Quantity of *Candida* Colonies in Saliva: A Diagnostic Evaluation for Oral Candidiasis

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Objective: *To investigate the relationship between the quantity of Candida colonies in saliva and oral candidiasis (OC), as well as to identify the threshold for distinguishing oral candidiasis from healthy carriage.*
Methods: *A diagnostic test was conducted in 197 patients with different oral problems. The diagnosis of OC was established based on clinical features. Whole saliva samples from the subjects were cultured for Candida species. Receiver operating characteristic (ROC) curve analysis was used in this study.*
Results: *OC patients had significantly more Candida colony-forming units per millilitre saliva (795 cfu/ml) than asymptomatic carriers (40 cfu/ml; $P < 0.05$). Among different types of candidiasis, the quantity of Candida colonies differed. The number of Candida colonies in pseudomembranous type was significantly higher than that in the erythematous type ($P < 0.05$). Candida albicans was the predominant species of Candida. The cut-off point with the best fit for OC diagnosis was calculated to be 266 cfu/ml. The sensitivity and specificity were 0.720 and 0.825, respectively. Analysis of the ROC curve indicated that Candida colonies had a high diagnostic value for OC, as demonstrated by the area under the curve (AUC = 0.873).*
Conclusion: *Based on this study, the value of 270 cfu/ml was considered a threshold for distinguishing OC from carriage.*
Key words: *quantity, candidiasis, Candida, saliva, diagnosis*
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Oral candidiasis (OC), the most common opportunistic infection in the human oral cavity, results from the overgrowth and invasion of *Candida* spp in the superficial epithelium of the oral mucosa[1]. It is usually associated with disorders such as human immunodeficiency virus (HIV) infection, anaemia, diabetes mellitus, neutropenia, Sjögren syndrome, or autoimmune polyendocrine syndrome I (APS-I), etc. With the ageing of the population, the incidence of OC has shown a parallel increase. Patients with OC present with a dry mouth, a burning sensation and a bad taste or loss of taste discrimination. Severe *Candida* infection may cause life-threatening dissemination through the blood circulation and may involve visceral organs. In addition, *Candida* spp localised in the oral mucosa may increase the risk of malignant transformation of abnormal epithelium[2]. Therefore, the disease must be identified early and accurately, which would be beneficial for early and appropriate intervention of the underlying diseases, as well as for the prevention of infectious exacerbation.

Clinical features are fundamental for the diagnosis of oral candidiasis. However, OC often accompanies other oral mucosal diseases, which interferes in clinical differentiation. Therefore, microbiological examinations

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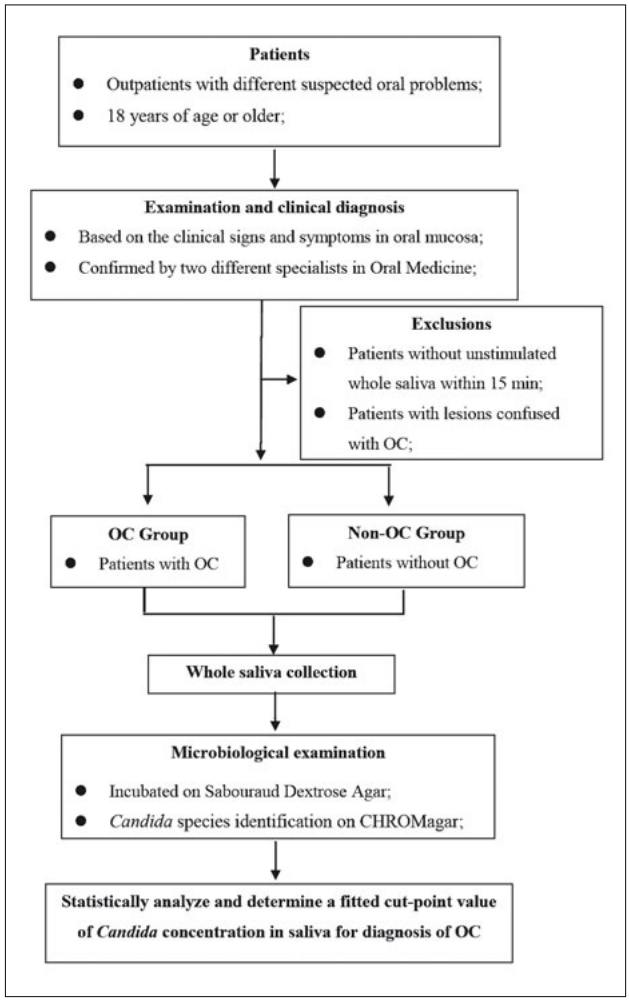

