



Prevalence and Genotypes of *Candida albicans* from Necrotising Periodontal Disease and the Tongue

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Abstract

Objectives: The objectives were to determine the prevalence of *Candida albicans* in lesions of necrotising periodontal disease (NPD) and on the tongue of patients and to compare the fingerprinting patterns of *C. albicans* isolates from the two oral sites.

Methods: Microbiological specimens were taken from NPD lesions and the tongue of 87 patients and cultured on Sabouraud Dextrose and CHROMagar, followed by treating patients according to a standard protocol. DNA fingerprinting, using restriction fragment length polymorphism (RFLP) with a ³²P-labelled Ca3 probe, was performed on paired isolates of *C. albicans* simultaneously isolated from NPD lesions and the tongue.

Results: *C. albicans* was isolated from 47 (54%) patients in total, from only the tongue in 39 (44.8%), simultaneously from the tongue and diseased sites in 7 (8.0%) and only from diseased site in 1 patient (1.1%). The DNA fingerprinting patterns of the isolates were similar within each pair but differed between the 7 pairs, without any evidence of a predominant genetic subtype among the isolates. The clade affiliation resembled that of previously fingerprinted isolates obtained from the region. No statistically significant correlation was demonstrated between the extent ($p = 0.4621$) or severity ($p = 0.3365$) of NPD lesions and the presence of yeasts (Fisher's Exact Test). NPD in all patients presenting for a follow-up visit had resolved with conventional treatment and without the addition of antifungal agents.

Conclusion: No association between *C. albicans* and NPD could be demonstrated and evidence is presented that it is unlikely that the *C. albicans* isolated from NPD represent a pathogenic subgroup and are more likely to be contaminants from elsewhere in the mouth.

Keywords

Candida albicans, Ca3 fingerprinting, Necrotising ulcerative gingivitis, Necrotising ulcerative Periodontitis, Necrotising periodontal disease, South Africa, HIV

Abbreviations

NPD: Necrotising Periodontal Disease; NUG: Necrotising Ulcerative Gingivitis; HIV: Human Immunodeficiency Virus; RFLP: Restriction Fragment Length Polymorphism

Introduction

Necrotising ulcerative gingivitis (NUG) and necrotising ulcerative periodontitis (NUP) have been classified as necrotising periodontal diseases (NPD) in the absence of sufficient data that they constitute separate diseases [1]. NPD is a distinct category of periodontal diseases and differs from chronic periodontitis in that it presents as an acute inflammatory condition and patients experience pain and bleeding, with clinically detectable ulceration and necrosis of the interdental papillae and gingival margin [2]. Patients may also present with a fever, bad breath and lymphadenopathy, requiring prompt treatment [3]. While NPD is commonly associated with HIV-infection [4], it is not an uncommon condition among HIV-negative patients, since other systemic disorders and socio-economic factors may play a role [5-7].

Oral candidiasis, with *C. albicans* as the most commonly associated yeast spp., is an equally well recognized comorbidity of HIV-infection [8,9]. *C. albicans* colonizes the oral cavity of approximately 50% of healthy individuals [10,11] but is restricted as a commensal organism by a competent host immune response [12-14]. Much interest has also been displayed in the association between yeasts and periodontal disease [15,16].

DNA fingerprinting methods have evolved as reliable tools in the molecular epidemiology of *C. albicans* [17,18], delineating the organism into 5 major genetic subgroups or clades [17,19,20] and demonstrating a geographic predilection of particular *C. albicans* clades [19,21,22]. Despite several hundred clinical isolates of *C. albicans* having been fingerprinted, no evidence emerged of a highly virulent or pathogenic genotype of *C. albicans* that is associated with disease [17,21,23,24]. *C. albicans*, propagating in mainly a clonal nature [25], can exist under a wide range of environmental conditions, including co-existing with a variety of other commensal and pathogenic microorganisms through the expression of relevant phenotypic and genotypic characteristics [26-28]. This ability of *C. albicans* to adapt is also referred to as plasticity [26,29] with the behavior of the organism under conditions of biofilm formation evoking much interest [30-32]. More recently the 'parasexual cycle', with the absence of meiosis, loss of heterozygosity and "extensive karyotypic rearrangement" was suggested as yet another survival mechanism under conditions of environmental stress and it is proposed as a mechanism that may contribute towards genotypic diversity [28,29].

