Original Research
Periodontics

Detection and sampling methods for isolation of *Candida* spp. from oral cavities in diabetics and non-diabetics

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Abstract: The purpose of this study was to detect *Candida* spp. on the tongue and in the subgingival sites in healthy and type 2 diabetes (T2D) patients with chronic periodontitis (CP), and to compare the accuracy of sampling methods. This study included 131 patients divided into four groups: healthy control (group A), nondiabetics + CP (Group B), diabetics with good metabolic control + CP (group C) and diabetics with poor glycoregulation + CP (Group D). Cotton swab samples from tongue and subgingival samples were obtained from each patient with help of sterile paper points and a sterile curette. Swab cultures were made on Sabouraud dextrose agar. The number of CFUs was counted. The sampling methods for subgingival plaque were compared by Receiving Operator Curve (ROC). The presence of *Candida* spp. on the tongue was statistically significant among groups (group D *vs*. others three groups: χ^2: $p < 0.005$ for each group). Positive findings of subgingival *Candida* spp. did not differ among the groups. There were no significant differences in the quantification of *Candida* spp., neither on the tongue, nor in the subgingival samples. 17.2% of diabetic patients revealed the presence of *Candida* spp. in the subgingival samples, with negative finding on tongue. There was a significant difference in the sampling methods for subgingival plaque ($p = 0.000$). *Candida* spp. is more prevalent on the tongue of diabetics. The sampling of subgingival plaque by a sterile curette is more accurate than with paper points. Subgingival plaque may represent a reservoir of commensals. It is necessary to standardize the sampling of subgingival plaque.

Keywords: Diabetes Mellitus, Type 2; Chronic Periodontitis; Candida; Periodontal Pocket.

Introduction

Type 2 diabetes mellitus (T2D) is a chronic metabolic disorder that leads to progressive defects in insulin secretion based on insulin resistance.[1] For thirty years periodontitis has been acknowledged as the sixth chronic complication of diabetes.[2,3] *Candida* infections are chronic opportunistic infections related to diabetic patients. The presence of *Candida* spp. in oral cavities of diabetics varies between 50-80%.[4,5,6,7] Yeasts commonly inhabit tongue, palate and buccal mucosa, and it has recently been found in the subgingival sites.[8] The periodontium may represent a reservoir of opportunistic microorganism, especially in *immunocompromised*

patients.[9] The presence of yeasts in subgingival sites was examined in relationship to general health and periodontal status. It varies between 30-50% in the case of diabetics.[4,7,10] Yeasts[11] and viruses[12] could have a significant role in the pathogenesis of periodontal diseases. The immunological response around hyphae of *Candida* spp. is similar to the response to periopathogens of bacterial origin, and consists of chronic mononuclear inflammatory cells with sporadic neutrophil leucocytes.[11] The potential role of yeasts in the pathogenesis of periodontitis is especially important for diabetic patients, because antibiotics are commonly used in the treatment of periodontitis.

The purpose of this study was to detect *Candida* spp. on the tongue and in subgingival sites in healthy and T2D patients with chronic periodontitis (CP), and to compare the accuracy of sampling methods.

Methodology

Subjects, Ethical approval

This cross-sectional study was approved by the Ethical Committee of the School of Dentistry, University of Belgrade (Ethics Approval no. 36/8). It included 131 patients divided into four groups. Group A (n = 35) consisted of healthy volunteers without clinical signs of CP. Group B (n = 30) consisted of healthy subjects diagnosed with CP. Group C (n = 26) included T2D patients with good glycoregulation and diagnosed CP and group D (n = 40) consisted of T2D patients with poor metabolic control and diagnosed CP.

Inclusion and exclusion criteria

The exclusion criteria were: presence of any disease except T2D, aggressive periodontitis, usage of medication that might affect the periodontium, *e.g.* corticosteroids, antibiotics, antiseptics, history of oral candidiosis treatment, pregnancy, lactation and periodontal treatment in the last 1.5 year.

Anamnesis data and biochemical/hematological analysis

Self-reported information about blood type, everyday intake of sweets and smoking habits were recorded. According to the blood type, patients were divided into O *vs.* A+B+AB blood type.[13] Patients were classified according to their smoking status as "non-smokers" and "smokers". Fasting plasma glucose levels (FPG), glycated hemoglobin (HbA_1c), hematological parameters (RBC, Hgb, HCT, MCV, MCH, MCHC, RDW) and sedimentation rate were measured.