Fig 1 The flowchart of study design.

are necessary for confirming the diagnosis. The microbiological methods most frequently used are smear and salivary culture[3]. Salivary culture is more sensitive than smear for *Candida* detection. The reported sensitivity of smear is 51.6%[4], which is less than that of salivary culture. *Candida* spp at a low concentration of 200 to 500 cells per milliliter of saliva may be detected by salivary culture, but not by smear[5]. Of the asymptomatic healthy population, between 20% and 40% carry *Candida* in their saliva[6]. Therefore, it is necessary to identify a threshold amount of *Candida* spp to distinguish candidiasis from healthy carriage. A receiver operating characteristic (ROC) curve analysis was used in this study to identify the best-fitted cut-off value for the number of *Candida* colonies indicating OC.

Materials and methods

Experimental design

A diagnostic test was performed to explore a fitted value of the number of *Candida* colonies in saliva for diagnosing OC. The study was conducted as shown in Figure 1.

Study population

Between 2013 and 2015, adult patients who visited the Department of Oral Medicine, Peking University School and Hospital of Stomatology, were enrolled. Detailed information was taken, including age, gender, general

Table 1 Inclusion and exclusion criteria

Inclusion criteria
Outpatients with different suspected oral problems
18 years or older
Exclusion criteria
Patients without unstimulated whole saliva within 15 min
Patients who had taken antifungal agents within 1 month
Patients with atrophic and erosive oral lichen planus, intraoral discoid lupus erythematosus, erythema multiforme, oral erythema, pemphigus vulgaris, or mucous membrane pemphigoid
Patients with vitamin B12 deficiency

Table 2 Oral candidiasis types of the OC-group patients.

Type	Number	Percentage
Pseudomembranous candidiasis	8	8%
Erythematous candidiasis	69	69%
Chronic hyperplastic candidiasis	1	1%
Denture stomatitis	19	19%
Median rhomboid glossitis	3	3%
Total	100	

health and medical and family history. Based on the clinical signs and symptoms, the clinical diagnosis was established by two different specialists before patients were divided into two groups. Patients diagnosed with OC were classified as the OC-group; the others were considered the non-OC group. The oral carriage of *Candida* refers to the normal, symptom-free presence of *Candida* in the mouth without any clinically visible disease[7]. The inclusion and exclusion criteria are listed in Table 1.

This study was approved by the Institutional Review Board of Peking University School and Hospital of Stomatology (PKUSSIRB-201311090). Each participant provided informed consent form before enrolment.

Saliva collection and microbiologic procedures

Whole unstimulated saliva was collected from the subjects, who had consumed no solid food or liquids, or performed any oral hygiene procedures for at least 2 h before saliva collection. At least 1 ml of unstimulated whole saliva from each subject was collected into a sterilised falcon tube. The samples were stored on ice, and cultured within 1 h. Before culture, the samples were gradient diluted (1:1, 1:10, 1:100) with sterilised distilled water and mixed entirely using a vortex mixer. Then, 0.5 ml of the diluted sample was incubated on Sabouraud Dextrose Agar (BioMérieux, Shanghai, China) at 37°C for 48 h. The number of colony forming units (CFU) per millilitre of saliva was counted by visual inspection and confirmed by two researchers. For each sample, colonies of different morphology were smeared on to CHROMagar plates (Jin Zhang Technology, Tianjin, China) and incubated at 37°C for 48 h. The species identification of yeast isolations was determined according to the colours of the colonies growing on CHROMagar.