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HIV/AIDS and associated mortality and morbidity place a significant demand on scarce health care resources in developing countries where the prevalence of the disease is high [33]. While several previous studies have focused on genotyping of *C. albicans* isolates obtained from patients presenting with chronic periodontal disease [34-37], uncertainty remains whether antifungal treatment should routinely be included in the treatment of acute periodontal conditions as there is paucity of information on a pathogenic role of *C. albicans* in such conditions [38]. The absence of fingerprinting studies on *C. albicans* from NPDP prompted this investigation. The objectives of the study were to determine the prevalence of *C. albicans* in lesions of NPDP and to compare that to the prevalence with which the organism occurs on the tongue of the same patient, as well as establishing whether a particularly prevalent genetic subtype of *C. albicans* is associated with NPDP lesions.

Materials and Methods

Patients

The study was approved by the Research, Ethics and Publications Committee of the former University of Limpopo, while informed consent was obtained from the 87 patients included in this study and who presented to the MEDUNSA Oral Health Centre with NPDP. Individuals who received antifungal or antibiotic treatment in the 3 months preceding the first visit were excluded from the study, while nevertheless treated for the presenting condition. Oral examinations and treatment were conducted by a single oral medicine-trained examiner using established, presumptive, clinical diagnostic criteria for candidiasis and NPDP [1,4]. The extent and severity of NPDP was recorded as described by Robinson, et al. [39], with the extent of the NPDP lesions categorized as 'few' when ≤ 4 tooth sites were involved and 'many' when ≥ 5 tooth sites were involved, while severity was categorized as 'mild' with tissue necrosis extending ≤ 3 mm from the gingival margin, and 'severe' when tissue necrosis extended ≥ 4 mm from the gingival margin. Treatment of NPDP was administered according to an established protocol and patients were urged to return after 5-7 days for a follow-up visit [6]. It was not an objective of the study to establishing a relationship between HIV-status and either the prevalence of *C. albicans* in lesions of NPDP or HIV-status and a particular genetic subtype of *C. albicans*, but because government policy urges patients to undergo testing [40], the HIV-status of patients is included. All patients of unknown HIV-serostatus were offered HIV-testing with those who agreed to testing providing written consent and receiving pre- and post-test counseling. Based on the test results HIV-positive patients were subsequently referred to a regional HIV-clinic where they were managed according to standard guidelines [41].

Yeast sampling and culture

Before any medication or periodontal treatment was administered, and after the extent and severity of the disease were noted, local anaesthesia was administered and a sterile curette tip was passed along the entire length of the NPDP affected sites to collect oral yeasts. During the process the curette tip was frequently shaken in a tube containing 2.5 ml sterile saline before finally dislodged into the tube. A swab was simultaneously taken from the dorsal surface of the

tongue by twirling it while stroking across the dorsal surface and also around the border of the tongue. The tube with the curette tip was vortexed at low speed to release any material, the tip removed and the tube again vortexed at full speed for 60 sec to further disrupt the collected material. This was followed by centrifugation at $1\ 800 \times g$ for 8 min to sediment the collected material. Following this, 2 ml of the supernatant was drawn off and discarded and the sediment was resuspended in the remaining 500 μ l, with equal volumes spiral plated on Sabouraud's Dextrose (SD) and CHROMagar plates [42]. The swab taken from the dorsal surface of the tongue was also plated on SD agar and CHROMagar, ensuring contact between the agar and all surfaces of the swab. Plates were incubated at 30 °C for 72 hours. Presumptive identification of *C. albicans* and other yeasts was performed on colony color on CHROMagar [36,43] and confirmed with the germ tube test. At least 5 colonies presumptively identified as *C. albicans* were picked from plates that yielded positive yeast growth and stored in sterile water at room temperature until utilised for fingerprinting.

