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Original Article

Microbiology Section

Prevalence and Virulence Potential of *Candida* Species for Proteinase and Phospholipase Activity in Head and Neck Cancer Patients: A Cross-sectional Pilot Study

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ABSTRACT

Introduction: Head and neck cancer patients undergoing Chemotherapy (CT) and Radiation Therapy (RT) are at a high-risk of oral *Candida* infections, mainly due to immune suppression and mucosal damage. *Candida* spp. most commonly implicated in such conditions are *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida* parapsilosis, and *Candida krusei*. *Candida* spp. also has some virulence factors, which include secreted aspartyl proteinase and phospholipases. They can enhance the ability of *Candida* spp. to colonise and penetrate host tissue along with evasion of the host immune system.

Aim: To determine the prevalence of *Candida* species and evaluate their virulence markers, specifically proteinase and phospholipase activity, in head and neck cancer patients.

Materials and Methods: This hospital-based cross-sectional pilot study was conducted in the Department of Microbiology, Fakhruddin Ali Ahmed Medical College and Hospital, Barpeta, Assam, India, from January 2022 to December 2022. Forty-three patients with histologically confirmed head and neck cancer, planned for 3-4 cycles of CT, RT, or both, and willing to provide informed consent were included. Saliva samples were collected using the spitting technique and inoculated with chloramphenicol on Sabouraud Dextrose Agar (SDA) slants. Germ tube tests identified *Candida albicans* morphologically, while Chrom agar incubation for 48 hours in the dark aided speciation. Proteinase activity was assessed using the modified SDA with 1% bovine serum albumin method, and phospholipase activity was evaluated using the modified SDA with egg yolk emulsion method. Demographic parameters, including age, gender, and tumour stage, were recorded. Statistical analysis employed Pearson's Chi-square tests, with significance set at p-value <0.05

Results: Among 43 patients, *Candida* spp. were isolated in 35 (81.3%), with *Candida albicans* being the most prevalent, 32 (74.4%). Non NCAC (Non–*Candida albicans Candida*)3 (6.9%) included two *Candida krusei* and one *Candida tropicalis*. Proteinase and phospholipase activities were detected in 34/35 (97%) and 33/35 (94%) of isolates, respectively, with all C. *albicans* isolates expressing both enzymes. Concurrent chemoradiation was often accompanied by *Candida* positivity 17/35 (48.6%), but this association did not reach statistical significance.

Conclusion: The high prevalence of *Candida* species, particularly *C. albicans*, with significant expression of virulence factors underscores their pathogenic potential in immunocompromised patients with head and neck cancer. These findings emphasise the need for early detection and antifungal prophylaxis to manage infections in this population.

Keywords: Head and neck neoplasms, Oral candidiasis, Prevalence, Saliva

INTRODUCTION

In relation to mortality and morbidity, one of the most prevalent non communicable diseases at the moment is cancer [1], with one in three people expected to get cancer by the age of 75 [2]. Head and neck cancer is the sixth most prevalent type of cancer worldwide, including in India, and is linked to significant morbidity and mortality [3]. Nicotine, tobacco-related products, alcohol consumption, and nitrosamines are all potential risk factors [4]. *Candida* spp. is also suspected of producing carcinogenic nitrosamines through its catalytic activity [5]. The prognosis of this disease has significantly improved as a result of the introduction of newer imaging modalities and treatment options. Nevertheless, surgery continues to be the primary treatment option for the buccal cavity. RT and CT are also employed as adjuvant therapies [6]. Concurrent chemoradiation is frequently employed in subsites such as the oropharynx, larynx, and hypopharyngeal tumours. CT and RT disrupt the oral microbiota, impairing the immune system and increasing the risk of fungal infections, particularly candidiasis, in cancer patients [7].

