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Assessment of Virulence Factors and Antifungal Susceptibility Pattern of Candida Species Isolated from Clinical Samples

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Abstract

Candida species are important opportunistic pathogens commonly associated with oral lesions, particularly in individuals with tobacco-related mucosal changes. The present study was conducted to assess the virulence factors and biofilm formation in Candida species and to determine their antifungal susceptibility patterns. A total of 40 patients with oral lesions were included in the study. Clinical details and oral manifestations were recorded, and samples were processed by Gram staining, culture, germ tube test, and antifungal susceptibility testing using disc diffusion and microbroth dilution methods. The majority of patients belonged to the 31–40 years age group (37.5%), with a mean age of 35.75±11.07 years. Tobacco pouch keratosis (42.5%), leukoplakia (37.5%), and pseudomembranous white lesions (20%) were the most common oral lesions observed. Candida species were isolated in 70% of cases. Among the 28 Candida isolates, 64.28% showed germ tube positivity, indicating predominance of *Candida albicans*, while 35.71% were germ tube negative. Antifungal susceptibility testing revealed that fluconazole (0.5 µg/mL) and voriconazole (0.06 µg/mL) were the most sensitive antifungal agents against Candida isolates. The study highlights the importance of identifying Candida virulence factors and performing antifungal susceptibility testing for effective management of oral candidiasis and prevention of antifungal resistance.

Keywords: *Candida albicans*, Biofilm formation, Oral lesions, Antifungal susceptibility testing, Virulence factors, Leukoplakia

Introduction

In recent decades, fungal infections have emerged as a significant global health concern, particularly among immunocompromised individuals. Among these, infections caused by Candida species are the most prevalent, ranging from superficial mucosal infections to life-threatening systemic conditions. While *Candida albicans* has traditionally been recognized as the primary pathogenic species, there has been a notable shift toward non-albicans Candida species, many of which exhibit increased resistance to commonly used antifungal agents [1].

The pathogenicity of Candida species is largely attributed to a variety of virulence factors, including adhesion to host tissues, secretion of hydrolytic enzymes, phenotypic switching, and biofilm formation [2]. Biofilms are structured microbial communities encased in an extracellular matrix that adhere to both biotic and abiotic surfaces, such as medical devices. These biofilms play a crucial role in persistent infections by enhancing resistance to antifungal drugs and protecting the organisms from host immune responses [3].

Superficial candidiasis is one of the important mycotic infections reported by the medical community worldwide. This includes some of mucocutaneous infections like vulvovaginal candidiasis, cutaneous candidiasis, onychomycosis and chronic mucocutaneous candidiasis [3, 4]. Superficial candidiasis is quite specific and usually self-limiting in immunocompetent individuals. This form of candidiasis can be easily treated by maintaining basic hygiene and with local therapy. However, at times superficial candidiasis indicates the beginning of severe form of the disease. Oral candidiasis is one of the most common clinical manifestations of candidiasis encountered in medical and dental practice [5].

The increasing incidence of antifungal resistance further complicates the management of Candida infections. Resistance mechanisms vary among species and are often associated with biofilm-forming which significantly reduces drug susceptibility. Therefore, understanding the relationship between virulence factors, particularly biofilm formation, and antifungal susceptibility patterns is essential for effective clinical management and therapeutic strategies [6].

This study aims to assess the virulence factors and biofilm-forming ability of various Candida species and to evaluate their antifungal susceptibility patterns. Insights gained from this research may contribute to improved diagnostic approaches and guide appropriate antifungal therapy, ultimately helping to reduce morbidity and mortality associated with candidiasis.

Methodology

Study Design and Population

This study was conducted on a total of 40 individuals. Patients included in the study were those clinically diagnosed with oral lesions.

Exclusion Criteria

Patients with a history of antifungal treatment for fungal infections were excluded from the study.

Sample Collection and Processing

Two oral swabs were collected from each participant under aseptic conditions.

Microscopy: One swab was used for smear preparation and subjected to Gram staining. The stained smear was examined under oil immersion for the presence of yeast cells with or without pseudohyphae.