Diagnosis of CP and T2D

T2D was diagnosed according to the criteria of the American Diabetes Association[1] by measuring glycaemia during 2 h 75 g oral glucose tolerance test (OGTT), as well as HbA_1c values. Non-diabetics exhibited normal parameters on OGTT and $HbA_1c < 6.5\%$. Glycoregulation was classified, according to HbA_1c, as satisfactory ($HbA_1c \leq 7.5\%$) and as with poor metabolic control ($HbA_1c > 7.5\%$).

Full mouth clinical examinations were performed at six sites per tooth and evaluated on each tooth in order to access periodontal parameters: plaque index-Silness Loe (PI), dichotomous bleeding on probing (BOP), probing pocket depth (PPD) and clinical attachment loss (CAL). Two calibrated doctors performed examinations using periodontal probe (XP 23/UNC-15, Hu-Friedy, Chicago, USA). Periodontitis was diagnosed if subject exhibited CAL > 1 mm and PPD > 3 mm at least at three sites in two quadrants.[14,15] Patients without clinical signs of periodontitis exhibited PPD < 3 mm and CAL = 0 mm.

Sample collection and cultivation

Samples were collected a day after periodontal examination. Oral swabs were collected by swabbing ten times from the dorsum of the tongue with the help of a dry sterile cotton stick. Swab cultures were immediately inoculated on Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, UK). The tooth with deepest PPD was isolated by means of sterile cotton rolls and the supragingival plaque was removed by using sterile gauze and a curette. Two sterile paper points with a size of 30 were placed into the pocket for 30s until a mild resistance appeaed. Paper points contaminated by blood were not included in the analysis. Subgingival samples were obtained from the same pocket by means of a sterile curette (S4L/4R SS G.Hartzell&Son, Concord, California). Both subgingival samples were

inoculated in sterile plastic tubes containing 1 mL of Sabouraud dextrose broth (Oxoid, Basingstoke, UK). The plastic tubes were vortexed for 1 min and 20ml of suspended broth was streaked on SDA. Samples were inoculated at 37°C for 48 h. The cultural and microscopic qualities of the yeasts were examined, and the germ-tube production test, as well as the carbohydrate and potassium nitrate assimilation tests, were performed when needed. After incubation, one calibrated microbiologist counted the growth density and number of Colony Forming Units (CFUs). The yeast growth density from tongue samples was defined as rare, medium or dense. The number of CFUs was measured for samples taken by means of paper points. Depending on the CFU/ml there were defined three groups: 1: < 500, 2: 500 - 2500 and 3: > 2500.

Statistical analysis

Statistical analyses were carried out by using SPSS 18.0 software package for Windows (SPSS inc., Chicago, USA) and MedCalc for Windows, version 13.3.30 (MedCalc Software, Mariakerke, Belgium) for the Receiving Operators Curve (ROC) analysis.

Descriptive data were presented as Mean ± SD or the percentage for discrete measures. t-test and One Way ANOVA were used for normally distributed data. Non-parametric data were analyzed using by using the Kruskall-Wallis and Mann-Whitney test. Categorical variables were compared using the Chi Square Test (χ^2). The relationship between CFU and clinical parameters was determined by Spearman's correlation coefficient. The linear regression model was used to determine predictors of the presence of *Candida* spp. ROC analysis was carried out in order to compare sampling methods for subgingival plaque collection for isolation of *Candida* spp. Differences were considered significant when p-value was < 0.05.

Results

Demographic, clinical and biochemical data are presented in Table 1. Groups were matched by age, gender and smoking status.

Differences between groups C and D were observed for HbA1c (Mann-Whitney, p = 0.000) and FPG (Mann-Whitney, p = 0.000). Hematological parameters did not differ between groups. Groups were matched according to their blood type (χ^2, p = 0.541).

PI differed between groups (Table 2). BOP was different between group A and other groups (Bonferroni: B *vs.* C = B *vs.* D = C *vs.* D: p = 1.000). PPD did not differ significantly between groups B, C and D (Bonferroni: B *vs.* C = B *vs.* D = C *vs.* D, p = 1.000).

