *Candida* colonisation of the oral mucosa

In diabetic patients, salivary glucose concentrations are significantly higher than in controls,[7,8,42] while another study claims that diabetic patients who wear dentures have even higher salivary glucose levels[14] and that increased salivary glucose levels are directly related to blood glucose concentration.[14] Others have found no correlation between salivary glucose and blood glucose levels.[7,42]

Newly diagnosed type I DM patients were also found to have high salivary glucose concentrations and low salivary flow rates. Subsequent to therapy salivary glucose concentrations decreased and salivary flow rate increased.[43] During periods of hyperglycaemia the salivary glucose levels correlated with the mean blood glucose levels but salivary flow rates and glucose levels did not correlate with the $HbA_{1c}$ levels.[43]

Diabetic patients who carry *Candida* intra-orally have significantly higher salivary glucose concentrations than those in whom *Candida* cannot be isolated.[14] However, no conclusive effect of increased salivary glucose concentration on the oral carriage of *Candida* could be demonstrated.[14]

Increased glucose in saliva seems to make it an ideal culture medium for *Candida,* as was shown by the increased growth of *Candida* in saliva with increased glucose levels, from diabetic patients.[11] *Candida albicans* does not grow in sugar-depleted saliva, but the addition of glucose produces exceptional growth.[11] It is concluded that persons with DM tend to have increased concentrations of glucose in saliva, and that this is associated with an increased growth of *Candida*.[11] *Candida* species cultured in a medium supplemented with glucose will also exhibit enhanced adhesion to epithelial cells.[12]

From these studies it is clear that diabetic patients show increased salivary glucose levels, and *in vitro* studies show saliva with increased glucose levels to be an ideal culture medium for *Candida*.

### 1.2.6 Isolation of *Candida* from the oral cavity

There are many methods available to isolate oral *Candida* including smears, swabs, imprint or impression cultures, mucosal biopsy and estimates of CFU from either saliva or from oral rinses.[44,45] Some of the researchers investigating the association between DM and *Candida* used a swab to retrieve and identify *Candida*,[9] while others quantitatively measured the *Candida* load.[4,10,35,]

The variety of isolation techniques used makes comparisons between studies difficult if not impossible.

A smear is a non-quantitative technique which is widely used and has the benefit of demonstrating hyphae, but it may be less sensitive than other methods.[45] *Candida* pseudohyphae and hyphae as demonstrated by cytology, rather than the yeast form, are considered more likely to be indicators of clinical infection.[9,46]

The oral rinse is a technique that allows for the quantification of microbes including *Candida* but does not localise the site of any putative infection.[45]

Imprint culture has been advocated as the most sensitive quantitative method for determining oral yeast carriage, but Samaranayake et al., 1986, showed the method of concentrated oral rinse compares favourably.[47] The oral rinse has the additional benefits of being easy to perform, and of sampling the whole mouth instead of just the sites selected by the clinician.[47]

Assessing *Candida* load may be important in establishing a diagnosis of *Candida* infection. Epstein, Pearsall and Truelove, 1980, demonstrated that patients with clinically diagnosed candidiasis had more than 400 CFU of *Candida* per millilitre of saliva, while carriers had less than 400 CFU per millilitre of saliva.[48] Oral carriers of *Candida* and patients with oral *Candidiasis* may thus be reliably differentiated on the basis of quantitative culture.[48,49]

*Candida* species can be differentiated through incubation on chromogenic agar, by using morphological and physiological criteria, by using commercially available identification systems, instrument-based identification systems or by genetic analysis.[45] This study will attempt to identify only *Candida per se*, and not to distinguish between different species.

# CHAPTER 2: AIM AND OBJECTIVE

The aim of this study is to attempt to determine whether there is any relationship between oral *Candida* colonisation and blood glucose levels, in a diabetic population.

The primary aim will be further explored through investigation of the following relationships:

- between plasma glucose and the identification of *Candida;*
- between $HbA_{1c}$ and the identification of *Candida* ;
- between differences in *Candida* identification through culture and cytology;
- between *Candida* identification and clinical infection and
- between *Candida* identification and the use of xerostomia inducing drugs

## 2.1 HYPOTHESIS

There is a relationship between oral *Candida* colonisation and blood glucose levels in a diabetic population.

## 2.2 NULL HYPOTHESIS

There is no relationship between oral *Candida* colonisation and blood glucose levels in a diabetic population.

# CHAPTER 3: MATERIALS AND METHODS

Study description: A cross-sectional study of oral colonisation by *Candida* and the blood glucose levels of a diabetic population.

Ethics approval to conduct this study was obtained from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria.

## 3.1 SETTING

This study was conducted at the Diabetic Clinic, Kalafong Hospital, Pretoria. Patients were seen during the months of September, October and November 2010.

## 3.2 PATIENT SELECTION

Diabetic patients were selected between and including the ages of 40 and 60 years. All gave their consent to be included in the study. A non-diabetic control group was not selected because the correlation between *Candida* colonisation and blood glucose levels were investigated. A comparison between diabetic and healthy controls was not the aim of this study.

Patients were excluded from the study if they:

- were edentulous, or wore a dental prosthesis;
- had used systemic antibiotics, glucocorticosteroids or a chlorhexidine containing mouthwash in the preceding three months;
- currently used tobacco;

- reported a subjective sensation of a dry mouth;
- were known to be infected with the Human Immunodeficiency Virus (HIV), or any other known diseases or treatments which could impair the immune system;
- were on treatment for either oral mucosal or salivary disorders.