Culture

The second swab was inoculated onto two sets of Sabouraud Dextrose Agar (SDA):

1. SDA without antibiotics
2. SDA supplemented with chloramphenicol and cycloheximide

One set of inoculated slants was incubated at room temperature and the other at 37°C. The cultures were observed daily for growth. Colonies suggestive of *Candida* were identified based on their characteristic pasty, smooth, creamy appearance with a typical odor. Suspected colonies were further confirmed by Gram staining, and isolates showing Gram-positive budding yeast cells were subjected to further identification tests.

Identification of Candida Species

Germ Tube Test

The germ tube test was performed using pooled human serum (screened negative for HIV and hepatitis).

- A loopful of yeast colony was inoculated into 0.5 mL serum and incubated at 37 °C for 2 hours.
- A drop of the suspension was examined microscopically for germ tube formation.
- The presence of at least five germ tubes without constriction at their origin was considered positive.
- Germ tube-positive isolates were presumptively identified as *Candida albicans* or *Candida dubliniensis* and were further differentiated by additional tests.

Morphotyping

Morphotyping was performed using Sabouraud-Triphenyltetrazolium (STTZ) agar as described by Quindos *et al*. (1992). Isolates were inoculated onto STTZ agar plates and colony characteristics were observed.

Corn Meal Agar Morphology

All isolates were inoculated onto corn meal agar supplemented with Tween-80.

- Plates were incubated at 25 °C for 72 hours.
- Microscopic examination was carried out to observe morphological features such as pseudohyphae, true hyphae, blastoconidia, arthroconidia, and chlamydospores.
- Species identification was based on characteristic morphological patterns.

Urease Test

Isolates were inoculated onto Christensen's urea agar slants and incubated at 25 °C for up to 72 hours. A color change indicated urease production.

Assessment of Virulence Factor: Biofilm Formation

Biofilm formation was assessed using the tube method described by Branchini *et al*. (1994).

- A loopful of 24-hour-old culture was inoculated into Sabouraud dextrose broth supplemented with 8% glucose.
- Tubes were incubated at 37 °C for 24 hours without agitation.
- After incubation, the broth was discarded and tubes were stained with 1% safranin for 7 minutes.
- Formation of a visible adherent film on the walls and bottom of the tube indicated biofilm production.
- *Candida albicans* ATCC 90028 and ATCC 10231 were used as positive and negative controls, respectively.
- Biofilm production was graded based on intensity of staining.

Antifungal Susceptibility Testing

Disc Diffusion Method

Antifungal susceptibility testing was performed using the Kirby–Bauer disc diffusion method on Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/mL methylene blue.

- Antifungal agents tested included amphotericin B (0.10 µg/mL), fluconazole (0.5 µg/mL), itraconazole (0.10 µg/mL), and ketoconazole (0.10 µg/mL), Voriconazole (0.06 µg/mL).
- Inoculum was prepared by suspending colonies in sterile saline to achieve a concentration of 1×10^6 to 5×10^6 cells/mL.
- Lawn culture was prepared using sterile swabs.
- Plates were allowed to dry for 15 minutes before placing antifungal discs.
- Plates were incubated and zones of inhibition were measured and interpreted as per standard guidelines.

LSI Broth Microdilution Method

Antifungal susceptibility was also determined using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method to determine minimum inhibitory concentrations (MICs) of antifungal agents.

Results and Discussion

Demographic profile of subject included in the study

In the present study, a total of 40 patients included with oral lesions who fulfilled the inclusion criteria. The age and sex

wise distribution are shown in Table 1. Majority of male patients were from age group 31-40 years (n=10, 25%) followed by 21-30 years (n=6, 15%). The mean ±SD age of male patients was 35.5 ± 11.2 years. Majority of female patients from belonged to age group 31-40 years (n=5, 12.5%) followed by 21-30 years (n=4, 10%). The mean ±SD age of female patients was 36.2 ± 11.6 years.

Table 1: Age and sex wise distribution

Age group	Male (%)	Female (%)	Chi square test (*P* value)
11-20 years	2	1	> 0.5 (non-significant)
21-30 years	6	4	
31-40 years	10	5	
41-50 years	4	3	
51-60 years	3	2	
TOTAL	25 (62.5%)	15 (37.5%)	

Table 2: Type of oral lesions seen in patients

S. No	Type of oral lesions	Percentage %
1.	Leukoplakia	15 (37.5%)
2.	Tobacco pouch keratosis	17 (42.5%)
3.	Pseudomembranous white lesion	8 (20%)
	Total	40

The details of oral lesions seen in patients included in the study (n=40) is shown in Table 2, Leukoplakia (n=15, 37.5%), tobacco pouch keratosis (n=17, 42.5%) and pseudomembranous white lesion (n=8, 20%) were the major oral lesions seen in the present study.