These patients may experience xerostomia, oral mucositis, dysgeusia, dysphagia, and ulcerations as a result of radiotherapy (RT). Nevertheless, it has been reported that these conditions, in conjunction with an immunocompromised state, promote the proliferation of *Candida* [8]. Conversely, chemotherapeutic medicines have the capacity to eliminate both cancerous and healthy cells within the body. Mucositis is the most prevalent oral reaction to CT, characterised by diffuse mucosal erosions and ulcerations. *Staphylococci*, *streptococci*, and *Candida* spp. are the most common secondary bacteria and fungi that infect these ulcerations [8]. Additionally, CT was determined to be the most effective treatment for locally advanced laryngeal, hypopharyngeal, and nasopharyngeal carcinoma in the neoadjuvant setting. The most frequent cause of infection by fungi worldwide is *Candida* species [9].

As they colonise the epidermis and mucosal surfaces as commensals, the preponderance of infections is attributable to the following five *Candida* spp., as revealed by long-term global surveillance studies:

Candida albicans, diploid, polymorphic yeast, is considered the most severe cause of candidiasis, generating yeast cells, pseudo hyphae, and real hyphae, three distinct morphologic forms [10]. *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, and *Candida parapsilosis* are among the NCAC isolates that cause candidemia, after *Candida albicans* [11].

Candida spp. possess certain virulence factors that are capable of promoting proliferation, which in turn leads to epithelial adhesion and the invasion of host tissue. Aspartyl proteinase and phospholipases are examples of extracellular hydrolytic enzymes that function as virulence factors. Pathogenic *Candida* species secrete aspartyl proteinases while an infection is in-vivo [12]. In vitro, the organism releases the same enzymes when it is grown with an exogenous protein-based nitrogen source, usually bovine serum albumin. Immunoglobulins (IgGs) and proteins of the extracellular matrix are degraded by aspartyl proteinase and phospholipases. They also elicit inflammatory reactions and inhibit the phagocytosis of Polymorphonuclear Neutrophils (PMNs). Producing proteinase is thought to increase the capacity of the organism to infiltrate and penetrate host tissues, allowing it to avoid detection by the host immune system [13]. Phospholipase enzymes are linked to the adherence, penetration, and membrane injury of host cells.

Antifungal resistance is rising in NCAC, particularly *Candida glabrata*, being resistant to the azoles and echinocandins [14], *Candida krusei* being intrinsically resistant to fluconazole [15], and *Candida auris* being multidrug-resistant [16]. It has been noted that different genotypes of *Candida* species may differ in their pathogenicity. Despite the abundance of research on pathogenic fungi, there have been suggestions that additional studies of virulence factors are necessary. The novelty of this study lies in its comprehensive evaluation of *Candida* species prevalence, their virulence factors (proteinase and phospholipase activity), and their association with treatment modalities in head and neck cancer patients in a specific region of India (Assam), providing insights into regional variations and treatment-related risk factors.

This study aimed to identify the prevalence and virulence factors of *Candida* species in head and neck cancer patients undergoing RT and/or CT. The primary objective was to determine the prevalence of *Candida* species in saliva samples and assess their proteinase and phospholipase activity. The secondary objectives included evaluating the association between *Candida* colonisation and treatment modalities (RT, CT, or concurrent chemoradiation) and assessing regional prevalence variations in India.

MATERIALS AND METHODS

This hospital-based cross-sectional pilot study was conducted in the Department of Microbiology, Fakhruddin Ali Ahmed Medical College and Hospital, Barpeta, Assam, India, from January 2022 to December 2022. The study was approved by the Institutional Ethical Committees (IEC) of VISTAS, Chennai (letter no. VISTAS-SPS/IEC/I/2022/05) and Fakhruddin Ali Ahmed Medical College and Hospital, Assam (letter no. FAAMC&H/P. Est./I.E.C./26/2021/346). Dual approvals were obtained to comply with the ethical requirements of the academic Institution (VISTAS, Chennai) and the clinical site (Fakhruddin Ali Ahmed Medical College and Hospital, Assam), ensuring adherence to regional and Institutional Ethical standards. Informed consent was obtained from all participants, with a waiver approved for secondary data use in line with Institutional guidelines.