## 3.3 SAMPLE SIZE

A total of 89 subjects were included in this study. An oral rinse specimen was collected from all 89 patients and smears were collected from 81. It was not possible to take smears from 8 subjects due to the unavailability of these patients for recall appointments.

## 3.4 DATA COLLECTION

The following information was obtained from patient files:

- age;
- gender;
- blood glucose levels;
- $HbA_{1c}$ levels and
- current medication.

Specimens were taken with a wooden spatula from the right buccal mucosa, palate and the tongue of each subject. Smears were then applied to glass slides and fixed. The slides were stained with Periodic Acid Schiff (PAS), examined by the principle investigator and checked by a senior oral pathologist of the department of Oral Pathology, Oral and Dental Hospital, Pretoria. Upon cytological examination, the presence or absence of *Candida* hyphae and spores were noted for each slide.

In addition, an oral rinse was collected from each subject according to the technique described by Samaranayake *et al.,* 1986.[47] Subjects rinsed their mouths with 10 ml phosphate buffered saline for 30 seconds, and then expectorated into a sterile container. The samples were placed on ice and delivered within 4 hours to the Department of Medical Microbiology, Faculty of Health Sciences, University of Pretoria, for culturing. The rinse was vortexed for one minute for even suspension and 0.1 ml of each sample was evenly plated onto Sabouraud's dextrose agar (SDA) plates (Gibco, Paisley, Scotland) using a flamed rod. Each SDA plate contained 50 mg/ml chloramphenicol in order to prevent bacterial colonisation (Sigma, Poole, Dorset, England). The plates were incubated (Prolab incubator) at 30 °C for 48 hours after which they were visually examined by one technician for the presence/absence of yeast colonies. The number of CFU per plate were counted and expressed as CFU/ml.

Each subject's mouth was also examined for clinical signs of *Candida* infection or other mucosal disease. A periodontal screening (Community Periodontal Index of Treatment Needs) was performed on each subject, and affected subjects were referred to the Oral and Dental Hospital, Pretoria, for management.

## 3.5 STATISTICAL ANALYSIS

Data were captured in Microsoft Office Excel 2007 spreadsheets. The initial design of the study anticipated a normal distribution of data so that correlations could be used. However, analysis of the data revealed that for *Candida* culture, blood glucose levels, $HbA_{1c}$, clinical

*Candida* infection and the use of xerostomia-inducing drugs the variables were not normally distributed. Therefore, in the case of *Candida* culture it was decided to use "presence/absence" as the data distribution was extremely skewed. Analysis by parametric statistical tests such as the Student's-t test or Pearson test would therefore not have been appropriate for analysis. The Mann-Whitney test, a non parametric test, was used which assumes that the variables are not normally distributed. Where associations were made between data presented as "presence/absence", the Chi-square test was used.

The Biomedical and Dental Program (BMDP) was used to calculate the Mann-Whitney tests, and the SAS-statistical program was used for the Chi-square tests.

# CHAPTER 4: RESULTS

Categorical data: The mean age of the subjects was 51 years. There were 53 females and 36 males. The level of diabetic control among the subjects was categorized according to Kumar et al., 2005,[4] as:

- Good ($HbA_{1c}$ < 8%): 35 subjects (40.7%);
- Fair ($HbA_{1c}$ 8% to 10% inclusive): 30 subjects (34.9%), and
- Poor ($HbA_{1c}$ > 10%): 21 subjects (24.4%).

## 4.1 RELATIONSHIP BETWEEN THE LEVEL OF DIABETIC CONTROL AND *CANDIDA* COLONISATION OF THE ORAL MUCOSA

### 4.1.1 Relationship between plasma glucose and *Candida* identification

The mean plasma glucose was 10.35 mmol/L (standard deviation 5.3, minimum 3, maximum 30.2) and the median plasma glucose 8.9 mmol/L.

#### 4.1.1.1 Culture

Culturing of the oral rinse samples yielded mean *Candida* CFU of 253.87 (standard deviation 649.76, minimum 0, maximum 4000).

There was no relationship between plasma glucose and the presence or absence of *Candida* in cultured samples (Mann-Whitney U test $p = 0.6458$).

##### 4.1.1.2 Cytology

Cytology positively identified *Candida* in 53 subjects. There was no relationship between plasma glucose and the presence or absence of *Candida* identified on smears (Mann-Whitney U test $p = 0.2295$).

### 4.1.2 Relationship between $HbA_{1c}$ and *Candida* identification

Measure of the subjects' long term diabetic control revealed a mean $HbA_{1c}$ of 8.76% (standard deviation 2.2%, minimum 5.4%, maximum 14.7%)

#### 4.1.2.1 Culture

There was no relationship between $HbA_{1c}$ and the presence or absence of *Candida* CFU (Mann-Whitney $p = 0.4732$).

#### 4.1.2.2 Cytology

There was no relationship between $HbA_{1c}$ and the presence or absence of *Candida* identified by cytology (Mann-Whitney U test $p = 0.7351$).

## 4.2 ASSOCIATION BETWEEN *CANDIDA* IDENTIFIED BY CULTURE AND CYTOLOGY

Oral rinse samples for culture were collected from all 89 subjects in the study: 42 (47%) yielded positive *Candida* cultures.

Smears for cytological examination were collected from 81 of the 89 subjects in the study: of these, 53 (65%) were positive.