Statistical analyses of the collected data and yeast growth was conducted with SAS[®], Release 9, run under Microsoft[®] Windows[®] XP for a personal computer. Fisher's Exact Test was performed and p values ≤ 0.05 were considered as significant.

DNA fingerprinting

C. albicans, obtained from both the tongue and NPDP of 7 patients, was revived on SD agar.

DNA extraction from these isolates, as well as the reference strain, 3153A, was performed using the method described by Scherer and Stevens [44]. All reagents used were molecular grade, unless otherwise specified. In brief, washed cells were digested with 0.1% β -mercaptoethanol and 20 μ l Zymolyase, before the addition of proteinase K and lysed with 200 μ l potassium acetate, purified with phenol:chloroform and DNA in the supernatant was precipitated with 500 μ l of 100% isopropanol. RNA was removed with 5 μ l of 10 mg/ml RNAase and incubated at 37 °C for 1 hour, followed by a final phenol:chloroform purification and overnight digestion of the extracted genomic DNA at 37 °C with EcoRI restriction enzyme.

DNA was loaded in the wells of a 0.8% agarose gel and electrophoresed at 65 V over a distance of 16 cm. DNA from the reference strain 3153A was run in the right hand lane. The DNA was transferred from the agarose gel to a nylon membrane through capillary blotting and cross-linked by ultra-violet light. The membrane was hybridized with a ³²P-labelled Ca3 probe [45] and radiographed on XO-MAT film, at -20 °C for 24 hrs, before development.

Results

Patients

Demographic and yeast carriage data of 50 (57.5%) females and 37 (42.5%) males are summarised in table 1. Forty patients (46%) declined HIV-testing. Of the 34 confirmed HIV-positive patients, 24 (70.6%) were female, with approximately equal numbers of males and females in the HIV-negative group, as well as the group of unknown HIV-status. Eighty three patients were classified as having extensive lesions (more than 5 teeth involved)

Table 1: Demographic data, HIV-status and isolation of *C. albicans* from 87 patients presenting with necrotising periodontal disease (NPDP).

	HIV+ (CD4 ⁺)	HIV- (CD4 ⁺)	Unknown HIV-status	Total (%)
Gender				
Female	24 (29-693 cells/ μ l)	6 (612-929 cells/ μ l)	20	50 (57.5)
Male	10 (21-318 cells/ μ l)	7 (475-1157 cells/ μ l)	20	37 (42.5)
Age (years)				
Mean	29.6	21.7	28.8	
Range	18-44 yrs	14-31 yrs	19-51 yrs	
<i>C. albicans</i> isolated				
Only tongue	15	2	22	39 (44.8)
Only NPDPs	0	0	1	1 (1.1)
Tongue & NPDPs	4	0	3	7 (8)
None isolated	15	11	14	40 (46)

and 22 patients classified as severe, with the lesions extending vertically > 3 mm from the gingival margin (Table 2). None of the patients received antiretroviral (ARV) treatment at the time of the first visit and those who tested positive for HIV-infection were referred and managed accordingly at an HIV-clinic of their choice. All of the 35 (40.2%) patients who returned for follow-up, including the 3 patients who were diagnosed with concomitant pseudomembranous candidiasis, presented with complete resolution of their NPD as evidenced by patients reporting absence of pain and bleeding and the absences of gingival ulceration on clinical examination.

Yeast culture

C. albicans was cultured from the tongue of 39 (44.8%) patients, while cultured from both the tongue and NPD sites in 7 (8.0%) patients, and from the NPD site but not from the tongue in only one patient (1.1%) (Table 1). Non-*C. albicans* spp., namely *C. krusei* and

Table 2: *C. albicans* isolated from lesions of NPD in 87 patients in relation to the severity and extent of their NPD.