This hospital-based cross-sectional study was conducted as a pilot study by randomly selecting a cohort of 43 head and neck cancer patients attending Barpeta Cancer Centre.

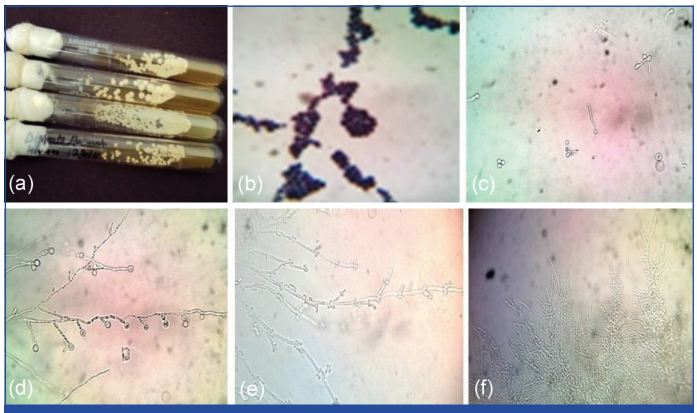
Inclusion criteria: Inclusion criteria of participants were patients with histologically confirmed malignancy of the larynx, hypopharynx, oropharynx, or oral cavity who were scheduled to receive RT, CT, or concurrent chemoradiotherapy and who provided written informed consent. Additionally, a comprehensive history of substance abuse with tobacco and related products was compiled.

Exclusion criteria: This included refusal to participate, current antifungal therapy, use of medications that may alter immune function, and documented candidiasis at baseline (before treatment initiation).

Study Procedure

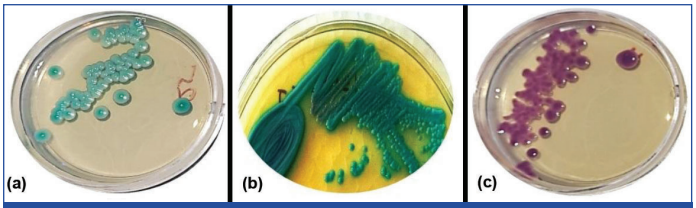
The study comprised 43 consecutive head and neck cancer patients who were planned to receive RT, CT, or both. The patients had clinical characteristics and histologically identified malignancies of the larynx, hypopharynx, oropharynx, and oral cavity. Tumour staging was classified according to the American Joint Committee on Cancer (AJCC) 8th edition [17]. The study's goal, nature, and significance of conducting therapeutic exams of the buccal cavity at different intervals following RT, CT, or combined therapy were thoroughly communicated to the participants. The participants were required to provide written informed consent and indicate their willingness to participate in the study.

Using the spitting technique, 5 mL of saliva samples were collected from the study participants into sterile wide-mouthed sample containers at the conclusion of four weeks of RT treatment or after three weeks in the case of three-weekly systemic CT. Saliva samples were selected due to their non invasive collection method and ability to reflect microbial colonisation in the oral cavity. The samples were then inoculated with chloramphenicol into Sabouraud dextrose agar (SDA) slants [Table/Fig-1a]. Gram stain was performed for morphological identification of budding yeast cells [Table/Fig-1b]. Using germ tube assays, *C. albicans* [Table/Fig-c] and *C. dubliniensis* were quickly identified morphologically. The generation of blastoconidia and chlamydospores was measured using cornmeal agar [Table/Fig-1d-f]. Following inoculation into Chrom agar medium for the differentiation and identification of *Candida* spp., since the medium contains chromogenic substrates that change colour in response to specific enzymes produced by different *Candida* spp, [Table/Fig-2a-c]. The isolates on the SDA media were cultured for 48 hours at 37°C in the dark. To satisfy the requirements, a number of biochemical tests were also carried out, including fermentation and glucose assimilation.