There was a statistically significant relationship between *Candida* identification by culture and by cytology (Chi-Square test $p = 0.0023$).

The frequency distributions of *Candida* isolated cytologically from different parts of the mouth was:

- tongue: 46/80 (57.5%)
- palate: 23/80 (28.75%)
- right buccal mucosa: 27/80 (33.75%)

## 4.3 ASSOCIATION BETWEEN IDENTIFICATION OF *CANDIDA* AND CLINICAL ORAL *CANDIDA* INFECTION

All subjects were examined for clinical signs of oral *Candida* infection: 34 of the 89 subjects (38.2%) demonstrated clinical signs of *Candida* infection. Median rhomboid glossitis was the most commonly seen *Candida* lesion (33 of the 89 subjects or 37%) (Figure 1). Two of these patients also showed erythematous candidiasis of the palate. One patient had erythematous candidiasis only. No other *Candida* lesions were noted.

**Figure 1: Dorsal surface of the tongue showing the clinical appearance of median rhomboid glossitis**

### 4.3.1 Culture

Patients with clinical diagnoses of oral candidiasis had a mean CFU of *Candida* of 368.88/ml, while patients without any clinical signs of candidiasis had a mean CFU of 182.76/ml. This association between clinical intra-oral *Candida* infection and CFU was statistically not significant (Chi-square $p$ = 0.6033).

### 4.3.2 Cytology

Of the 29 subjects with median rhomboid glossitis from whom smears were taken, 19 (65.5%) of the tongue smears demonstrated the presence of *Candida* (n=19). Of these 19 smears, hyphae were found in 6, hyphae and spores in 1, and spores only in 12.

The association between intra-oral *Candida* infection and cytological isolation of *Candida* was statistically not significant (Chi-Square $p$ = 0.3238).

## 4.4 ASSOCIATION BETWEEN IDENTIFICATION OF *CANDIDA* AND THE USE OF XEROSTOMIA INDUCING DRUGS

**Table 1: Xerostomia inducing drugs taken by subjects in the study population**

| Drug | Number of patients taking drug |
|---|---|
| Amitriptyline | 33 |
| Carbamazepine | 11 |
| Fluoxetine | 1 |
| Glyceryl trinitrate | 3 |
| Isosorbide mononitrate | 7 |
| Losec (Omeprazole) | 2 |
| Methyldopa | 16 |
| Phenergan | 1 |

(Xerostomia is a known side effect of these drugs according to the South African Medicines Formulatory, 2010)

### 4.4.1 Culture

There was no association between the use of xerostomia-inducing drugs and the identification of *Candida* by culture (Chi-Square $p = 0.7456$).

### 4.4.2 Cytology

There was no association between the use of xerostomia-inducing drugs and the identification of *Candida* by cytology (Chi-Square $p = 0.3828$).

# CHAPTER 5: DISCUSSION

## 5.1 IDENTIFICATION OF *CANDIDA* IN A DIABETIC POPULATION

*Candida* could be identified by culture in 47% and by cytology in 65% of diabetic subjects in this population. This is similar to what was found in a previous study in dentate diabetic patients (63.6%), and is greater than the prevalence in non-diabetic persons (37.5%).[2] A high prevalence of *Candida* colonisation was also found in other studies.[3,4,10,22,23,41]

The mean CFU (253.87SD±649.76 CFU/ml) in this population was however far lower than that obtained by Lamey et al., 1988, (406.7SD±726 CFU/ml).[2] Kumar et al., 2005, reported even higher mean CFU among type I (1251.37 CFU/ml) and type II (830.37 CFU/ml) diabetic patients.[4] The study by Lamey et al., 1988, failed to demonstrate a greater density of candidal growth in diabetic subjects compared with non-diabetic subjects.[2]

Of the 65% of subjects in our study from whose mouths *Candida* could be identified by cytology, 52.83% demonstrated the presence of hyphae. This is much more than reported by Guggenheimer et al., 2000, who identified *Candida* pseudohyphae in only 23% of type I DM patients.[35]

### 5.1.1 Exposition of high prevalence of *Candida* colonisation in diabetic patients

Many local and systemic factors may predispose to oral candidiasis, such as poor bacterial plaque control, denture wearing, oral keratotic lesions, smoking habits, orthodontic appliances and mouth breathing.[19] Systemic conditions such as disseminated malignancy, immune deficiency, and exposure to antibiotics[19] and/or corticosteroids may further predispose to *Candida* infection.[17,18] Our study has sought to eliminate certain independent predisposing factors by excluding denture wearing, the smoking habit, known HIV infection, xerostomia, and the use of systemic corticosteroids and/or antibiotics. Patients over 60 years of age were excluded as subjects because of conflicting views in the literature on the effect of age on oral *Candida* carriage.[41]

Within a diabetic population, known risk factors for *Candida* colonisation associated with the disease process of DM, include impaired host defence,[6] xerostomia, increased salivary glucose[7,14] decreased salivary flow[8] and possibly secretor status (secretion of the ABH antigens of the ABO blood group in saliva).[50]

Poorly controlled DM is associated with decreased neutrophil functions, such as phagocytosis, intracellular killing, bactericidal activity and chemotaxis.[51] Such neutrophil defects have been demonstrated in diabetic patients with oral candidiasis.[52] Poor glycaemic control, with resultant hyperglycaemia, may also exert an influence by impairing wound healing, granulocyte function and decreasing cellular immunity, complement function and lymphokine response.[6]