Statistical analysis

The sample size was calculated according to the following formulas[8]:

$$N_1 \text{ (OC group)} = \frac{Z\alpha^2 \, Sen \, (1 - Sen)}{\Delta^2}$$

$$N_2 \text{ (Non-OC group)} = \frac{Z\beta^2 \, Spe \, (1 - Spe)}{\Delta^2}$$

If both α and β values were set at 0.05, then Z_α was equal to Z_β at 1.96 (two tails). The anticipated sensitivity (Sen) and specificity (Spe) were both set at 0.6 in this study, based on the results of a preliminary experiment. The sample size of each group was calculated as 92 or more.

SPSS ver. 19.0 (IBM, Armonk, NY, USA) was used to analyse the data. Parametric and non-parametric statistical tests were used to compare the OC and non-OC groups. The independent sample *t* test and chi-square test were used in the analyses of age and gender, respectively, and a non-parametric Kolmogorov-Smirnov statistical test in the analysis of the number of *Candida* colonies in saliva. $P < 0.05$ was considered statistically significant.

ROC curve analysis was used to determine the optimal cut-off point of the number of *Candida* colonies for diagnosing OC. The cut-off value that maximised the Youden index was selected as the optimal threshold. Sensitivity, specificity, positive and negative predictive value and area under the ROC curve were also calculated.

Results

General information of the subjects

A total of 100 patients – 81 females (81/100, 81%) and 19 males (19/100, 19%) – were diagnosed with OC by two separate oral clinicians and classified in the OC group, while another 97 subjects – 79 females (79/97, 81.4%) and 18 males (18/97, 18.6%) – were classified in the non-OC group. The mean age was 60.6 ± 11.9 years in the OC-group, and 59.4 ± 12.4 years in the non-OC group. No significant difference was found in age ($P = 0.786$) or gender ($P = 0.937$) between the two groups.

Oral diseases in the subjects

Among the 100 OC-group patients, 8 were diagnosed with pseudomembranous candidiasis, 69 patients with erythematous candidiasis, and 1 with chronic hyperplastic candidiasis based on the classification developed by Holmtup and Axel3. Other *Candida*-associated lesions were also observed, including denture stomatitis (19) and median rhomboid glossitis (3) (See Table 2).

In the non-OC group, 35 of the 97 (36.1%) patients were diagnosed with oral lichen planus, 35 out of 97 (36.1%) with burning mouth syndrome, and 8 patients with recurrent aphthous ulcer (8.2%). Other cases included geographic glossitis and/or fissured tongue, traumatic ulceration, lichenoid reaction, labial discoid lupus erythematosus, foliate papillitis, chronic cheilitis and hemangioma. There were 3 healthy subjects included in the study (Table 3).

Table 3 Oral diseases of the non-OC group patients.

Oral diseases	Number	Percentage
Oral lichen planus	35	36.1%
Burning mouth syndrome	35	36.1%
Recurrent aphthous ulcer	8	8.2%
Geographic tongue and/or fissured tongue	4	4.1%
Traumatic ulceration	3	3.1%
Lichenoid reaction	3	3.1%
Discoid lupus erythematosus	2	2.1%
Foliate papillitis	2	2.1%
Chronic cheilitis	1	1.0%
Hemangioma	1	1.0%
Normal	3	3.1%
Total	97	100%

Table 4 *Candida* spp in saliva in both groups.

