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Antifungal Resistance Patterns, Virulence Attributes and Spectrum of Oral *Candida* Species in Patients with Periodontal Disease

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Authors' contributions

This work was carried out in collaboration between all authors. Author DAK was involved in literature search, experimental studies, data acquisition and manuscript drafting. Author SM was involved in designing of experimental studies, data analysis and manuscript editing. Author UB was involved in conceptualization, designing, data analysis and manuscript editing and author SFB was involved in experiment design and manuscript editing. Author LAK was involved in conceptualization, designing, data analysis, manuscript editing and review. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To observe the antifungal resistance pattern, virulence attributes and spectrum of *Candida* species in oral cavities of patients with periodontal diseases and healthy individuals.
Study Design: A total number of 52 patients with periodontal disease and 100 healthy subjects were included in the study.
Place and Duration: The study was carried out in the Apex Regional STD Teaching, Training and Research Centre, VMMC and Safdarjang Hospital and Department of Biosciences, Jamia Millia Islamia, New Delhi. Duration of the study was from December 2011 to June 2013.
Methodology: Oral swabs were collected from the patients and control group. The specimens

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were cultured for *Candida* and the species identified, according to standard protocols. Antifungal susceptibility testing and virulence tests were performed.
Results: Out of 52 patients screened 11 yielded (21%) different *Candida* species, with *Candida albicans* (83%) being the commonest and non *Candida albicans Candida* (NCAC) species accounting for 17 %. Among 100 healthy controls, 23 were colonized by various *Candida* species, with *Candida albicans* again as the predominant species. A significant difference (*P* =.002) was observed in the secretion of proteinase enzyme between the isolates from cases and controls. However, in the distribution of *Candida* species, antifungal resistance patterns, phospholipase secretion and biofilm formation there was no such significant difference.
Conclusion: Our results reveal that there is no significant difference in the distribution of *Candida* species among healthy subjects and patients with periodontal diseases. Antifungal resistance patterns and expression of some of the important virulence attributes also revealed no differences between the isolates from patients and control populations.

Keywords: Candida; periodontal disease; antifungal susceptibility test; proteinase; phospholipase; biofilm.

1. INTRODUCTION

Periodontal disease results due to inflammatory processes in the tissues surrounding the teeth in response to accumulation of bacteria or dental plaque [1]. It is characterized by many stages from an easily treatable gingivitis to severe periodontitis [1]. A wide range of subgingival micro organisms, including gram positive and gram negative bacteria (facultative or obligate anaerobes) and probably yeasts, are believed to be associated with etiology of periodontitis [2]. Though periodontal diseases are considered to be polymicrobial [3], the role of individual microbe in causing disease is affected by the composition of microbiota [2]. Any change in the cellular or humoral immune response may result in the colonisation of *Candida* species, opportunistic pathogenic yeasts, in the subgingival environment [4]. Studies have suggested a possible role of *Candida* species in causing periodontal lesions, due to the similarity in the distribution of yeast and some bacterial period on to pathogens in the periodontal pockets [5]. Ability of *Candida* species to coaggregate with bacteria in dental biofilm, and to adhere with epithelial cells, is supposed to be an important factor leading to the invasion of gingival connective tissue and, contributing to the progression of oral diseases. Also, various virulence factors of *Candida* species contributes to its proliferation [5]. Though *C. albicans* is the most common species responsible for various clinical conditions, other non *Candida albicans Candida* (NCAC) species such as *C. tropicalis, C. krusei, C. glabrata, C. parapsilosis etc.* are also reported as pathogens, especially in the era of increased reports on immune-compromised patients in various disciplines of medicine [6]. *Candida* species has been suggested to have a role in periodontitis, however, this finding is hardly ever conclusively proven in studies from India or elsewhere [2,3]. Hence, the present study was undertaken to explore the spectrum of *Candida* species in the oral cavities of individuals with periodontal lesions and healthy controls. Tests for Antifungal resistance patterns, hydrolytic enzyme production and biofilm formation, were also performed on the isolates to get a baseline data.