Decreased salivary gland function has been found in diabetic patients[8,53] and low salivary flow rate has independently been associated with oral candidiasis.[19] However, in the study by Guggenheimer et al., 2000, there were no reports on the subjective sensation of dry mouth or on decrease in the salivary flow rates of a diabetic population.[35] Sharon et al., 1985, also demonstrated a normal salivary flow rate in diabetic patients, despite the fact that up to a third of these patients complained of “dry mouth”.[42] Xerostomia in DM, which has been observed by some authors,[3,54] is explained by increased urinary flow and dehydration in uncontrolled patients.[42] In a previous study the presence of xerostomia could not be correlated with the oral carriage of *Candida* in a diabetic population.[3]

Salivary glucose concentration was found to be significantly higher in diabetic patients than in controls.[7,8,42] Some studies could correlate the raised salivary glucose levels with blood glucose levels[14] whereas others could not.[7,42] In diabetic patients with raised salivary glucose levels, *Candida* could more frequently be isolated from the mouth.[14] This can be explained by the fact that saliva with a high glucose concentration may favour *Candida* growth.[11,13,55]

The genetically determined inability to secrete the water soluble glycoprotein forms of the ABO blood group antigens has been associated with an increased predisposition to certain infections. The role of this secretor status in diabetic patients is however controversial. Diabetic patients who were secretors had equivalent *Candida* carriage and infection to non-secretors in one study,[2] in contrast to other studies where non-secretor diabetic patients showed a greater frequency of oral *Candida* carriage.[50,56] Aly et al., 1992, concluded that the density of *Candida* colonisation is influenced by secretor status, but that carriage *per se* does not depend on secretors status.[41]

## 5.2 RELATIONSHIP BETWEEN THE LEVEL OF DIABETIC CONTROL AND ORAL COLONISATION BY *CANDIDA*

### 5.2.1 Relationship between plasma glucose and identification of *Candida* in the mouth

*Candida* was identified by culture and by cytology.

#### 5.2.1.1 Culture

There was no relationship between blood glucose and *Candida* CFU. This is in agreement with other studies which found no relationship between diabetic control and the prevalence or density of *Candida* colonisation.[2,4,10,22]

#### 5.2.1.2 Cytology

In this study, no relationship was found between plasma glucose and the presence of *Candida* through cytology which is in agreement with other researchers.[3,9]

### 5.2.2 Relationship between $HbA_{1c}$ and *Candida* identification

#### 5.2.2.1 Culture

There was no relationship between $HbA_{1c}$ and *Candida* CFU. This is in agreement with other studies.[4,22,41]

#### 5.2.2.2 Cytology

There was no relationship between $HbA_{1c}$ and *Candida* identification through cytology. This differs from Aly et al., 1992, who found that diabetic patients were more likely to be palatal carriers of *Candida* in the presence of persistent glycosuria, higher random plasma glucose levels and higher $HbA_{1c}$.[41] Guggenheimer et al., 2000, correlated the presence of *Candida* pseudohyphae with elevated levels of $HbA_{1c}$.[35]

Many previous studies did not exclude local risk factors, but rather looked at local factors that may have contributed to *Candida* carriage. In those instances where *Candida* carriage could not be attributed to hyperglycaemia, direct correlations were found to local predisposing factors.[3,10,33,35,41,57]

It is apparent that *Candida* may be more frequently isolated from the oral cavity of diabetic patients, but cannot be related to glycaemic control. Local factors may put the diabetic patient more at risk of *Candida* colonisation than an otherwise healthy patient.

## 5.3 ASSOCIATION BETWEEN IDENTIFICATION OF *CANDIDA* BY CULTURE AND BY CYTOLOGY

In this study, oral *Candida* was demonstrated by cytology in 65% of patients. The tongue was found to be most frequently colonised (86.8%). Comparison between the techniques for *Candida* isolation revealed that cytology was more reliable in identifying *Candida*. With cytology as the gold standard, culturing had a specificity of 64% and sensitivity of 71%.

Oral yeasts have been isolated most frequently from the tongue, followed by the palate, then floor of mouth and then angles of the mouth.[41,57] This supports the suggestion that the tongue might act as a reservoir for yeasts.[58]

According to Silverman et al., 1990, a smear is of value in differentiating between yeast and hyphal forms but is less sensitive than cultural methods.[49] This study is not in agreement with this and demonstrated a greater prevalence of oral *Candida* when utilizing cytology

(65%) than when utilizing culture (49%). The reason for this disparity in our study is not certain, but might be ascribed to the fact that oral rinse samples were not centrifuged. According to Samaranayake et al., 1986, concentrating the rinse through centrifugation increases the sensitivity from 88% to 92%.[47]

The oral rinse technique used for collecting *Candida* for culturing is known to be a sensitive technique for estimating the oral carriage of yeasts.[47] Counts and cultures lack the sensitivity to discriminate between oral *Candida* infection and C*andida* carriage.[35] Even so, the number of CFU may correlate with clinical infection.[48] The distinction between oral *Candida* carriage and infection is not altogether clear, but it is proposed that the presence of *Candida* pseudohyphae and hyphae, rather than the spore form, is more likely to be associated with clinical infection.[59] Hyphae have greater pathogenic potential because of their ability to adhere to epithelial cells, a feature that is not shared by the spore forms.[55]

## 5.4 ASSOCIATION BETWEEN THE IDENTIFICATION OF *CANDIDA* AND CLINICAL *CANDIDA* INFECTION

### 5.4.1 Culture

In subjects with clinical diagnoses of oral candidiasis we found mean *Candida* CFU of 368.88/ml and patients without any clinical signs of candidiasis 182.76/ml.