[Table/Fig-1]: Culture and morphology of *Candida* isolates: (a) Sabouraud dextrose agar with chloramphenicol (slants) showing *Candida* spp. Growth; (b) Gram-stained smear showing budding yeast cells (100×); (c) Germ-tube test positive- *Candida albicans* (40×); (d) Corn-meal agar (Dalmau plate) showing chlamydospore formation- C. *albicans* (40×); (e) Corn-meal agar showing blastoconidia- *Candida tropicalis* (40×); (f) Corn-meal agar showing blastoconidia with "match-stick" appearance - *Candida krusei* (40×).
SDA: Sabouraud dextrose agar; CMA: Corn-meal agar; Magnifications as indicated

Isolates were used to prepare yeast suspensions to identify virulence determinants. The stock culture was first inoculated in a small amount, applying chloramphenicol using the sterile loop method on SDA (HiMedia), and then incubating for 24 to 48 hours at 37°C. The yeasts were then collected and reconstituted at a turbidity that

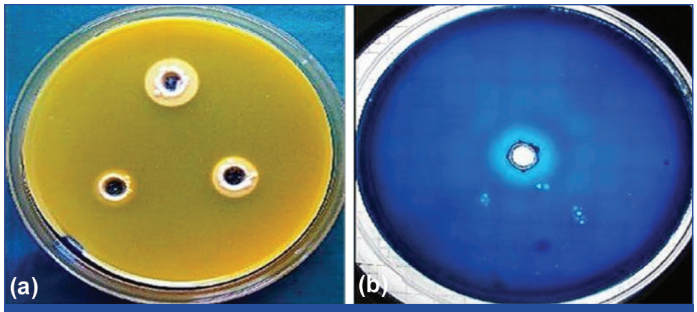


[Table/Fig-2]: Species differentiation on HiCrome *Candida* differentiation agar showing characteristic colony colours: (a) light-green colonies typical of *Candida albicans*; (b) steel-blue colonies typical of *Candida* tropicalis; (c) lavender/rose-lilac colonies typical of *Candida krusei*. Plates were photographed after routine incubation (≈24-48 hour at 35-37°C); while surface morphology may vary, the diagnostic hues are consistent.

matched that of a Phosphate-buffered Solution (PBS; pH=7.4) to approximately 0.5 McFarland Optical Density (OD). A 1×10^7 yeast cell/mL was thus the final suspension added to the solution.

The modified technique by Price MF et al., was used to identify *Candida phospholipase* [18]. About 13g SDA, 11.7 g NaCl, 0.111 g $CaCl_2$, and 184 mL of sterile distilled water were then added and sterilised. This mixture was added to a 20 mL yolk emulsion, which was centrifuged for 10 minutes. The final composite was poured aseptically into a sterile petri dish and allowed to set. Small wells were drilled in the medium with a sterile well borer. Using a micropipette, aliquots of yeast suspension (10 µL) were added to each well. Phospholipase production was measured after 48 hours of incubation at 37°C. A precipitation zone encircling the wells indicated positive phospholipase production [Table/Fig-3a]. The phospholipase, in turn, indicated their participation in the degradation of phospholipids. It was thought that the absence of a precipitation zone around the wells, which is a sign of phospholipase synthesis, was detrimental to phospholipase production. Phospholipase was not detected, indicating that the phospholipids were not degraded. To calculate the phospholipase activity (Pz value), the diameter of the well was compared to the overall diameter of the precipitation zone. When Pz=1, the strain produced no phospholipase activity. Hence, a low Pz value indicates that a high enzyme level is synthesised. Proteinase and phospholipase activities were classified based on Pz values, with lower values indicating higher enzymatic activity. To ensure accuracy, all assays were performed in triplicate.