Species	OC-group (n = 100)	Non-OC-group (n = 54)
Candida albicans	86	46
Candida glabrata	3	1
Candida krusei	3	1
Candida tropicalis	1	1
Unidentified species	0	2
Multiple *Candida* species	7	3

Number of Candida colonies in saliva

Candida spp were isolated from 100 OC-group subjects and 54 non-OC-group subjects. The *Candida* species were identified based on the colour of colonies grown on CHROMagar medium. *Candida* albicans was the predominant species, accounting for 86% (86/100) in the OC-group and 85.2% (46/54) in the non-OC group, respectively. *Candida tropicalis*, *Candida glabrata* and *Candida krusei* were also isolated in both groups. Some of the subjects in both groups carried more than one type of *Candida* spp (Table 4). The number of *Candida spp* in saliva differed greatly between the OC and non-OC groups. In the candidiasis patients, the median count of *Candida* colonies was 795 (range 4 to 88,800) cfu/ml, which was significantly higher than that of the asymptomatic carriers [40 (range 4 to 2,732) cfu/ml] in the non-OC group ($P < 0.05$) (Table 5). Further analysis of the different types of OC showed that the patients with pseudomembranous candidiasis carried the highest load of *Candida* colonies in saliva, a median of 25,120 cfu/ml, followed by the patients with denture stomatitis (median 3,060 cfu/ml) and erythematous candidiasis (median 654 cfu/ml).

Threshold of Candida colonies for a diagnosis of OC

To identify the fitted threshold of diagnosis for OC, an ROC curve was plotted based on the sensitivity and specificity of all the possible cut-off values of *Candida* colonies counted in saliva. The area under the curve (AUC) was 0.873 (95% CI: 0.827, 0.920), indicating highly significant diagnostic accuracy (Figure 2). The statistical optimal threshold was calculated as 266 cfu/ml, corresponding to the maximal Youden index of 0.545. Under the optimal threshold, the sensitivity and specificity of saliva culture were 0.720 and 0.825, respectively; the positive and negative predictive values were 0.809 and 0.741, respectively, with a diagnostic odds ratio of 12.10.

Discussion

Candida is a commensal organism and inhabits the oral mucosa in 20% to 40% of normal subjects[3]. Under the surveillance of the host immune system, a balance is achieved between the mucosal epithelium and *Candida*. The host epithelial defence mechanisms, the intact epithelial structure, innate antimicrobial peptides secreted by the mucosa, and normal saliva flow, are responsible for the prevention of *Candida* adherence, overgrowth, and invasion of the underlying tissues[9]. If this balance is disrupted, the mucosal epithelium is destroyed by

the excessive proliferation of *Candida*, and candidiasis occurs.

The quantity of *Candida* spp reflects pathogenic virulence. With increasing numbers of *Candida* colonies, the risk of developing *Candida* infection increases[10,11]. Vargas et al demonstrated a significant increase in the number of organisms in the progression from asymptomatic carriage to pseudomembranous oral candidiasis[10]. A remarkable difference was also noted in the present study. The count of *Candida*-colonising oral cavities of OC patients was 795 cfu/ml, while it was 40 cfu/ml in the asymptomatic carriers. The remarkable difference suggests it is possible to find out a quantitative cut-off point of *Candida* colonies in distinguishing asymptomatic carriage from oral candidiasis.

Nevertheless, the quantitative determination of *Candida*-marking OC is still not clear due to the widely differing results in different studies. Epstein et al investigated subjects with oral problems, but excluded those with factors predisposing to OC, such as diabetes, and the use of oral contraceptives, steroids or antibiotics. Consequently, they determined a value of 400 cfu/ml of *Candida* in saliva as the threshold[12]. By contrast, in the study of Xu et al, a value of 200 cfu/ml was considered as the cut-off point to *Candida* infection and the diagnosis of OC patients was based on concurrent positive *Candida* culture and smear[13]. Unlike these studies, we included subjects predisposed to candidiasis because many of the OC patients in clinic had taken to antibiotics, or steroids, or had diabetes. The reported incidence of candidiasis among patients treated with topical immunosuppressive agents was reported to be 11.4%[14]. In addition, microscopic examination of smears was not conducted in the present study due to its insensitivity to low *Candida* concentration of 200 to 500 cfu/ml, as organisms may grow in cultures at this low concentrations[5]. In order to determine an optimal threshold for the quantity of *Candida* colonies required for a diagnosis of OC, the diagnostic test was performed based on an ROC curve analysis. Unlike other traditional diagnostic tests, this provides tools to select possibly optimal cut-off points and to discard suboptimal ones independently from (and before specifying) the class distribution. Consequently, the result of an ROC curve analysis is relatively close to the true circumstance. The cut-off point that was best fit for distinguishing OC from carriage was 266 cfu/ml, with a sensitivity and specificity of 0.720 and 0.825, respectively. The area under the curve (AUC) was 0.839 (95% CI: 0.785, 0.892).