On average, the *Candida* load was at least twice as high in patients with clinical candidiasis as in those without. The mean values in this study were far lower than reported by Torres et al., 2003, who demonstrated that patients with a clinical diagnosis of oral candidiasis had mean *Candida* CFU of 10 100/ml.[19] However, the oral rinse technique was not used in their study and stimulated whole saliva was collected.

The mean value of *Candida* CFU obtained in patients with clinical candidiasis (368.88/ml) is slightly less than the 400/ml proposed to be the arbitrary cut-off point between health and disease.[48] Considering that 45.45% (15/33) of patients with median rhomboid glossitis, had negative *Candida* cultures, such an arbitrary cut-off point might need to be questioned for use in diagnosing patients with median rhomboid glossitis.

This also confirms that candidal counts from carriers of *Candida* and from individuals showing *Candida* infection show overlap, and that the isolation of *Candida* cannot be regarded as evidence of infection but should rather be considered relative to the clinical findings.[25]

### 5.4.2 Cytology

The prevalence of median rhomboid glossitis is greater among diabetic patients than in the general population.[2,35] Median rhomboid glossitis had a substantially higher prevalence in diabetic subjects this study (37%) than has been previously reported (1.8-24%).[2,35,60]

The aetiology of median rhomboid glossitis was formerly attributed to a developmental abnormality in which the tuberculum impar persists as a superficial structure. This theory has fallen away, and it is now generally accepted that a localized *Candida* infection is to blame.[61]

From the 29 patients with median rhomboid glossitis from whom smears were taken, 65.5% of the tongue smears could demonstrate the presence of *Candida* (n=19). Of the 19 smears, 6 demonstrated the presence of hyphae, 1 the presence of hyphae and spores, and 12 the presence of spores only. While the identification of *Candida* is in agreement with other studies,[35,60] the study by Guggenheimer et al., 2000, focused solely on the identification of *Candida* pseudohyphae.[35]

This challenges the concept that blastospores are associated with healthy individuals who are carriers of *Candida*.[18] Instead, in this study; blastospores were also associated with the clinical presentation of median rhomboid glossitis.

The low prevalence of *Candida* hyphae in patients with clinically diagnosed *Candida* infection is surprising, considering that this is the form most commonly associated with clinical infection.[9,46,59]

Median rhomboid glossitis has been associated with smoking and with the use of dentures in a type I DM population, and could be correlated with the diabetic complications of nephropathy and retinopathy.[35]

*Candida* may either commensally colonise or truly infect the oral cavity. The distinction between these two entities is not clear, but the number of CFU in saliva (> 400/ml)[48] or the demonstration of hyphae trough cytological smears has been suggested as diagnostic criteria.[59] Both these criteria lacked diagnostic accuracy in this study. We propose that the term 'infection' be reserved for patients who present with clinical signs and symptoms of Candidiasis.

## 5.5 ASSOCIATION BETWEEN IDENTIFICATION OF *CANDIDA* AND THE USE OF XEROSTOMIA INDUCING DRUGS

No association could be demonstrated between the use of drugs with xerostomia as a side effect, and the clinical or laboratory identification of *Candida*. This is in agreement with Guggenheimer et al., 2000, who also pointed out that the use of these drugs is more common among diabetic patients.[35]

Amitriptyline is known for its xerostomia inducing effects due to its anticholinergic activity. Thirty-three (37%) patients in our study were on amitriptyline but none reported any subjective sensation of dryness of the mouth.

Xerostomia is a known risk factor for candidiasis, with *Candida* isolated from 30-70% of patients with xerostomia caused by systemic diseases or medication.[62] Decreased salivary gland function has been reported to be associated with type I DM.[53] However, Guggenheimer et al., 2000, could not demonstrate more dry mouth symptoms or a decrease in salivary flow rates in their diabetic study population.[35]

## CHAPTER 6: CONCLUSION

In this study, no relationship was found between oral *Candida* colonisation and blood glucose levels in a diabetic population.

Cytology proved to be a more accurate tool for the detection of intra-oral *Candida* than culturing.

Median rhomboid glossitis was the most common clinical presentation of oral candidiasis. However, even in cases with clinically evident candidiasis, *Candida* could not always be identified.

The use of drugs with xerostomia as a side effect did not further predispose diabetic patients to oral *Candida* carriage or infection.