Positive finding of *Candida* spp. on tongue were found in 38/131 (27.3%) patients. The presence of *Candida* spp. on tongue was significantly higher in group D (χ^2: group A *vs.* D: p = 0.033, B *vs.* D: p = 0.007, C *vs.* D: p = 0.046) (Figure 1). When comparing *Candida* spp. findings on

Table 1. Demographic and biochemical data of patients.

	Group A	Group B	Group C	Group D	p-value
Age	43.57 ± 3.389	47.07 ± 10.869	48.31 ± 6.851	47.55 ± 7.527	[1]0.056
Sex (m/f)	14 (28.6%) / 21(71.4%)	14(40%) /16(60%)	14(53.8%) / 12(46.2%)	26(65%) /14(35%)	[2]0.164
Smokers N(%)/ Nonsmokers N(%)	8(22.9%) / 27(77.1%)	11(36.7%) / 19(63.3%)	8(30.8%) / 18(69.2%)	10(25.0%) / 30(75.0%)	[2]0.606
FPG (mmol/l)	4.65 ± .539	4.73 ± 0.624	7.49 ± 1.875	11.247 ± 4.061	[3]0.000
HbA1c (%)	4.81 ± 0.623	4.86 ± 0.635	7.09 ± 0.578	10.84 ± 1.366	[3]0.000
RBC (x10^{12})	4.53 ± .467	4.68 ± .703	4.58 ± .363	4.69 ± .601	[1]0.587
Hgb	137.50 ± 10.276	138.43 ± 12.263	138.88 ± 11.669	137.19 ± 14.731	[3]0.883
HCT (l/l)	0.41 ± .0538	0.43 ± .709	0.41 ± .032	0.42 ± .064	[3]0.423
MCV (fl)	91.52 ± 7.516	91.72 ± 7.755	89.22 ± 3.520	89.28 ± 10.486	[1]0.077
MCH (pg)	30.71 ± 2.243	30.30 ± 2.613	29.58 ± 3.667	28.98 ± 3.062	[3]0.060
MCHC (g/l)	333.88 ± 29.170	329.66 ± 28.846	339.01 ± 14.495	327.49 ± 22.803	[1]0.196
RDW(%)	14.26 ± 1.501	14.28 ± 1.64	13.79 ± 1.546	14.68 ± 1.789	[1]0.170

All values are presented as Mean ± SD.
[1]One Way ANOVA; [2]Pearson Chi Square Test; [3]Kruskal-Wallis; [4]Independent Sample t-test.

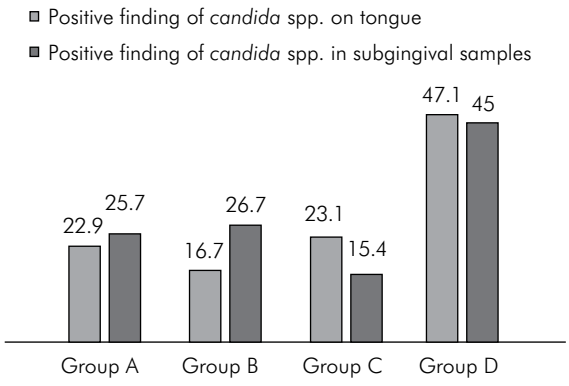

Figure 1. Presence of *Candida* spp. on tongue and in subgingival samples.

tongue of non-diabetics (20.3%) and diabetics (37.9%), a statistical difference was observed (χ^2, p = 0.028).

The quantification of *Candida* spp. on the tongue did not differ between groups. The univariate logistic regression model was applied, in order to identify parameters that could predict positive finding of *Candida* spp. on tongue. Age, gender, blood type, everyday intake of sugars, smoking habits, number of teeth, diabetes duration, treatment mode, FPG level, HbA1c, RBC, Hgb, MCV, HCT, MCH, MCHC and RDW were analyzed as potential predictors. There was found no predictor for the positive finding of yeasts on tongue.

The subgingival findings of *Candida* spp. were positive in 41/131 (29.50%). There was no difference in the presence of yeast in subgingival sites between groups (χ^2, p = 0.060) (Figure 1).

There was no relation between presence of subgingival *Candida* spp. and clinical periodontal parameters. The quantification of subgingival *Candida* spp. was not different between groups.

