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EVALUATION OF BIOFILM FORMING ABILITY AMONG CANDIDA SPECIES ISOLATED FROM VARIOUS TYPES OF ORAL AND MAXILLOFACIAL LESIONS.

Microbiology

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ABSTRACT

Background: *Candida* is the most common cause of fungal oral lesion. Only few decades back, role of *Candida* in the process of infection was considered as passive and only host's organic weakness was the known mechanism. However, this concept is revamped recently and it is now stated that *Candida* can actively participate in the process of disease establishment and progression by aggression known as virulence factors. Biofilm is one of the important virulence factors of *Candida* spp. The present study was conducted with an aim to evaluate biofilm forming ability among *Candida* species isolated from various types of oral and maxillofacial lesions. **Methodology:** *Candida* spp. isolated fromoral lesionswere identified upto species level by standard mycological protocol. Biofilm formation was studied by Confocal Scanning Laser Microscope. **Results:** A total of 104 *Candida* spp. were isolated from patients with oral lesions. Leukoplakia, tobacco pouch keratosis and pseudomembranous whitish lesion were the common oral lesions. *C. albicans* were isolated from 83 (79.8%) patients with oral lesions whereas non-albicans*Candida* (NAC) spp. were isolated from 21 (20.2%) cases.A total of 80 (76.9%) *Candida* isolates from oral lesions showed biofilm formation. As compared to other *Candida* spp., *C. tropicalis* showed high ability to form biofilm. *Candida* isolates from oral lesions like leukoplakia and pseudomembranous whitish lesions showed maximum biofilm formation. **Conclusion:** Study of virulence factors like biofilm formation will aid in better understanding of association between oral lesions and *Candida* spp. It may be also facilitate to explore new therapeutic modalities in future.

KEYWORDS

Biofim formation, Candida, oral lesions, virulence factors.

INTRODUCTION.

In recent years, scientific communities have developed a keen interest in the mechanism responsible for establishment and progression of infection by opportunists. Only few decades back, the role of opportunist microorganisms was considered to be passive and organic weakness in host was the only mechanism considered for opportunistic infection. Recently this concept is revamped, as few opportunistic pathogens like *Candida* spp. can directly participate in the process of establishment of infection by production of virulence factors.[1]

Candida, a fungal opportunist, is capable of causing a broad spectrum of manifestations ranging from muco-cutaneous overgrowth to serious and life threatening disseminated infections.[2]Oro-pharyngeal candidiasis (OPC) is the most common clinical manifestations of candidiasis encountered in medical and dental practice.[3] Clinically, OPC presents in multitude forms. Host factors inclusive of ill-fitting dentures, antibiotic, immunosuppressive therapy, HIV/AIDS, malignancy and impaired granulocyte function and virulence attributes of *Candida* spp. like extracellular hydrolases, adhesion, phenotype switching and biofilm formation contribute to development of oral and maxillofacial lesions.[3,4]

Among various virulence properties attributing to pathogenicity of *Candida*, biofilm is considered to be the most important. Biofilms are defined as specific and well organized communities of cells that are under the control of signaling molecules.[5] Formation of biofilm confers certain properties on *Candida* spp. These include evasion of host immune mechanisms, antifungal resistance and resistance to competitive pressure from other micro-organism.

The present study was conducted at teaching Dental hospital of Pune with an aim to evaluate biofilm forming ability among *Candida* species isolated from various types of oral and maxillofacial lesions.

MATERIALAND METHODS.

Study design:

The present descriptive cross-sectional study was conducted in the Department of Microbiology of Mahatma Gandhi Institute of Medical Sciences (MGIMS), Sevagram in collaboration with Department of Microbiology and Department of Oral Medicine and Radiology of Sinhagad Dental College, Pune. The protocol of the study was approved by Institutional Ethics Committee (MGIMS), Sevagram (MGIMS\IEC\27\2010).

Study population:

The study included a total of 460 patients presenting with oral lesions to outpatient department (OPD) of Department of Oral Medicine and Radiology, Sinhgad Dental College and Hospital, Pune. Additionally, age and sex matched healthy individuals without any oral lesions were recruited as controls. All participants (cases and controls) were explained about the study and procedure of sample collection in local language. Participants those were willing to give informed signed consent were only included in the study. Clinical and demographic data were collected using structured questionnaire.