Different fungal burdens were observed among the types of OC. The number of *Candida* colonies in the patients with pseudomembranous candidiasis was 2.5 × 104 cfu/ml of saliva, which was higher than that in the patients with denture stomatitis (3 × 103 cfu/ml) or the patients with erythematous candidiasis (650 cfu/ml). These results imply that the mean number of *Candida* colonies in a cohort of OC was influenced by

Table 5 The numbers of *Candida* colonies in saliva in different patients carrying *Candida*.

	Number of ***Candida*** colonies (cfu/ml)	
	Range	Median
OC patients		
Pseudomembranous type	56–88,800	2,5120
Erythematous type	4–86,400	654
Chronic hyperplastic type	10	10
Denture stomatitis	42–25,000	3060
Median rhomboid glossitis	62–4,540	760
Total	4–88,800	795
Asymptomatic carriers	4–2,732	40

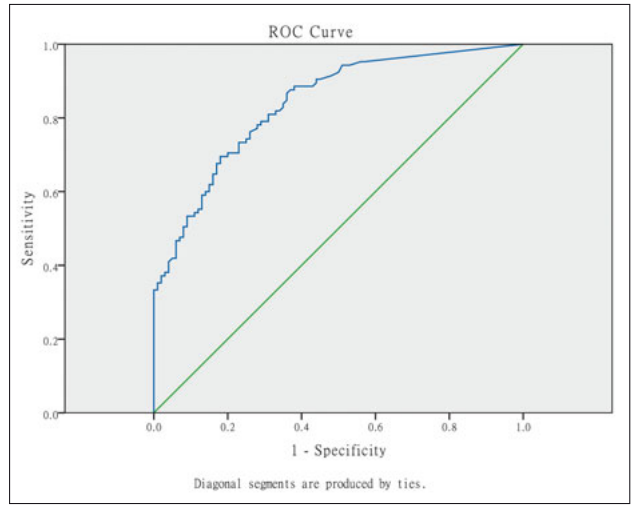

Fig 2 ROC curve of the numbers of Candida in saliva for the OC diagnosis.

the OC types. The diagnostic threshold varied in different proportion of OC types investigated. Consistent with other research[3], the erythematous type of OC was the most common type in our study, accounting for 69%, which warranted the application of the cut-off value obtained in this study for the diagnosis of OC.

In the present study CHROMagar, which is always a medium for the primary identification of *Candida* species, was used. According to the colours of the yeast colonies growing on the medium, the common pathogenic *Candida* species could be differentiated, including *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida krusei* and *Candida parapsilosis*. CHROMagar was effective in determination due to the high sensitivity and specificity to the common pathogenic *Candida* species, which were 71% to 100% and 92% to 100%, respectively[15]. In comparison to PCR and API 20C AUX system for *Candida* species confirmation, CHROMagar is low-cost and easily performed, which fulfilled the need in the present study.

In summary, the quantity of oral *Candida* in the saliva of OC candidiasis patients was significantly higher than that in asymptomatic carriers. The value of 266 cfu/ml was identified as a microbiological marker that should suggest the possibility of *Candida* infection to clinicians.

Conclusion

The value of 270 cfu/ml may be a fitted microbiological marker reminding the clinicians the possibility of *Candida* infection.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Dr Pei Ru ZHOU carried out the study and prepared the manuscript; Dr Xiao Song LIU designed the study; Dr Hong HUA supervised the study.

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