NPD	Patient numbers		
	No yeasts isolated	Yeasts isolated	Total
Extent ^a (p ≥ 0.05)			
Many	68	15	83
Few	4	0	4
Severity ^b (p ≥ 0.05)			
Severe	20	2	22
Mild	52	13	65

^aFisher's Exact Test: p= 0.4621 (no significant relationship); ^bFisher's Exact Test: p = 0.3365 (no significant relationship).

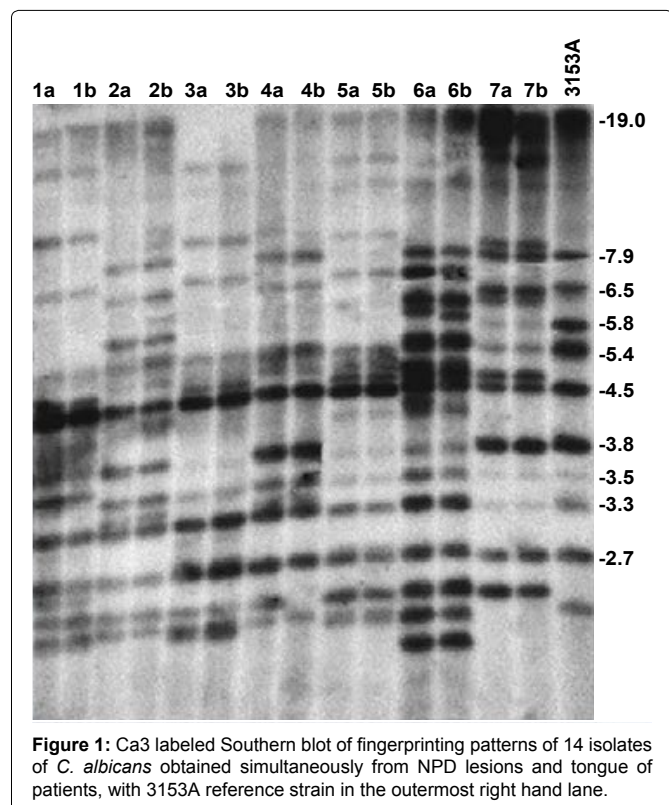


Figure 1: Ca3 labeled Southern blot of fingerprinting patterns of 14 isolates of *C. albicans* obtained simultaneously from NPD lesions and tongue of patients, with 3153A reference strain in the outermost right hand lane.

C. parapsilosis, occurred together with *C. albicans* in two patients and one patient respectively, based on presumptive identification on colony colour on CHROMagar (results not shown).

The relationship between the extent and severity of NPD and yeast carriage among the 87 patients is summarised in table 2. No statistically significant relationship was demonstrated between either the extent (Fisher's Exact Test: p = 0.462) or severity (Fisher's Exact Test: p = 0.3365) and the presence of *C. albicans*.

DNA fingerprinting

The DNA fingerprinting patterns of the 14 isolates obtained through Southern blot hybridization with the ³²P-labeled Ca3 probe, are displayed in figure 1, with the reference strain 3153A in the outermost right hand lane. All the isolates are *C. albicans* as evident from hybridising with the Ca3 probe. The fingerprinting patterns of 14 (7 pairs) *C. albicans* isolates revealed genotypic similarity within each pair of isolates from the tongue and from NPD lesion. In assigning a clade affiliation to the pairs the frequency with which bands occur at a particular position, were considered [21]. Isolates 3a and b, and 5a and b displayed a similar dense banding pattern in the 540 and 450 bp region, which is characteristic of the South African clade [21], while the fingerprinting patterns of isolates 2a and b and 4a and b, which are also similar, correspond with that of clade I. Paired isolates 6a and b corresponded to clade II and 7a and b to clade III while 1a and b were not affiliated to any of the known 4 clades that occur among South African oral *C. albicans* isolates [21,22]. Clinical data and clade affiliation of *C. albicans* from the 7 patients from whom isolates were obtained from both the tongue and diseased sites, are summarised in table 3.