2. MATERIALS AND METHODS

Samples were collected from 52 patients with periodontal diseases, (gingivitis-5, chronic gingivitis- 22 and chronic periodontitis- 25), attending the Department of Dental Surgery, Safdarjang Hospital, New Delhi. A detailed history-taking preceded the collection of samples. All subjects included in the study were explained about the procedures and possible outcomes, and their samples were collected after an informed written consent. Two swabs were collected by gently rubbing gingival and supra gingival area of the affected teeth using sterile cotton swabs. First swab was inoculated on Sabouraud's Dextrose Agar (SDA) and incubated at 37ºC for up to 7 days, and observed daily for growth, while and the second swab was used to prepare a smear for Gram staining.

One hundred healthy volunteers served as control group. Two swabs were collected from each subject by depressing the tongue and gently rubbing the surface of gum, tooth, tonsils and tongue, and processed as described above.

All isolates were identified by their morphology on Gram smear, cultural characteristics on SDA, Chrom Agar (colour, texture etc.), Corn Meal Agar with 1% Tween 80, germ tube test and carbohydrate assimilation/ fermentation tests [7]. To differentiate *C. albicans* from *C. dubliniensis*, all *C. albicans* isolates (confirmed by conventional methods) were subjected to growth at 45ºC and Tween 80 hydrolysis test, along with the ATCC control strains [8]. Subsequent to species identification, antifungal susceptibility testing was performed on all the isolates using antifungal agents including Fluconazole (FL), Ketoconazole (KE), Voriconazole (VO) and Amphotericin B (AP) by E- test strip method. This was performed by suspending a portion of the isolated colony in normal saline, and adjusting its turbidity to 0.5 McFarland standard. The suspension was then spread over the surface of a pre dried RPMI-1640 + 2% glucose agar media, using a sterile cotton swab. E test strips were applied using a sterile forceps and MICs were determined after 24 h and 48 h of incubation at 37ºC [9]. In case of doubt in the results by E test, MIC was confirmed by micro broth dilution method, to determine the discordance, if any.

2.1 Assay for Extracellular Proteinase and Phospholipase

A single colony of *Candida* species was inoculated in 10 ml of yeast extract peptone dextrose (YEPD) broth and incubated at 37ºC for 18 h; the inoculum was then transferred to a centrifuge tube and centrifuged at 3000 rpm for 5 min. The supernatant was discarded and the pellet obtained was washed with sterile distilled water. The pellet was re-suspended in sterile normal saline and centrifuged to remove any residual media and 1x 10^6 yeast cells (1.25 OD_{550} /ml) were re-suspended in sterile normal saline and processed further.

Proteinase production by the isolates was determined on a medium containing Bovine Serum Albumin (BSA) according to standard method. Nine hundred millilitres of media was prepared which contained yeast nitrogen base without amino acid, 2 g; ammonium sulphate, 1.45 g; glucose, 20 g; agar, 20 g. The media was autoclaved at 121ºC for 20 min. Two grams BSA was dissolved in 100 ml of distilled water and sterilized by filtration. Sterilized BSA was slowly added to the autoclaved and cooled media, and poured into petridish. On this media 1 µL of the inoculum was deposited at equidistant points and allowed to dry at room temperature. The plates were incubated at 37ºC for 3-4 days [10]. Each isolate was inoculated in triplicate. *Candida albicans* ATCC 10231 and ATCC 10261 were used as positive controls. *Candida parapsilosis* ATCC 22019 was used as a negative control.

Proteinase activity was determined by calculating the zone of Precipitation (P_z) using the formula- Ratio of the diameter of the colony to the sum of diameter of the colony and the zone (in mm) , a method described by Price et al. [11].

Candida isolates were screened for production of extracellular phospholipase activity by growing them on egg yolk agar and measuring the size of the zone of precipitation by the method described by Price et al. [11]. Briefly, the egg yolk medium consisting of agar, 20 g; peptone, 10 g; glucose,30 g; NaCl, 57.3 g; $Cacl_2$, 0.55 g; distilled water added to 900 ml, was autoclaved at 121ºC for 20 min. One hundred millilitres of egg yolk was slowly added to the autoclaved and cooled media and poured into sterile petridish. On this media 2 µL of the inoculum was deposited at equidistant points and allowed to dry at room temperature. The plates were incubated at 37ºC for 3- 4 days [12]. *Candida albicans* ATCC 10231 and ATCC 10261 were used as positive controls. *Candida glabrata* ATCC 90030 was used as a negative control. P_z was measured by dividing the diameter of the colony by the sum of diameter of the colony and the zone of precipitation [12].