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# CHAPTER 8: ADDENDA

## 8.1 MEASURES OF GLYCAEMIC CONTROL

**Measures of glycaemic control**

| Patient Number | mmol/l Blood glucose | % HbA1 | |
|---|---|---|---|
| 1 | 4.8 | 9.7 | moderate |
| 2 | 10.2 | 7.9 | good |
| 3 | 9.3 | 6.2 | good |
| 4 | 8.1 | 6.1 | good |
| 5 | 8 | 9.6 | moderate |
| 6 | 20.1 | 11.5 | poor |
| 7 | 4.8 | | |
| 8 | 3.9 | 5.7 | good |
| 9 | 18.7 | 9.4 | moderate |
| 10 | 10.5 | 7.3 | good |
| 11 | 16.8 | 6.3 | good |
| 12 | 11.8 | 9.1 | moderate |
| 13 | 10.3 | 7 | moderate |
| 14 | 4.4 | 12 | poor |
| 15 | 11 | 12.3 | poor |
| 16 | 8 | 10.9 | poor |
| 17 | 3.7 | 8.6 | moderate |
| 18 | 8.8 | 7.4 | good |
| 19 | 17 | 8.5 | moderate |
| 20 | 12.2 | 11.4 | poor |
| 21 | 7 | 6.1 | good |
| 22 | 8.2 | 9.2 | moderate |
| 23 | 9.7 | 14.7 | poor |
| 24 | 5.6 | 5.6 | good |
| 25 | 5.3 | 6.2 | good |
| 26 | 6.5 | 9.9 | moderate |
| 27 | 7.8 | 10 | moderate |
| 28 | 18.1 | 8.2 | moderate |
| 29 | 10.2 | 5.6 | good |
| 30 | 8.7 | 5.9 | good |
| 31 | 7 | 8.6 | moderate |
| 32 | 22.7 | 9.3 | moderate |
| 33 | 5.7 | 5.8 | good |
| 34 | 7.1 | 6.9 | good |
| 35 | 7.4 | 6.2 | good |
| 36 | 8.4 | | |
| 37 | 5.3 | 7.9 | good |

| | | | |
|---|---|---|---|
| 38 | 4.8 | 7.3 | good |
| 39 | 16.1 | 13.5 | poor |
| 40 | 10.3 | 9.3 | moderate |
| 41 | 13.5 | 12.1 | poor |
| 42 | 14.8 | 11.2 | poor |
| 43 | 5.2 | 9.7 | moderate |
| 44 | 8.2 | 9.5 | moderate |
| 45 | 12.4 | 7.8 | good |
| 46 | 6.5 | 7.8 | good |
| 47 | 4.3 | 7.8 | good |
| 48 | 8.9 | | |
| 49 | 10.3 | 9.2 | moderate |
| 50 | 13.2 | 9.6 | moderate |
| 51 | 6.6 | 5.5 | good |
| 52 | 11.7 | 9.6 | moderate |
| 53 | 13.4 | 5.9 | good |
| 54 | 7.4 | 5.9 | good |
| 55 | 5 | 9.9 | moderate |
| 56 | 13.8 | 7.6 | good |
| 57 | 11.9 | 8.8 | moderate |
| 58 | 16.5 | 9.5 | moderate |
| 59 | 4.2 | 5.8 | good |
| 60 | 5.8 | 7.5 | good |
| 61 | 15.8 | 11.8 | poor |
| 62 | 3 | 9.2 | moderate |
| 63 | 12.6 | 11.6 | poor |
| 64 | 20.8 | 7.4 | good |
| 65 | 17.9 | 12.9 | poor |
| 66 | 5.9 | 6.5 | good |
| 67 | 7.1 | 7.6 | good |
| 68 | 6.7 | 9.2 | moderate |
| 69 | 24.2 | 9.4 | moderate |
| 70 | 14.8 | 10.6 | poor |
| 71 | 5.9 | 8.2 | moderate |
| 72 | 7.8 | 7.1 | good |
| 73 | 8.9 | 10.6 | poor |
| 74 | 15.3 | 11.2 | poor |
| 75 | 3.4 | 10.7 | poor |
| 76 | 5.3 | 5.4 | good |
| 77 | 13.7 | 10.3 | poor |
| 78 | 12.4 | 8.8 | moderate |
| 79 | 16.3 | 10.6 | poor |
| 80 | 12 | 11.6 | poor |

| | | | |
|---|---|---|---|
| 81 | 20.2 | 10 | moderate |
| 82 | 4.7 | 8 | moderate |
| 83 | 6.2 | 7.9 | good |
| 84 | 15.6 | 11.5 | poor |
| 85 | 9.4 | 5.9 | good |
| 86 | 30.2 | 13.6 | poor |
| 87 | 13.7 | 9 | moderate |
| 88 | 8.2 | 7.5 | good |
| 89 | 5.5 | 5.8 | good |