Discussion

HIV-infection in developing countries contributes significantly towards morbidity and mortality, placing a high demand on scarce health care resources [33]. Denial and resistance to HIV-testing remains a major stumbling block in curbing the disease [46]. No reliable conclusions can be made regarding HIV-infection and any presenting pathology when approximately half of the patient population is of unknown HIV-status, hence the exclusion thereof from the objectives of this study. The 60% loss to follow-up among this cohort corresponds with elsewhere in South or sub-Saharan Africa [47]. Despite these particular socio-demographic factors, the unnecessary administration of medication cannot be afforded for economic as well as reasons of sound clinical practice. The clinical findings of this study, excluding the 3 patients who initially presented with pseudomembranous candidiasis and received antifungal treatment, as well as previous studies, confirm that NPD resolved without the administration of antifungal agents [5,6,48]. Because both NPD and oral candidiasis are associated with immunosuppressive conditions [9] it is likely that immunosuppressed patients would either carry a higher load of *C. albicans* on the oral mucosa [49] or that the two disease conditions occurred simultaneously [15,39] and *C. albicans* being perceived as playing a causative role in NPD.

This was the first fingerprinting, or genotyping study to compare *C. albicans* from lesions of NPD with those obtained from elsewhere in the mouth, namely the tongue. Restriction fragment length polymorphism (RFLP), combined with the Ca3 labeled probe, is accepted as a reproducible fingerprinting method with high discriminatory power [18,29], as demonstrated by significant

Table 3: HIV-status, clinically diagnosed oral candidiasis, severity and extent of NPD and clade affiliation of paired *C. albicans* isolates obtained from NPD lesions and the tongue of 7 patients.

Patient	HIV status (CD4 ⁺ count)	Oral candidiasis	NPD extent	NPD severity	<i>C. albicans</i> clade affiliation
Male	HIV+ (293 cells/μl)	-	many	mild	No group
Female	unknown	+	many	mild	Clade I
Female	unknown	+	many	mild	Clade SA
Male	unknown	-	many	severe	Clade I
Female	unknown	+	many	mild	Clade SA
Male	unknown	-	many	mild	Clade III
Female	HIV+ (73 cell/μl)	-	many	mild	Clade II

numbers of clinical *C. albicans* isolates from different geographic areas or patient cohorts that were fingerprinted according to this method [22]. In this study clearly discernable fingerprinting patterns were obtained (Figure 1), revealing similar patterns between the two isolates of each pair for all of the 7 pairs. This is strongly suggestive of contamination between the two sites and because the organism had a much lower prevalence in NPD sites that the tongue was the most likely the primary site of colonisation [10]. Assigning a clade affiliation to the fingerprinted isolates was readily achieved due to the similarity within the pairs and the high degree of resolution that the Southern blot (Figure 1) provided. The representation of the different clades in this study corresponded with the findings of a previous study on South African oral *C. albicans* isolates in which the majority of isolates from both HIV-positive and healthy subjects clustered in clade SA and clade I [21]. Taking the prevailing clade distribution of *C. albicans* in a particular geographic locale into consideration is required in not mistaking isolates from particular anatomical sites or disease conditions as being 'unique' or 'enriched'.

The authors are confident that the 9.2% prevalence of yeasts from NPD sites is not an underestimation as specimens were collected along the entire affected gingival margin that involved more than 5 teeth in 83 of the 87 patients. The prevalence obtained in this study corresponds well with the 10%, 13% and 15.6% reported respectively by Moore, et al. [50], Lamster, et al. [35] and Reynaud, et al. [51]. As it is not possible to standardise the collection of yeast specimens when using a curette as opposed to paper points, no attempt was made to quantify or make deductions on the number of colony forming units (cfu) obtained from the various patients or the two sites. The oral rinse method cannot be used when comparing the prevalence with which yeasts can be isolated from two different oral sites, as in this study, due to the swishing action resulting in an 'artificial' spread of organism throughout the mouth. To have fingerprinted several *C. albicans* isolates from the same patient is unlikely to have changed the findings as several studies have proven that the same genotype of the organism was repeatedly isolated from the same individual [17,18], with possible microevolutionary changes in the hypervariable region of the genome [52]. It is therefore unlikely that the genotypic similarity of isolates from two sites in all 7 patients can be attributed to chance. Alternatively, if it was common that patients are colonised by a variety of distinctly different genetic subtypes of *C. albicans*, it would be unlikely that the two isolates, one obtained from the tongue and the other from NPD, would be identical. Fourteen fingerprinted isolates therefore constitute a sufficient number to make deductions from. In addition, a most recent study, using the same fingerprinting method as in this study, demonstrated the colonisation by a similar genetic subtype of *C. albicans* from oral mucosa and carious dentine [53]. The lack of association between *C. albicans* and NPD is further substantiated by the fact that no statistically significant association between either the severity or the extent of NPD and the carriage of oral yeasts among this cohort could be established (Table 2). The 52.8% of patients from whom isolates were obtained from the tongue also fall within the normal range of oral *Candida* carriage reported for both healthy and HIV-positive patients without clinically detectable candidiasis [10,11,37].