To identify *Candida* proteinases, a modified technique by Staib F was implemented [19]. SDA containing 2% dextrose, 0.1% KH_2PO_4, and 0.05% $MgSO_4$ was added and sterilised. After cooling to 50°C, the melting agar mixture was mixed with 1% bovine serum albumin. After that, the methyl agar was transferred into a sterile petridish under aseptic conditions. Minute wells in the medium were then bored out using a sterile well borer. An aliquot of 10 bµL of yeast suspension was added to the well using a micropipette. After that, the containers were incubated at 37°C for 48 hours. The plates were taken out after 48 hours and fixed for 20 minutes with 20% Trichloroacetic Acid (TCA). Trichloroacetic acid was eliminated from the plates after they were cleaned with deionised distilled water. Deionised distilled water was used to rinse them after they had been stained for 10 minutes with 1.25% amido black. Following that, the plate was treated with 15% glacial acetic acid and incubated at 25°C for 18 hours. Proteinase production was assessed on the plates following an 18-hour washing with deionised distilled water. A transparent zone of proteolysis encircling the wells that was impervious to amido black staining indicated the production of proteinase [Table/Fig-3b]. This is a sign of proteinase-mediated protein breakdown. The entire plate was uniformly stained with amido black stain, indicating positive proteinase synthesis. This means this protein was not broken down due to the lack of proteinase. The width of the proteolytic unstained region in the well divided by the well's diameter was used to calculate the proteinase activity (Pz). The strain exhibited no proteinase activity when Pz=1. Consequently, high enzyme production is associated with low Pz.



[Table/Fig-3]: Phenotypic detection of extracellular enzymes in *Candida* isolates on solid media: (a) Phospholipase activity on SDA supplemented with egg yolk produces an opaque precipitation halo around colonies, (b) while proteinase activity on SDA containing bovine serum albumin yields a clear zone after Amido Black staining; in both assays, larger halos indicate stronger enzymatic activity (smaller Pz index).

STATISTICAL ANALYSIS

The Pearson's Chi-square test was used to assess the association between tobacco consumption and cancer incidence, prevalence of *Candida* species across cancer sites, and the correlation between candidal growth and the treatment modality. Statistical significance was defined as a two-sided p-value <0.05. All analyses were performed using IBM Statistical Package for Social Sciences (SPSS) version 25.0.

RESULTS

The demographic profile of the 43 head and neck cancer patients included in the study is presented in [Table/Fig-4], of which the majority, 25 (58.1%) were between the ages of 50 and 70, with a male preponderance of 27 (62.8%), and 34 (79%) were using tobacco and related products.

Demographics	n (%)
Age	
<50 years	11 (25.6%)
50-70 years	25 (58.1%)
>70 years	7 (16.3%)
Gender	
Male	27 (62.8%)
Female	16 (37.2%)
Tobacco and tobacco-related products	
Yes	34 (79%)
No	9 (21%)

[Table/Fig-4]: Demographic profile of head and neck cancer patients (N=43).

Among these head and neck cancer cases, 15 (35%) were Oropharyngeal carcinomas (OP), 11 (26%) were Laryngeal carcinomas (LG), 10 (23%) were Hypopharyngeal carcinomas (HP), and 7 (16%) were Oral carcinomas (OL) [Table/Fig-5]. At the time of sample collection, all 43 study participants exhibited the presence of white regions and erythema on their buccal mucosa. Among the stage-wise distribution, 70% of cases belonged to stage III and stage IV as per the AJCC 8th edition classification. of the 43 head and neck cancer patients, 24 (55.8%) were undergoing concurrent chemoradiation treatment, 12 (28%) were undergoing only RT, and 7 (16.3%) were undergoing only CT.