## 8.2 CYTOLOGY RESULTS

**Cytological results of smears**

| Patient | Tongue | | | Palate | | | Buccal R | | |
|---|---|---|---|---|---|---|---|---|---|
| Number | Y/N | Hyphae | Yeast cell | Y/N | Hyphae | Yeast cell | Y/N | Hyphae | Yeast cell |
| 9 | N | | | N | | | N | | |
| 10 | Y | | Y | Y | | Y | Y | | Y |
| 11 | Y | Y | | Y | Y | | Y | Y | Y |
| 12 | N | | | Y | | Y | N | | |
| 13 | Y | | Y | N | | | Y | | Y |
| 14 | y | y | | N | | | N | | |
| 15 | N | | | N | | | N | | |
| 16 | N | | | N | | | N | | |
| 17 | N | | | N | | | N | | |
| 18 | Y | | Y | N | | | N | | |
| 19 | N | | | N | | | N | | |
| 20 | Y | | Y | Y | | Y | Y | | Y |
| 21 | Y | | Y | N | | | N | | |
| 22 | N | | | N | | | N | | |
| 23 | N | | | N | | | N | | |
| 24 | Y | | Y | Y | | Y | Y | | Y |
| 25 | Y | | Y | N | | | N | | |
| 26 | N | | | N | | | N | | |
| 27 | Y | | Y | Y | | Y | N | | |
| 28 | Y | | Y | Y | | Y | N | | |
| 29 | N | | | N | | | N | | |
| 30 | N | | | N | | | N | | |
| 31 | N | | | N | | | N | | |
| 32 | N | | | N | | | N | | |
| 33 | Y | | Y | N | | | Y | | Y |
| 34 | N | | | N | | | N | | |
| 35 | N | | | N | | | N | | |
| 36 | N | | | Y | Y | | Y | Y | Y |
| 37 | N | | | N | | | N | | |
| 38 | Y | Y | | N | | | Y | Y | |
| 39 | Y | | | N | | | N | | |
| 40 | Y | Y | | N | | | N | | |
| 41 | Y | | Y | N | | | Y | | Y |
| 42 | N | | | N | | | N | | |
| 43 | N | | | N | | | Y | Y | |
| 44 | Y | Y | Y | Y | | Y | Y | | Y |
| 45 | Y | Y | Y | N | | | N | | |
| 46 | Y | | Y | N | | | N | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 47 | Y | | Y | Y | | Y | Y | Y | Y |
| 48 | Y | Y | Y | Y | Y | | Y | Y | |
| 49 | Y | | Y | N | | | N | | |
| 50 | N | | | N | | | N | | |
| 51 | Y | Y | | Y | Y | | Y | Y | |
| 52 | N | | | Y | Y | | Y | Y | |
| 53 | N | | | N | | | Y | | Y |
| 54 | Y | | Y | N | | | N | | |
| 55 | Y | | Y | N | | | N | | |
| 56 | N | | | N | | | N | | |
| 57 | N | | | N | | | N | | |
| 58 | Y | Y | | Y | Y | | Y | Y | |
| 59 | Y | Y | | N | | | N | | |
| 60 | Y | Y | | N | | | Y | Y | |
| 61 | Y | Y | | N | | | N | | |
| 62 | Y | | Y | Y | Y | Y | Y | Y | |
| 63 | Y | Y | | N | | | Y | Y | |
| 64 | Y | Y | Y | Y | | Y | N | | |
| 65 | Y | | Y | Y | | Y | N | | |
| 66 | Y | | Y | N | | | N | | |
| 67 | Y | | Y | Y | | Y | Y | | Y |
| 68 | Y | Y | Y | N | | | N | | |
| 69 | N | | | N | | | Y | Y | Y |
| 70 | N | | | N | | | N | | |
| 71 | Y | Y | Y | Y | Y | | N | | |
| 72 | N | | | N | | | N | | |
| 73 | Y | Y | Y | N | | | Y | Y | |
| 74 | N | | | N | | | N | | |
| 75 | Y | | Y | N | | | Y | Y | |
| 76 | N | | | N | | | N | | |
| 77 | N | | | N | | | N | | |
| 78 | Y | Y | | N | | | N | | |
| 79 | N | | | N | | | N | | |
| 80 | Y | Y | Y | N | | | N | | |
| 81 | Y | Y | | N | | | N | | |
| 82 | N | | | Y | Y | Y | Y | | Y |
| 83 | Y | | Y | N | | | N | | |
| 84 | Y | Y | Y | Y | | Y | Y | Y | Y |
| 85 | N | | | N | | | N | | |
| 86 | N | | | N | | | N | | |
| 87 | Y | | Y | Y | | Y | N | | |
| 88 | Y | | Y | Y | | Y | Y | | Y |
| 89 | N | | | N | | | N | | |

## 8.3 RESULTS OF *CANDIDA* CULTURE AND PRESENCE OF CANDIDIASIS

**Results of *Candida* culture and presence of candidiasis**

| Patient Number | Colony forming units of *Candida* | Clinical presence of Candidiasis |
|---|---|---|
| 1 | 0 | Absent |
| 2 | 92 | Median rhomboid glossitis |
| 3 | 0 | Median rhomboid glossitis |
| 4 | 0 | Absent |
| 5 | 52 | Absent |
| 6 | 0 | Erythematous candidiasis |
| 7 | 0 | Median rhomboid glossitis |
| 8 | 0 | Median rhomboid glossitis |
| 9 | 0 | Median rhomboid glossitis |
| 10 | 0 | Absent |
| 11 | 1410 | Median rhomboid glossitis, erythematous candidiasis |
| 12 | 0 | Absent |
| 13 | 0 | Absent |
| 14 | 10 | Median rhomboid glossitis |
| 15 | 1130 | Median rhomboid glossitis |
| 16 | 20 | Absent |
| 17 | 0 | Absent |
| 18 | 0 | Median rhomboid glossitis |
| 19 | 0 | Absent |
| 20 | 0 | Median rhomboid glossitis |
| 21 | 0 | Absent |
| 22 | 0 | Absent |
| 23 | 30 | Median rhomboid glossitis |
| 24 | 0 | Median rhomboid glossitis |
| 25 | 630 | Median rhomboid glossitis |
| 26 | 0 | Absent |
| 27 | 1410 | Median rhomboid glossitis |
| 28 | 70 | Median rhomboid glossitis |
| 29 | 0 | Absent |
| 30 | 10 | Absent |
| 31 | 0 | Absent |
| 32 | 0 | Absent |
| 33 | 260 | Median rhomboid glossitis |
| 34 | 0 | Median rhomboid glossitis |
| 35 | 0 | Median rhomboid glossitis |
| 36 | 3940 | Median rhomboid glossitis |