In the case of diabetic patients, there was a positive correlation between the presence of subgingival *Candida* spp. and HbA1c (Spearman correlation coefficient, r = 0.276, p = 0.025).

Logistic regression analysis did not identify any parameter that could predict the presence of *Candida* spp. in subgingival samples.

18/131 (12.9%) patients presented negative *Candida* spp. findings on the tongue and positive findings in subgingival samples.

There was a statistical difference regarding sampling methods for subgingival plaque collection and yeast detection (Table 3). The ROC curve was used to compare the diagnostic techniques of both collecting methods. The referent sampling method was by means of a sterile curette. There was a difference between methods (p = 0.000). Sensitivity was 0.576 and specificity was 0.919. The area under the curve was 0.747. Asymptomatic 95% Confidence Interval was 0.638-0.857 (Figure 2).

Discussion

The proposed microbiological etiologies of the periodontal disease have been changing for decades. There is increasing evidence about the involvement of microorganisms other than bacteria (*e.g.* viruses[11] and yeasts[8,12]) in the pathogenesis of periodontal disease.

Candida spp. is a common oral saprophyte. Yeast may form biofilm, which is an essential strategy for their survival in oral milieu.[16,15] Beside biofilm formation, this genus is able to produce exoenzimes, proteinases and metabolites in order to adhere to epithelial cells and inhibit the function of polymorphonuclears.[17,18,19] It can be isolated in about 50% of healthy population without clinical signs of infection.[13] In the case of diabetics, this prevalence is higher.

The prevalence and quantification of *Candida* spp. on tongue and subgingival samples were examined in diabetic and non-diabetic patients. To the best of our knowledge, this is the first study that examined patients with a clinically healthy periodontium, subjects diagnosed with periodontitis and diabetics. In our study, the overall prevalence of *Candida* on the tongue was 27.3%. The most frequent finding was in a group of poorly controlled diabetics. Differences in the prevalence of *Candida* spp. on tongue in the case of diabetic patients is in accordance with other studies,[6,20,21] but the percentage (37.9%) was lower compared to other studies, where findings of *Candida* on tongue varied between 59-77%.[4,5,7] The quantification of yeast growth on tongue did not differ between groups, which is contrary to other studies[6,22] probably because denture wearers were included and sampling methods were different.

Candida spp. was a commonly occurring microorganism in samples from subgingival sites.

Table 2. Clinical periodontal parameters.

Clinical parameter	Group A	Group B	Group C	Group D	p-value
PI	0.82 ± 0.423	1.72 ± 0.767	2.65 ± 0.458	2.23 ± 2.32	[1]0.000
BOP (%)	39.61 ± 19.273	62.057 ± 24.288	64.24 ± 24.043	64.41 ± 28.86	[2]0.000
PPD (mm)	2.02 ± 0.524	2.89 ± 0.944	2.85 ± 0.932	2.69 ± 0.756	[2]0.000
CAL (mm)	0	3.56 ± 2.142	3.98 ± 1.947	4.12 ± 2.104	[1]0.661

All values are presented as Mean ± SD.
[1]Kruskal-Wallis Test; [2]One Way ANOVA.

Table 3. Comparison of sampling methods for subgingival plaque collection.

Paper point method	Sterile curette method		Total	p-value
	Negative finding	Positive finding		
Negative finding	90 (68.7%)	14 (10.7%)	104 (79.4%)	[1]p < 0.000*
Positive finding	8 (6.1%)	19 (14.5%)	27 (20.6%)	
Total	98 (74.8%)	33 (25.2%)	131 (100%)	

All values are presented as N (%).
[1]Pearson Chi Square Test.

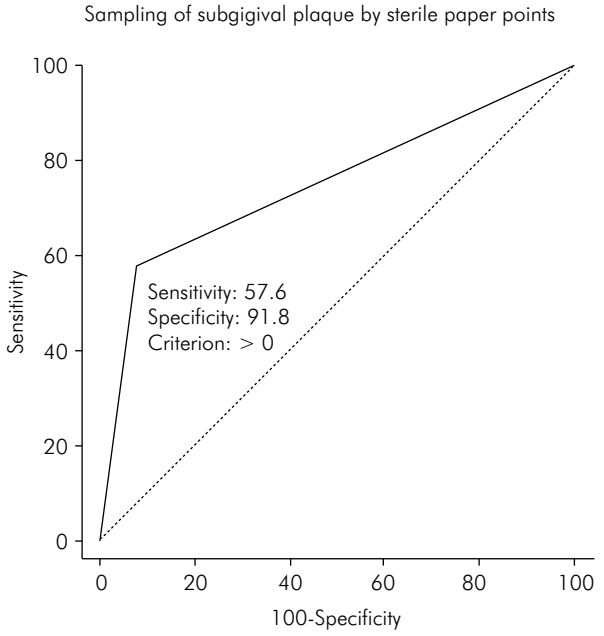

Figure 2. ROC curve for sampling methods of subgingival plaque by sterile curette and by sterile paper points.