Table 3: Gram staining and culture positive isolates (n=40)

No. of candida species detected in Gram staining and culture (n=40)	Percentage (%)
28	70%

Germ Tube Formation

Out of 28 Candida isolates, a total of 18 (64.28%) Candida isolates recovered from cases showed germ tube formation whereas in 10 (35.71%) isolates the test was negative and this difference was significant (Fisher's Exact test P value 0.0001). Only germ tube positive Candida isolates showed chlamydospore formation. The germ tube positive Candida isolates also showed colony color resembling *C. albicans* on HiChrom Candida agar.

Fig 1: Colonies of *C. albicans* on SDA

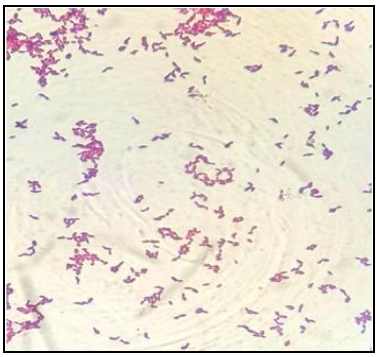

Fig 2: Gram staining showing Gram positive yeast cells

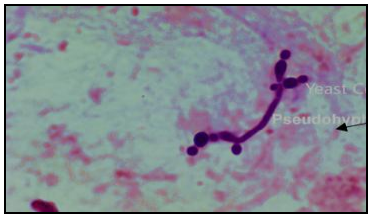

Fig 3: Germ tube production in *Candida albicans*

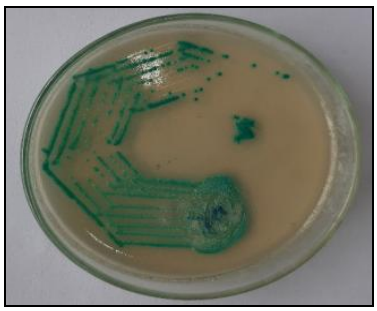

Fig 4: Colony color of *Candida albicans*

Antifungal susceptibility testing

Antifungal susceptibility testing of *Candida albicans* was studied by disc diffusion and microbroth dilution methods. The results of these methods were compared. Antifungals like ketoconazole, amphotericin B, fluconazole, Voriconazole were tested fluconazole (0.5 µg/mL) and Voriconazole (0.06 µg/mL) were found most sensitive.

Conclusion

The present study demonstrated a high prevalence of Candida species among patients presenting with oral lesions, with Candida isolation observed in 70% of the study population. The majority of patients belonged to the 31–40 years age group, indicating increased susceptibility in the active adult population. Tobacco pouch keratosis, leukoplakia, and pseudomembranous white lesions were the predominant oral manifestations associated with Candida colonization. Among the Candida isolates, *Candida albicans* was the most frequently identified species, as indicated by germ tube positivity in 64.28% of isolates. The study further highlighted the role of virulence factors, particularly biofilm formation, in enhancing the pathogenic potential of Candida species. Biofilm-producing isolates are known to contribute to persistent infection and reduced susceptibility to antifungal agents, thereby posing a therapeutic challenge.

Antifungal susceptibility testing performed by disc diffusion

and microbroth dilution methods demonstrated that fluconazole and voriconazole exhibited the highest sensitivity against Candida isolates, followed by amphotericin B and ketoconazole. The findings indicate that azole antifungals remain effective therapeutic agents against most Candida isolates in the present study.

In conclusion, the study emphasizes the clinical significance of identifying Candida species, evaluating their virulence characteristics, and determining antifungal susceptibility patterns for appropriate management of oral candidiasis. Early diagnosis and targeted antifungal therapy may help reduce disease progression, prevent complications, and improve patient outcomes. Further large-scale studies are recommended to monitor changing antifungal resistance trends and the emerging role of non-albicans Candida species in oral infections.

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The research was self-funded. The authors' independently design the study, analyze and interpret the data, prepared the manuscript.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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