Sample collection and processing:

Oral swabs were obtained from the lesion site with sterile cotton swab by firmly swabbing the lesion.In case of denture wearers swabs were obtained from oral mucosa, tongue (including dorsum of tongue) and soft palate. Scrapping with sterile cement spatula was taken in non-scrapable lesions like leukoplakia.

In controls, samples were obtained by swabbing the dorsum of the tongue and oral mucosa. A total of 2 swabs were collected per patient and control. The swabs were immediately transported to laboratory for further processing.

Out of two swabs collected per patient and control, one was used for preparation of smear for Gram staining. The Gram stained smear was examined under oil immersion for presence of yeast cells with or without pseudohyphae. The second swab was inoculated on to two slants each of Sabouraud dextrose agar (SDA) without antibiotics and SDA with antibiotics (Chloramphenicol and Cycloheximide). One from each of inoculated slant was kept for incubation at room temperature and other at 37ºC.

The slants were examined daily for evidence of growth of *Candida*. If colonies resembling *Candida* was observed on any of the four inoculated SDA slopes then Gram stained smear of the colony was examined. If the stained smear showed gram positive yeast cells, the colony was further subjected for the identification of yeast including germ tube test, carbohydrate assimilation test and colony color and morphology on HiChrom*Candida* differential agar. After identification the isolate was sub-cultured on SDA slant and maintained for further test.

Biofilm formation:

Biofilm formation was studied by Confocal Scanning Laser Microscope (CSLM) method.[6]In this method yeast suspension was prepared from 24 h *Candida* isolates from on SDA slants.Suspension was prepared in 200 ml of Yeast Nitrogen Broth (YNB with 0.9%glucose) and incubated overnight in fetal bovine serum at 37^{0}C.

The cells were Centrifuged and washed with Phosphate Buffer Saline (PBS).Again the cells were vortexed and re-centrifuged at 5000 rpm for 5 min and resuspended in YNB broth and used for biofilm formation.A 30 ml of the ready suspension (OD_{600}=1.0) was inoculated into polystyrene petri dishes (Laxbro company).The plates were incubated at 37^0C for 90 min to allow adhesion of cells to polystyrene surface. The petri dishes were washed twice with PBS then YNB was added in petridishes. Then petridishes were incubated at 37^0C for 48 hrs and the material was fixed with Paraformaldehyde(PFA) in PBS for better results.For staining 50 mL of calcofluor white was added to the biofilm surface and observed under Confocal Scanning Laser Microscope (CSLM) for biofilm formation. Stained biofilms were observed with a LSM 510 META confocal scanning laser microscope mounted on Axiovert 200 M inverted microscope (both from Carl Zeiss, Jena, Germany).A 543nm line of He–Ne laser line for excitation, 545 nm dichroic mirror and 565–615 nm band-pass emission filter for fluorescence detection were employed. A 5X /0.75 PlanApochromat dry objective was used, with the confocal pinhole opening of 1 airy unit.Images were further processed in the LSM IMAGE EXAMINER software (Carl Zeiss). Biofilm images were displayed individually as 2-D plots.

RESULTS.

A total of 104 (22.6%) *Candida* spp. were isolated from patients with oral lesions whereas a total of only 10 (2.2%) healthy individuals showed growth of *Candida* isolate. The isolation of *Candida* was significantly high from patients with oral lesions (cases) as compared to healthy controls (Fisher's Exact Test *P*=0.001). The oral lesion wise distribution of *Candida* spp. is shown in table 1. The rate of isolation of *Candida* spp. was significantly high from pseudomembranous whitish lesion compared to other oral lesions (Chi square test *P* value<0.0001).

Table 1: Oral lesion wise distribution of *Candida* spp.