| | | |
|---|---|---|
| 37 | 30 | Absent |
| 38 | 340 | Median rhomboid glossitis |
| 39 | 0 | Absent |
| 40 | 0 | Median rhomboid glossitis |
| 41 | 150 | Absent |
| 42 | 0 | Absent |
| 43 | 0 | Absent |
| 44 | 530 | Absent |
| 45 | 370 | Absent |
| 46 | 0 | Absent |
| 47 | 0 | Absent |
| 48 | 30 | Absent |
| 49 | 10 | Median rhomboid glossitis |
| 50 | 0 | Median rhomboid glossitis |
| 51 | 240 | Absent |
| 52 | 40 | Absent |
| 53 | 0 | Absent |
| 54 | 190 | Absent |
| 55 | 0 | Median rhomboid glossitis |
| 56 | 0 | Median rhomboid glossitis |
| 57 | 630 | Absent |
| 58 | 4000 | Absent |
| 59 | 0 | Median rhomboid glossitis |
| 60 | 970 | Median rhomboid glossitis |
| 61 | 20 | Absent |
| 62 | 1270 | Median rhomboid glossitis |
| 63 | 680 | Absent |
| 64 | 170 | Absent |
| 65 | 0 | Absent |
| 66 | 0 | Absent |
| 67 | 30 | Median rhomboid glossitis |
| 68 | 10 | Absent |
| 69 | 320 | Absent |
| 70 | 0 | Absent |
| 71 | 460 | Median rhomboid glossitis, erythematous candidiasis |
| 72 | 0 | Absent |
| 73 | 220 | Absent |
| 74 | 0 | Median rhomboid glossitis |
| 75 | 250 | Absent |
| 76 | 230 | Absent |
| 77 | 0 | Absent |
| 78 | 10 | Absent |

| | | |
|---|---|---|
| 79 | 230 | Absent |
| 80 | 0 | Absent |
| 81 | 0 | Absent |
| 82 | 230 | Median rhomboid glossitis |
| 83 | 250 | Median rhomboid glossitis |
| 84 | 450 | Absent |
| 85 | 0 | Absent |
| 86 | 0 | Absent |
| 87 | 580 | Absent |
| 88 | 590 | Absent |
| 89 | 0 | Absent |

## 8.4 XEROSTOMIA INDUCING DRUGS TAKEN BY THE STUDY POPULATION

**Xerostomia inducing drugs taken by study population**

| Patient number | Amitriptyline | Carbamazepine | Fluoxetin | Methyldopa | Omeprazole | Phenergan |
|---|---|---|---|---|---|---|
| 1 | | | | | | |
| 2 | X | X | | | | |
| 3 | x | | | | | |
| 4 | | | | x | | |
| 5 | | | | | | |
| 6 | x | | | | | |
| 7 | | | | | | |
| 8 | | | | | | |
| 9 | | | | | | |
| 10 | | | | | | |
| 11 | | | | | | |
| 12 | x | x | | | | |
| 13 | | | | | | |
| 14 | | | | | | |
| 15 | x | x | | x | | |
| 16 | x | x | | | | |
| 17 | | | | | | |
| 18 | | | | | | |
| 19 | x | x | x | | | |
| 20 | | | | x | | |
| 21 | | x | | | | |
| 22 | | | | | | |
| 23 | | | | | | |
| 24 | | | | | | |
| 25 | | | | | | |
| 26 | | | | | | |
| 27 | | | | x | | |
| 28 | x | | | | | |
| 29 | | | | | | |
| 30 | | | | | | |
| 31 | x | | | x | | |
| 32 | | | | | | |
| 33 | | | | | | |
| 34 | | | | x | | |
| 35 | | | | | | |
| 36 | x | x | | | | |
| 37 | | | | x | | |
| 38 | x | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 39 | x | | | x | | |
| 40 | x | x | | | | |
| 41 | | | | | | |
| 42 | | | | | | |
| 43 | | | | | | |
| 44 | x | | | | | |
| 45 | x | | | x | | |
| 46 | | | | | | |
| 47 | x | | | | | |
| 48 | | | | | | |
| 49 | | | | | | x |
| 50 | x | | | | | |
| 51 | | | | | | |
| 52 | x | x | | | | |
| 53 | x | | | | | |
| 54 | | | | | | |
| 55 | | | | x | | |
| 56 | | | | | | |
| 57 | | | | | | |
| 58 | x | | | | | |
| 59 | | | | | | |
| 60 | x | | | x | | |
| 61 | x | | | | | |
| 62 | | | | | | |
| 63 | x | | | | | |
| 64 | | | | | | |
| 65 | x | | | | | |
| 66 | | | | x | x | |
| 67 | x | x | | | | |
| 68 | x | x | | | | |
| 69 | | | | | | |
| 70 | | | | x | | |
| 71 | | | | | | |
| 72 | | | | | | |
| 73 | | | | | | |
| 74 | | | | | | |
| 75 | | | | | | |
| 76 | | | | x | | |
| 77 | x | | | x | | |
| 78 | | | | | | |
| 79 | | | | | | |
| 80 | | | | | | |
| 81 | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 82 | x | | | | | |
| 83 | | | | | | |
| 84 | x | | | | | |
| 85 | | | | | | |
| 86 | x | | | x | | |
| 87 | x | | | | | |
| 88 | x | | | | | |
| 89 | x | | | | | |