Site/stage/treatment detail	n (%)
Site	
Oropharynx	15 (34.9%)
Larynx	11 (25.6%)
Hypopharynx	10 (23.3%)
Oral cavity	7 (16.3%)
Stage	
I	1 (2.3%)

II	5 (11.6%)
III	8 (18.6%)
IVA	13 (30.2%)
IVB	9 (20.9%)
IVC	7 (16.3%)
Treatment	
Concurrent CT/RT	24 (55.8%)
Radiotherapy (RT)	12 (27.9%)
Chemotherapy (CT)	7 (16.3%)

[Table/Fig-5]: Site/Stage/treatment profile of head-and-neck cancer patients (N=43).

The study found no statistically significant association between tobacco use and *Candida* growth, despite high positivity in both users (76.5%) and non users (100%). Similarly, *Candida* growth remained high across treatment modalities- chemoradiotherapy (70.8%), RT alone (91.7%), and CT alone (100%)- with no significant association. Analysis by anatomical site also showed high positivity across groups (60-93.3%) without statistical significance. Tumour stage likewise demonstrated no meaningful association with *Candida* growth [Table/Fig-6].

Variables	Category	*Candida* growth		χ^2 value	p-value
		No n (%)	Yes n (%)		
Tobacco use	No (n=9)	0	9 (100)	2.60	0.11
	Yes (n=34)	8 (23.5)	26 (76.5)		
Tumour site	Oropharynx (n=15)	1 (6.7)	14 (93.3)	4.52	0.21
	Larynx (n=11)	2 (18.2)	9 (81.8)		
	Hypopharynx (n=10)	4 (40.0)	6 (60.0)		
	Oral cavity (n=7)	1 (14.3)	6 (85.7)		
Tumour stage	I (n=1)	0 (0)	1 (100)	3.38	0.64
	II (n=5)	1 (20.0)	4 (80.0)		
	III (n=8)	1 (12.5)	7 (87.5)		
	IVA (n=13)	4 (30.8)	9 (69.2)		
	IVB (n=9)	2 (22.2)	7 (77.8)		
	IVC (n=7)	0 (0)	7 (100)		
Treatment	Concurrent CT/RT (n=24)	7 (29.2)	17 (70.8)	4.20	0.12
	RT (n=12)	1 (8.3)	11 (91.7)		
	Chemotherapy (CT) (n=7)	0	7 (100)		

[Table/Fig-6]: Association of *candida* growth with tobacco use, tumour site, stage, and treatment in head and neck cancer patients.
Pearson's χ^2 test used; two-tailed; significance set at P <0.05. Concurrent CT/RT = concurrent chemoradiotherapy. Tumour stage I–IVC per AJCC clinical staging. None of the associations reached statistical significance (all p-values>0.05).

In the current study, the prevalence of *Candida* spp. among head and neck cancer patients was 81.3% (35/43), and that of *Candida albicans* was 74.4% (32/43) [Table/Fig-7]. The NCAC 3 (6.9%) were outnumbered by *Candida albicans* 32 (74.4%). Two *Candida krusei* and one *C. tropicalis* were detected among the NCAC.

Species	*Candida* Spp.	Prevalence rate (%)
Candida albicans	32	74.4
Candida krusei	2	4.6
Candida tropicalis	1	2.3
Total *Candida* species	35	81.3

[Table/Fig-7]: Prevalence of *Candida* species N=43.

The virulence factor profile of the 35 *Candida* spp. isolated is presented in [Table/Fig-8]. Proteinase and phospholipase were considered positive for this investigation when the Pz value was less than 1. *Candida* spp. exhibited the highest proteinase activity (97%), followed by phospholipase (94%).

Virulence factor	Total positive n (%)
Proteinase	34 (97.1%)
Phospholipase	33 (94.3%)

[Table/Fig-8]: Profile of virulence factors detected among the isolates (n=35).

The relationship of virulence factors, specifically proteinase and phospholipase, among the various *Candida* spp. that have been isolated is revealed in [Table/Fig-9]. Proteinase and phospholipase activity were expressed by all 32, *Candida albicans* isolates. One each of the two *C. krusei* isolates (n=2) was positive for phospholipase and proteinase, respectively, constituting 50% of the total. The proteinase activity was detected in the only *C. tropicalis* isolated (n=1), but no phospholipase activity was detected.

