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Adherence of *Candida* Species Isolated from Patients with Head and Neck Cancer Presented for Radiation Therapy and its Correlation with Hemogram

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Abstract

The objective of the current study was to quantify the adherence property of *Candida* species causing infections in head and neck cancer patients on radiation therapy (RT) or chemoradiotherapy (CRT). Additionally, the study aimed to establish a correlation between adherence properties and patients' hemograms. A prospective cohort study was done on head and neck cancer (HNC) patients with oropharyngeal candidiasis. All patients received radiation doses ranging from 60 to 70 Gy using Intensity Modulated Radiation Therapy. Inoperable patients received concurrent chemotherapy with cisplatin 40mg/m^2 once weekly during the course of RT. Swabs were collected from patients with signs and symptoms of candidiasis and microbiological confirmation and speciation were done according to standard protocol. An in vitro adherence test was done. Hemogram of all the patients at the start of infection was compared with the adherence. Candidiasis was detected in 58(35.3%) out of a total of 164 patients (Age 36-85). *Candida albicans* was the most prevalent 39(67.2%) species isolated. *C. albicans* showed the highest adherence which was statistically significant. The hemogram at the start of treatment and with *C. albicans* infection showed a statistically significant decrease in the absolute lymphocyte count (p-value 0.0001), RBC count (p-value 0.004) and haemoglobin level (p-value 0.003). Less adherence was seen with non-albicans *Candida* infection when compared to *C. albicans* (Odds 0.894, 95% CI -0.818-0.977), significant at 5 % (0.013). *C. albicans* showed significant adherence, which indicates the severity of infection. The occurrence of oral candidiasis is strongly associated with low lymphocyte count and severity of mucositis. Absolute lymphocyte count needs to be monitored in HNC patients who are undergoing RT or CRT.

Keywords: *Candida albicans*, Candidiasis, Chemo Radiotherapy

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INTRODUCTION

Head and neck cancer is a heterogenous term that includes a variety of malignancies of the oral, pharyngeal (including oropharynx, nasopharynx and hypopharynx), laryngeal, nasal and paranasal regions and of the salivary glands. It is prevalent in areas where there is a high rate of smoking and alcohol consumption. Head and Neck Cancer (HNC) ranks as the seventh most prevalent cancer globally, with more than 800,000 new cases reported each year. It is very common in India and accounts for 30% of the total cancer burden of the country,with the highest number of HNC patients in the world. Every year around 2,00,000 new cases occur in India.[1] Majority (90%) of these are squamous cell carcinomas.[1] Radiation therapy (RT) with concurrent chemotherapy is the preferred treatment option for these carcinomas. *Candida* is a commensal of the oral cavity in a large proportion of patients undergoing RT or chemoradiotherapy (CRT) for HNC. This carrier state increases as the treatment progresses and some develop oral candidiasis.[2,3] Direct mucosal damage is caused due to radiation as well as by cytotoxic drugs. The xerostomia caused due to glandular destruction causes these opportunistic yeasts to become infective. As a result of the radiation, the cell mediated immunity of the host is impaired which will favour the development of these infections.[4] *Candida albicans* is the predominant species identified in oropharyngeal infections and is commonly found as an asymptomatic carrier in these patients.[2,3]

Oral *candida* colonisation can intensify symptoms associated with oral mucositis and lead to a decline in the overall health of the patients. Changes in the structure of the mucosal layer due to oral mucositis often create openings for bacteria and fungi to invade injured tissue, leading to infections and raising the likelihood of oral candidiasis occurrence.[5] Adherence is the initial crucial step in the establishment of *Candida* infection which prevents the organism from getting washed away. The extent of adherence varies depending on the concentration, species/strain, and various environmental factors such as saliva and interactions with other oral microorganisms.[6,7] The incidence and severity of mucositis development vary depending on several factors like the cancer subsite, treatment modality used, dose, daily fractionation, chemotherapeutic agent, oral hygiene, overall immune status of the host, history of smoking and alcohol consumption, etc. The dosimetric parameters can predict the time and severity of side effects, but there are not many studies on the clinical parameters that can predict the severity of mucositis which may become complicated with *Candida* infection. The disruption of the equilibrium between fungal virulence factors and the host's immune defences leads to the onset of oral candidiasis. Patients with mucositis grade 2 or above are at higher risk of developing oropharyngeal candidiasis.[8-10] The primary objective of the current study was to quantify the adherence property of *Candida* species causing infections in head and neck cancer patients on radiation therapy or chemoradiotherapy. Furthermore, the study sought to determine a relationship between the adherence property and the patients' hemogram, investigating whether this correlation could serve as a predictive factor for initiating antifungal therapy in individuals with head and neck cancer.

METHODS

A prospective cohort study of the adherence property of *Candida* isolated from HNC patients with oropharyngeal candidiasis who were attending the Radiation oncology department of a tertiary care centre. The study was approved by the Institutional Research Committee and Ethics Committee (Ref No. 30/IEC/19/AIMS-11). All patients with Grade 3 and 4 cancer in the head and neck region, seen from December 2019 to December 2022, who satisfied the inclusion criteria, were enrolled. Before sample collection, patients were required to provide written consent, indicating their informed agreement.