Type of oral lesion	No. of patients	No. Candida isolates (%)
Leukoplakia	173	25 (14.4)
Tobacco pouch keratosis	60	07 (11.7)
Pseudomembranous whitish lesion	39	32 (82.1)
Oral cancer	28	14 (50)
Angular chelitis	14	05 (35.7)
Denture stomatitis	11	07 (63.6)
Coated tongue/Fissured tongue/Geographic tongue	24	-
Chemicalburn/smoker's Palate/Hyperkerotosis	05	-
Erythematous lesion	22	08 (36.4)
Ulcers	13	-
Oral submucosa fibrosis	17	03 (17.6)
Lichen planus	03	-
Multiple lesions	51	03 (5.9)
Total	460	104 (22.6)

The species distribution of *Candida* isolates from patients with oral lesions is shown in figure 1. *C. albicans*were isolated from 83 (79.8%) patients with oral lesions whereas non-albicans*Candida* (NAC) spp. were isolated from 21 (20.2%) cases. NAC isolates included C. *glabrata*, *C. tropicalis*C *guillermondi*and *Ckrusei*.

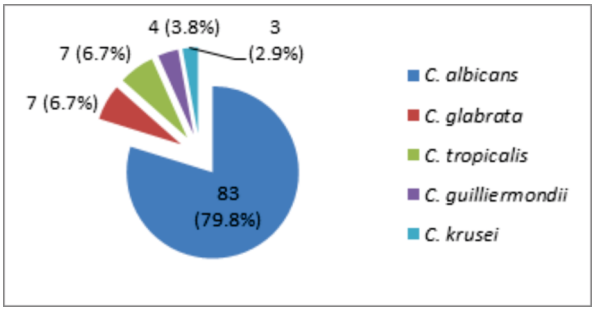

Figure 1: Species distribution of *Candida* isolates from patients with oral lesions.

In healthy control, out of 10 *Candida* isolates, a total of 7 were identified as *C. albicans.* NAC spp. included one each was *C. krusei, C. tropicalis*and *C. guilliermondii.*

A total of 80 (76.9%) *Candida* isolates from oral lesions showed biofilm formation. As compared to other *Candida* spp., *C. tropicalis* showed high ability to form biofilm. Biofilm formation was seen in 84.3% of *C. albicans*. Biofilm formation was not observed in *Candida* isolates from healthy controls.

Table 2: Biofilm formation in *Candida* species isolated from oral lesions.

Candida spp.	Total isolates	Biofilm formation (%)
C. albicans	83	70 (84.3)
C. glabrata	7	02 (28.5)
C. tropicalis	7	06 (85.7)
C. krusei	3	01 (33.3)
C. guilliermondii	4	01 (12.5)
Total	104	80 (76.9%)

Candida isolates from oral lesions like leukoplakia (100%) and pseudomembranous whitish lesions (84.4%) showed maximum biofilm formation (Table 3).

Table 3. Oral lesion wise biofilm formation in *Candida* species.

Type of oral lesion	N	Biofilm formation (%)
Leukoplakia	25	25 (100)
Tobacco pouch keratosis	07	03 (42.9)
Pseudomembranous whitish lesion	32	27 (84.4)
Oral cancer	14	09 (64.3)
Angular chelitis	05	03 (60)
Denture stomatitis	07	04 (57.1)
Erythematous lesion	08	06 (75)
Oral submucosa fibrosis	03	01 (33.3)
Multiple lesions	03	02 (66.7)
Total	104	80 (76.9%)

DISCUSSION

Among various fungal pathogen, *Candida* is unique as it can exist in both commensal and pathogenic forms in gastrointestinal tract of humans.[7]Oropharyngeal candidiasis (OPC) caused due to overgrowth of *Candida* spp., though rarely fatal can cause significant debility especially in immunocompromised individuals.[8]A search through available literature has revealed that most ofnational and international research studies are directedtowards prevalence of oral candidiasis in certain group of patients like denture users, diabetics and people living with HIV/AIDS and there is dearth of information regarding isolation and identification of *Candida* species from various other oral lesions.