Species	Proteinase (%)	Phospholipase (%)
C. albicans (n=32)	100%	100%
C. krusei (n=2)	50%	50%
C. tropicalis (n=1)	100%	0%

[Table/Fig-9]: The virulence factor profile of the different species of *Candida*.

To conclude, all *Candida albicans* isolates expressed proteinase and phospholipase, while 66.6% of non *Candida albicans* isolates expressed proteinase and 33.3% of them expressed phospholipase. Due to the small sample size of NCAC (n=3), inferences on their virulence potential (proteinase and phospholipase activity) are limited and should be interpreted with caution.

DISCUSSION

The present study found that 81.3% of head and neck cancer patients tested positive for *Candida* species. These findings closely align with previous studies reporting *Candida* prevalence in 70% of cancer patients with aerodigestive tract infections [20,21]. The high prevalence in the study may reflect regional factors in Assam or the impact of treatment modalities, as saliva samples effectively captured microbial colonisation.

The predominance of *Candida albicans* in the current study at 74.4% is consistent with Mohandas V and Ballal M's study on diabetic patients, which reported 74.19% C. *albicans* [22]. Similarly, in the current study, NCAC comprised 6.9% which closely resembled the findings by Dahiya MC et al., at 8% NCAC in irradiated patients [23].

All C.*albicans* isolates in this study expressed proteinase and phospholipase, critical virulence factors facilitating tissue invasion and immune evasion. In contrast, only 66.6% of NCAC isolates produced proteinase and 33.3% produced phospholipase. This differs from Mohandas V and Ballal M's findings, where NCAC showed comparable enzyme activity [22], possibly due to the small NCAC sample size (n=3) in the current study, which limits robust conclusions.

Concurrent chemoradiation was associated with the highest *Candida* growth (48.6%), followed by RT (31.4%) and CT (20%), though the association was not statistically significant (p-value=0.122), likely due to the small sample size. Jain M et al., reported a similar trend, with *Candida* carriage increasing by 50% in RT, 75% in CT, and 81.25% in combined therapy [6]. Silverman JS et al., noted *Candida* carriage in 49% of RT patients [24], while Samaranayake LP et al., found 70% carriage in CT patients [25]. These comparisons suggest that treatment modalities significantly influence *Candida* colonisation, with combined therapy posing the greatest risk.

Clinical implications include the need for routine antifungal susceptibility testing in head and neck cancer patients, given the rising azole resistance in NCAC species. Interventions such as oral care (rinses, toothbrushing), antifungal prophylaxis, and nutritional strategies (sugar-free diets, probiotics) could reduce *Candida* colonisation. Future

studies should focus on larger sample sizes, include diabetic status and HbA1c levels, and explore susceptibility patterns to optimise prophylactic antifungal strategies in high-risk cancer patients.

Limitation(s)

Adequately powered studies are needed to validate these findings and to better inform the prevention and management of candidiasis in patients with head and neck cancer. Also, data on the diabetic status and average HbA1c levels of the patients were not collected in the present study, as diabetes was not a primary focus. Future studies could include these parameters to explore their impact on *Candida* colonisation.

CONCLUSION(S)

The present study identified a high prevalence of *Candida* species (81.3%) in head and neck cancer patients, with *C. albicans* dominating (74.4%) and expressing significant proteinase and phospholipase activity. Concurrent chemoradiation was associated with increased *Candida* growth, highlighting the need for routine antifungal susceptibility testing and prophylactic strategies to manage infections in this population.

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Authors' contribution: RC: Carried out the study; GG: Supervising and guiding the research work; TS: Supported with clinical history and sample processing; SB: Guidance in Data gathering; DB and AKK: Data interpretation and Manuscript preparation.

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