2.2 Assay for Biofilm Formation

Biofilm formation was determined spectrophotometrically by one of the methods described by Thumbarello et al. [13], with minor modifications. Briefly, Sabouraud dextrose broth (SDB) was prepared with a final concentration of 8% glucose. *Candida* isolates were grown on SDA for 24 h and a saline washed suspension of each isolate was prepared. The turbidity of each suspension was adjusted to a concentration of 3 x 10^7 CFU/ml by spectrophotometer. To each well of the microtitre plate containing 180 µL of SDB, 20 µL of the suspension was added. After incubating at 37ºC for 90 minutes (adhesion phase), medium containing planktonic cells was discarded and each well was gently washed with PBS. For biofilm formation, fresh medium was added and the plates were incubated at 37ºC for 48 h. After incubation, the wells were washed twice with PBS to remove any planktonic cells and 200 µL of PBS was added. The biofilm was

measured directly by the spectrophotometric reading at 405 nm with a microtitre plate reader. The percent transmission (% T) was calculated by subtracting the % T value of each test sample from the % T of the reagent blank to obtain % T_{bloc}. Biofilm formation was scored based on their % T values as either negative (% T_{bloc} < 10) or graded as 1+ (% T_{bloc} 10 - 20), 2+ (% T_{bloc} 20 - 35), 3+ (% T_{bloc} 35- 50), and 4+ (% T_{bloc} ≥ 50). The isolates were further classified into low biofilm producers (1+) and high biofilm producers (2+,3+ or 4+) [13].

2.3 Data Analysis

Data analysis was performed by chi square test and t test, using graph pad prism software version 5.00. A *P*- value of <0.05 was considered significant.

3. RESULTS

Out of 52 patients with periodontal diseases, 11 yielded *Candida* species (Fig. 1). *Candida albicans* was the predominant species (83%) isolated. While 10 patients yielded single isolates, one had multiple NCAC species (one isolate each of *C. tropicalis* and *C. glabrata*), giving a total of 12 isolates.

Among 100 subjects in the control group 23, (23%) carried *Candida* species in their oral cavities, with a predominance of *C. albicans* (18) followed by *C. glabrata* (3), *C. parapsilosis* (1), *C. tropicalis* (1).

In vitro antifungal susceptibility testing revealed that all isolates from both healthy and diseased population were sensitive to all the antifungal agents tested (Table 1).

In the present study Pz value for proteinase ranged from 0.313 to 0.417 among the isolates from patients with periodontal disease and 0.222 to 0.375 from control group. Similar details for phospholipase producing isolates ranged from 0.421 to 0.455 in periodontal disease group and 0.400 to 0.478 in healthy controls (Table 2).

Of 10 *C. albicans* and 2 NCAC species, isolated from patients with periodontal diseases, tested for biofilm formation, positive findings were observed in two *C. albicans* and one NCAC species (Table 3). Among the isolates from control group, four isolates were positive for biofilm formation, which included three isolates of *C. albicans* and one *C. parapsilosis* (Table 3).