Oral lesions are attributed to multitude of aetiological agents including infectious agents like bacteria, viruses, fungi and parasites. [9]They negatively affect individual's life and lead to pain or discomfort that interferes with mastication, swallowing, and speech.[9, 10]As these lesions are often with halitosis, xerostomia or oral dysesthesia, they also have negative effect on individual's daily social activities.[9,10]

In this study, Leukoplakia (13.1%), tobacco pouch keratosis (8.4%) and pseudomembranous whitish lesion (6.1%) were the common oral lesions seen in patients. A total of 51 (11.1%) patients presented with multiple oral lesions. As clinical manifestations of many oral diseases can mimic oral manifestations of certain systemic disorders, laboratory diagnosis plays a crucial role in confirmation and/or supplementation of clinical diagnosis. The pattern of oral lesions vary as per country, gender, exposure to risk factors, general health status of the population, and criteria used for diagnosis.[11]

In field of dentistry, among various infective causes of oral lesions, fungal infections are of more concern, owing to its varied clinical presentation.[12] Fungal oral lesions may be sometimes superficial or at times may be indicative of a serious disseminated illness.*Candida* is considered to be the most common cause of fungal lesions. Beside *Candida* spp. other fungi like *Aspergillusfumigatus*, *Cryptococcus neoformans*, *Histoplasmacapsulatum*, *Blastomy cesdermatitidis*, Zygomycetes class, *Coccidioidesimmitis*, *Paracocci dioidesbrasiliensis*, *Penicilliummarneffei*, *Sporotrixschenckii* and *Geotrichumcandidum*can also produce oral lesions.[13]

In the present study, a total of 104 (22.6%) *Candida* spp. were isolated from patients with oral lesions compared to a total of 10 (2.2%) in controls. Being an opportunistic fungus, *Candida* is very closely associated with oral lesions. Therefore, presence of *Candida* species in

the patients with all the above lesions was also studied and compared with the isolation in normal controls. Similar to our observation Azevedo*et al.* reported high isolation of Candida spp. from patients oral lesions as compared to health individuals.[14]Intact oral mucosa is a robust physical barrier against ingress of various microorganisms including *Candida* spp. Any change in oral muosal epithelia such as atrophy, hyperplasia and dysplasia may compromise the mucosal barrier facilitating to candidal invasion.

Out of 104 *Candida* spp. isolated from patients with oral lesions, a total of 83 (79.8%) isolates were identified as *C. albicans*whereas 21 (20.2%) isolates were NAC spp. Many researchers reported findings similar to ours.[15, 16] Contrast to our finding Deorukhkar*et al.* (2012) andMulu*et al.* (2013) reported predominance of NAC spp.[17, 18]Therefore it can be commented that although, studies have documented the emergence of NAC spp. *C. albicans* still remains the major cause of oral candidiasis.

In the present study, *C. glabrata* (currently known as *Nakaseomycesglabrata*), *C. tropicalis*, *C. krusei*(currently known as *Pichiakudriavzevii*) and *C. gulliermondii* (currently known as Meyerozyma*gulliermondii*) were the NAC spp. isolated from patients with oral lesions. The frequency of colonization/infection of oral cavity by NAC spp. is gradually increasing. A clinician should suspect infection due to NAC spp., when patient fails to respond to routine antifungal therapy[19] because different *Candida* species show different sensitivity to antifungal drugs. As distribution of *C. albicans* and NAC spp. vary as per country, type of population studied and health-care setup, species identification plays an important role in designing local guidelines for selection of most appropriate antifungal drug for prophylaxis and treatment of *Candida* infections.[20]

Biofilms of *Candida* aid in maintaining its commensal and pathogenic properties.Microbial cells within biofilms possess distinct phenotypic characteristics compared to their planktonic counterparts.[2, 5]Understanding how the microbial communities that make up these biofilms communicate and compete for space can help give clues as to how microbes can persist and survive antifungal treatment.[2, 5] In the current study, a total of 80 (76.9%) *Candida* isolates from oral lesions showed biofilm formation. As compared to other *Candida* spp., *C. tropicalis* showed high ability to form biofilm. The variability in biofilm forming ability in can be attributed to the type of infection, infecting species and strain, the site and stage of infection as well as the host response to infection.[2,5]

Candida isolates from oral lesions like leukoplakia (100%) and pseudomembranous whitish lesions (84.4%) showed maximum biofilm formation. As none of the isolate from healthy individuals showed biofilm formation by CSLM method, our study demonstrates that biofilm formation in isolates from oral lesions can be can used as an important tool to distinguish infective strains from colonizers.

CONCLUSION.

Virulence factors are aggression due to which *Candida* species actively in establishment and progression of infection. Study of virulence factors like biofilm formation will aid in better understanding of association between oral lesions and *Candida* spp. It may be also facilitate to explore new therapeutic modalities in future.

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