Previous fingerprinting studies on *C. albicans* from sites of chronic periodontitis, healthy gingival sulci and, or oral mucosa, all used different fingerprinting methods [34-37,54]. Three of the studies reported unique *C. albicans* genotypes isolated from sub-gingival areas or periodontal pockets [35,36,55], one not finding any difference between genotypes and the different oral site they were isolated from [34] and yet another reporting an "enrichment" of a particular clade [54]. The latter finding might well be due to the natural clade distribution of *C. albicans* clades in that geographic area [17], while the other studies did not explain their findings apart from mentioning that these unique strains did not originate from elsewhere in the mouth [35]. These studies on *C. albicans* from chronic periodontitis sites were all performed before the most recent discoveries on how *C. albicans* adapts to harsh environmental conditions and in the process generates genomic variance [28]. Considering the conditions

in the mouth, it is attractive to speculate that genomic variance in *C. albicans* may have actually resulted in sites, or pockets of chronic periodontal disease. In periodontal pockets deeper than 3 mm there is low oxygen tension (anaerobiosis), oral biofilm is protected from disruption, allowing it to mature [56,57] while there is interaction between *C. albicans* and other oral microflora, including host defenses [28,29,58]. It is proposed that oral biofilm in periodontal pockets provide a good *in vivo* niche conducive to *C. albicans* mating and for the parasexual cycle to produce "tetraploid progenitors (which) can produce populations of progeny cells with a high degree of genomic diversity, ..." [58]. Future *in vivo* studies on oral biofilm, for example sub- or supragingival plaque in healthy gingival tissue or chronic periodontal disease, pseudomembranous or chronic hyperplastic candidiasis, could offer an opportunity to further elucidate findings from laboratory or mouse model studies on the occurrence of 'unique' genetic subtypes [28,58]. Longitudinal observations on isolates from a state of disease through to resolution thereof, may provide valuable answers regarding the "fitness" of *C. albicans* strains that have possibly undergone genotypic rearrangement, to survive in the human host, as well as offering information on a possible shift in the expression of relevant phenotypic characteristic at each point in time [17,28,31,57,58].

In conclusion it is emphasized that this is the first study in which *C. albicans* isolates from acute periodontal disease were fingerprinted and that the results differ from that found in chronic periodontitis or healthy tissue. It is possible that the dynamics of the acute inflammatory environment, with an abundance of necrotic, desquamating tissue, associated bleeding and mediators of acute inflammation [2,3], is not conducive to the undisturbed colonization, replication and biofilm formation by *C. albicans*. The low frequency with which *C. albicans* was isolated from sites of NPD, the absence of a statistically significant correlation between NPD and *C. albicans* carriage, the condition resolving without antifungal treatment, as well as the genotypic similarity between paired isolates from diseased sites and the tongue contribute to the conclusion that it is unlikely that highly virulent strains of *C. albicans* exist. No such strain or strains were demonstrated to have played a role or contributed to the pathogenesis of NPD in this instance. Furthermore, for the first time, recent sophisticated *in vitro* research findings on the parasexual cycle of *C. albicans* is discussed in a clinical context, namely chronic periodontal disease.

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