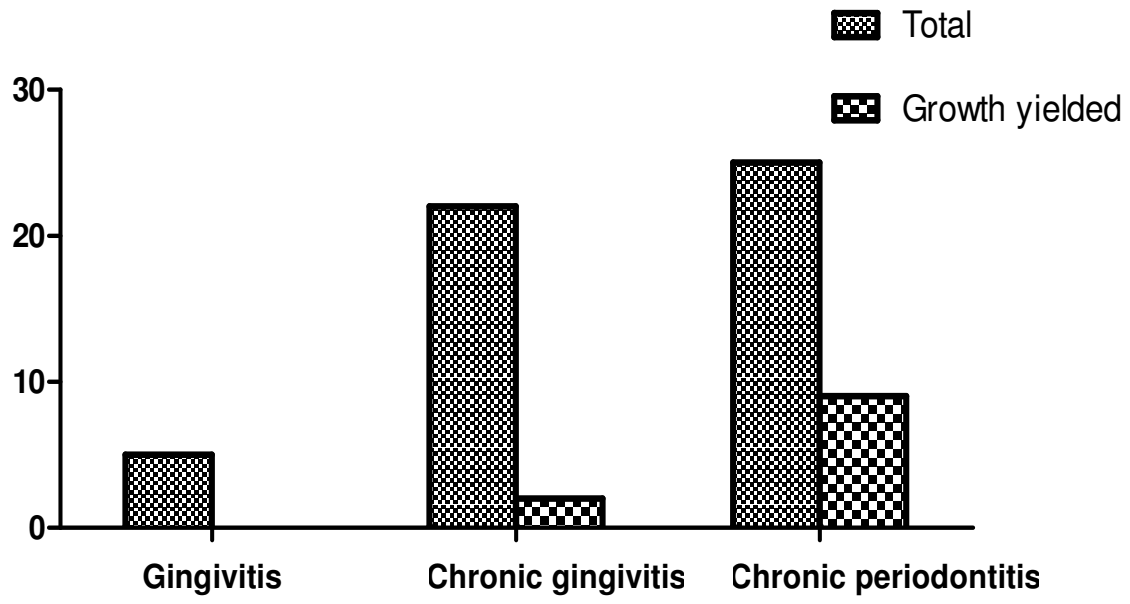

Fig. 1. Distribution of *Candida* species in patients with periodontal diseases

Table 1. Antifungal resistance patterns of *Candida species*

Species	Amphotericin B	Ketoconazole	Fluconazole	Voriconazole
***C. albicans* (n=28)**				
Chronic Periodontitis /Gingivitis:10	S-10 R- 0	S-10 R-0	S-10 R-0	S-10 R-0
Control:18	S- 18 R-0	S-18 R-0	S -18 R-0	S-18 R-0
***C. glabrata* (n=4)**				
Chronic Periodontitis:1	S-1 R-0	S-1 R-0	S-1 R-0	S-1 R-0
Control: 3	S-3 R-0	S-3 R-0	S-3 R-0	S-3 R-0
***C. tropicalis* (n=2)**				
Chronic Periodontitis:1	S-1 R-0	S-1 R-0	S-1 R- 9	S-1 R-0
Control: 1	S-1 R-0	S-1 R-0	S-1 R- 9	S-1 R-0

S- sensitive, R-Resistant

Table 2. Proteinase and phosopholipase activity of *Candida* species isolated from healthy controls and periodontal disease group

Hydrolytic enzyme activity	Healthy controls	Periodontal disease group
Proteinase (P_z)	0.302±0.040	0.348±0.036
Phospholipase (P_z)	0.436±0.025	0.438±0.016

Values expressed as Mean ± Standard Deviation (SD)

Table 3. Biofilm positive *Candida* isolates from patients with periodontal disease and Controls

Species	Total no. of isolates	Biofilm positive	Grade			
			4+	3+	2+	1+
C. albicans	**28**	**5**				
Chronic periodontitis/gingivitis	10	2	1	0	0	1
Control	18	3	0	0	0	3
C. tropicalis	**2**	**1**				
Chronic periodontitis	1	1	0	0	0	1
Control	1	0	0	0	0	0
C. glabrata	**4**	**0**				
Chronic periodontitis	1	0	0	0	0	0
Control	3	0	0	0	0	0
C. parapsilosis	**1**	**1**				
Chronic periodontitis/ gingivitis	0	0	0	0	0	0
Control	1	1	0	1	0	0

4. DISCUSSION

Periodontal diseases result due to an inflammatory, degenerative and/ or necrotic response in gingival and associated connective tissues, elicited by the colonization of various microbes in periodontal pockets [4]. Initiation and progression of periodontal disease depends mainly on the stimulation of host's immune system and release of immune factors, causing tissue damage [4]. Periodontitis is considered as a polymicrobial infection caused by an array of gram positive and gram negative bacteria and probably yeasts [2]. Among the yeasts inhabiting the oral cavity of humans, *Candida* species are the commonest. They cause overt infection in patients with various immunosuppressive conditions. Though *C. albicans* is suggested to be associated with periodontitis, role of this opportunistic pathogenic yeast in the pathogenesis of periodontal disease is yet to be confirmed [3].

In the present study, *Candida* species was isolated from 21% of patients with periodontal diseases (chronic gingivitis and chronic periodontitis). This is higher than the figure reported by Joshi et al. from India, where the authors isolated *Candida* species in subgingival plaque specimens from 7.5% of chronic periodontitis patients [2]. Another study by Urzua et al. from Chile, observed a total carriage rate of 69.2% patients with chronic periodontitis (oral mucosa - 38.5%, subgingival - 3.9% and both - 26.9%) [3]. This variance in observation may be due to the difference in the site of specimen collection or methods used for yeast recovery. *Candida albicans* (83%) was the commonest species isolated and NCAC species accounted for 17%. In the recent past, there have been reports on the emergence of NCAC species in causing various clinical outcomes [14,15]. However, our study revealed that C. *albicans* is still the commonest species isolated from healthy controls and patient group, corroborating with previous reports [2,3,16,17].