Statistical differences of yast presence were not observed between groups or between diabetics *vs.* non-diabetics. Sardi *et al.*[20] found differences in subgingival findings of yeasts between well-controlled insulin dependent T2D patients and control group of healthy CP patients. When comparing the prevalence of subgingival positive findings in good *vs.* poorly controlled diabetics, Melton *et al.*[4] found no significant differences. Studies examining the prevalence of subgingival yeasts in healthy patients according to their periodontal status, demonstrated the impact of the periodontal probing depth on the presence of subgingival yeasts[8] which was not the case in our study. *Candida* spp. is an opportunistic pathogen, and it is considered as marker of immunocompromised patients. Periodontitis itself has been recognized as a state of disturbed cellular and humoral immune local response[23] and patients with diagnosed diabetes are also considered immunocompromised. Our results, which show a similar prevalence of subgingival yeasts in healthy patients and healthy patients with diagnosed CP, are in contrast with these facts. We examined only the presence of yeasts which does not always lead to clinical infection. Some authors indicate that the presence of yeasts in subgingival sites is transient.[24] The reaction of host immunity around yeasts was not a subject of investigation or the exact species of *Candida* genus. *Candida* spp. is capable of adhering to epithelial cells and inducing inflammation.[25]

There is an increasing number of studies investigating subgingival prevalence of *Candida* spp. Some studies used a sterile curette as sampling method,[7,24] while others used sterile paper points.[8,9] To the best of our knowledge, there is no explanation in any of these studies about the choice of the sampling method. In an attempt to answer this question, we used both methods in the same pocket and ROC analysis was carried out. A sterile

curette method was defined as the "golden standard". Specificity, which represents true negative results, was excellent (0.919) but sensitivity, which represents the probability that test results will be positive when the disease is present, was 0.576. The area under the curve shows a fair accuracy of the test. According to this ROC analysis, it may be concluded that subgingival plaque sampling by means of a sterile curette is more accurate than sampling by means of sterile paper points. This is in agreement with the fact that *Candida* spp. forms its colonies on the surface of the epithelial cells[26], *i.e.* it is necessary to "scratch" with the help of a curette in order to ensure the accuracy of the results. On the other hand, sampling by sterile paper points is more appropriate if it is necessary to quantify *Candida* spp. Sampling with paper point can be standardized in terms of paper point size, duration of the presence of the paper point in the pocket and paper point pressure in the pocket. In different studies, there are differences in paper point size and insertion duration when sampling the subgingival plaque. Considering that numerous studies use paper points as sampling method, methodology should be standardized in order to compare results.

It has already been proven that subgingival sites may be a reservoir of *Candida* spp.[9] In our study 12.9% of all patients harbored yeast in subgingival sites with no presence on tongue. The subgingival area is beneficial for *Candida* growth.[16]

The detection of yeast prevalence and their quantification, as well as the recognition of different species, virulence factors and drug resistance in subgingival biofilm are important because of the emerging usage of antibiotics as adjuvant periodontitis therapy. The usage of a broad spectrum antibiotics may lead to *Candida* opportunistic infections and periodontal destruction. Some authors indicate that the presence of yeasts in the subgingival area is transient,[24] *i.e.* the study to be carried out should be rather longitudinal than cross-sectional.

Conclusions

Candida spp. is more prevalent on tongue in the case of diabetics than in the healthy control group, regardless of periodontal status. In addition to that, diabetics with poor glycoregulation exhibited more yeast than patients with good metabolic control. The subgingival area may represent reservoir of commensals. However, longitudinal studies are needed to confirm these results. Correspondingly, it is necessary to standardize sampling methods for the collection of subgingival plaque.

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