Reports on azole resistance among *C. albicans* isolated from periodontitis are available in literature [18,19]. Hence, *in vitro* antifungal susceptibility testing was performed on the isolates to determine the current scenario of resistance patterns among the isolates. Interestingly, none of the isolate (from neither the control or patient groups) showed resistance to the standard antifungal agents tested. This finding is partially in agreement with Koga- Ito et al., where the authors observed resistance in *Candida* species (isolated from controls and periodontitis) to some of the antifungal agents, but not to fluconazole. The exact mechanism responsible for absence of drug resistance in these isolates cannot be explained precisely, as several factors such as prior exposure to particular drugs, acquiring of resistance genes etc, are responsible for the development of drug resistance [20].

The human oral cavity provides a unique environment for microbial colonization with a multitude of ecological niches [4]. *Candida* species possess multiple virulence factors that help in the invasion of the host tissue and evasion of host defence mechanisms. These include, various mechanisms adopted by *Candida* species to adapt to the host environment, synergistic coaggregation or competition with other bacteria, adhesion, hyphal formation, phenotypic switching, secretion of hydrolytic enzymes such as proteinase and phospholipase [5,21].

Candida species can adapt to adverse host environment such as alteration in pH, oxygen concentration and availability of nutrients [5]. Also, some of the *Candida* species are capable of aggregating with other oral microorganisms, leading to the progression of oral diseases [22]. Ability of yeasts to coexist with commensal or pathogenic bacteria in oral cavity is considered an important virulence factor, as it helps in initial colonisation [5].

Secretion of hydrolytic enzymes is considered as an important virulence attribute of *Candida* species. These hydrolytic enzymes include secreted aspartyl proteinase (SAPs) and phospholipase. The main roles of SAPs are to provide nutrition for the cells, to help in the penetration and invasion and to evade immune responses [23]. Phospholipase helps the organism lyse the host cells and alter surface characteristics of the cells so that adherence and penetration are facilitated. In the present study, while a significant difference (P = .002) was observed in the secretion of proteinase enzyme, the same was not seen in case of phospholipase enzyme (P = .82) production by *Candida* species isolated from healthy controls and patient group. While, proteinase secretion was observed in all species of *Candida* (*C. albicans* and NCAC), phospholipase was secreted only by *C. albicans*. There is a difference in opinion regarding the phospholipase secretion by NCAC species. Various investigators demonstrated phospholipase production in NCAC species isolated from different clinical conditions [24,25]. However, our finding corroborates with the report by Samaranayake et al. [26], in which the investigators observed no phospholipase activity among NCAC species. This difference in observation may be due to factors such as strain to strain variation and differences in methods used for the preparation of media [25].

Biofilm is considered as a major virulent attribute of microbes as it helps in withstanding host defence mechanisms and resisting antifungal treatment [27]. It is a complex microbial ecosystem with microorganisms attached to a surface and embedded in an organic polymer matrix [28]. Once microorganisms have successfully attached to a surface, they form aggregates and produce extracellular polysaccharide matrix. Coaggregation is a specific type of cell- to - cell adherence which

leads to the formation of multi species biofilm [29]. It has been reported that *Candida* species have the ability to co - aggregate with other bacteria in dental biofilm and the qualitative and quantitative properties of other microbes may influence the biofilm formation by *Candida* [5]. In the present study no significant difference (P = .86) was observed in the biofilm formation among isolates from patients with periodontal disease and control subjects.

5. CONCLUSION

Our results reveal that there is no significant difference in the distribution of *Candida* species among healthy subjects and patients with periodontal diseases. Absence of Candida in direct smear from the periodontal cases may indicate that these isolates probably reside as commensal organisms. Antifungal resistance patterns and expression of some important virulence attributes also revealed no differences between the isolates from patients and control populations. Further studies may be required to connect these and other virulence attributes of Candida to the development and progression of periodontal diseases.

ETHICAL APPROVAL

The study was approved by Institutional Ethics Committee [No. 26-11-EC (21/31)].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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