Inclusion criteria

Histologically proven carcinoma of the head and neck region, stages III and IV as diagnosed by the Oncologist were included in the study.

Exclusion criteria

The presence of oral mucositis or candidiasis before RT or CRT or patients on antifungals 15 days before the start of treatment were excluded.

Sample size

Based on the previous studies prevalence of *Candida* among HNC patients who have undergone RT ranged from 25-56%.[11,12-15] Therefore using an average prevalence (P) of 41%, Za of 1.98 and precision (d) as 20% of prevalence, the minimum sample size was calculated as 144.

$n=(Za)^2 pq / d^2$

Study procedure

All patients received RT doses ranging from 60 to 70 Gy using the Intensity Modulated Radiation Therapy (IMRT) technique. Inoperable patients received concurrent chemotherapy with cisplatin 40mg/m^2 once weekly during the course of radiation. Mucositis was graded based on Radiation Therapy Oncology Group criteria (RTOG).[16] Samples were collected from patients who had clinical signs and symptoms of candidiasis. Sterile swabs were used to collect samples from visible lesions. This was then immediately transported to the microbiology laboratory for processing and identification of the yeasts. Swabs were inoculated onto a chromogenic HiCromeTM *Candida* differential agar media (HiMedia Laboratories Private Limited, India) plate and incubated at 37°C for about 48-72 hours. Initial identification was done based on the colour produced on HiCrome agar according to manufacturers' instructions and Gram staining was performed and colonies were confirmed to be gram-positive yeast-like budding cells. Further identification was done by tests like germ tube and chlamydospore production. All the strains were properly labelled and stored in Sabouraud's Dextrose Agar media (SDA) prepared as slants and stored at 4°C until further tested.

Germ tube test

A minimal concentration of the test organism was suspended in 0.5-1.0 ml of sterile serum in a test tube, followed by incubation at a temperature of 35-37°C for a maximum duration of 3 hours. Afterwards, a single droplet of the yeast-serum mixture was placed onto a slide and observed using a high-power objective lens, with a cover slip applied to examine the production of germ tubes. [17]

Chlamydospore production

A single line of the test organism, consisting of a fresh and actively growing yeast, was streaked down the middle of a corn meal agar plate. To dilute the inoculum, three or four additional streaks were made across the initial streak. The plate was then covered with a coverslip and incubated in darkness at room temperature for a duration of 3 days. Subsequently, the plate was examined by placing it on the microscope stage without its lid, and observations were made using both low-power and high-dry objectives. The most distinctive morphology, particularly the terminal chlamydospores of *C. albicans*, was predominantly observed near the coverslip's edge.[17]

Buccal epithelial cell(BEC) adherence assay

The test was based on the method described by Kimura and Pearsall *et al.*[18] Epithelial cells were collected from the mouths of healthy persons. The collected cells were then suspended in Phosphate-Buffered Saline (PBS), was washed and adjusted to a final concentration of 10^5 cells/ml.

The stored *Candida* was revived by culturing onto a fresh SDA culture plate and was incubated at 37°C for about 48 hours. Individual colonies were then inoculated into Sabouraud's glucose broth, incubated for 24 hours, centrifuged and yeast cells were harvested and washed with PBS. 1 ml of washed *Candida* suspension was then incubated with 1 ml of a BEC suspension and was placed in a water bath set to a temperature of 37°C and allowed to incubate for a duration of 1 hour. Subsequently, the mixture of BEC and fungal suspension was combined with 5ml of PBS and centrifuged at 3000rpm for 2 minutes. The supernatant, which contained the unattached *Candida*, was discarded, and the recovered BEC was re-suspended once again in 5ml of PBS. This process was repeated. The epithelial cells were then suspended in 15ml of PBS and gently filtered

through a membrane filter using suction, allowing any remaining free fungi to pass through while retaining the epithelial cells on the filter surface. The filtered epithelial cells were subsequently transferred onto a microscope slide, air-dried, fixed with alcohol, and Gram-stained. The slide was then examined under a high-power objective lens, and the number of Candida adhering to each epithelial cell was counted. A total of twenty-five epithelial cells were counted, and the average number of fungi per cell was determined along with the standard error of the mean for each sample.

Statistical analysis

Data was analysed using SPSS (v16, IBM, Illinois, US). Continuous measurements were reported as the mean Standard Deviation (SD). Categorical measurements were presented as numerical counts and corresponding percentages. The correlation between hemogram and adherence was analysed by the Pearson correlation coefficient. The association between *Candida* species variation and the type of treatment was analysed by Chi-square test. The risk of *C. albicans* development was analysed using logistic regression. Hemogram at the start of treatment and infection was analysed using a paired t-test. A significance level of $p<0.05$ was considered statistically significant.

RESULTS

Oropharyngeal candidiasis was detected in 58(35.3%) out of a total of 164 patients. Of the 58 patients, the majority, 41(81%) were men and 11(19%) were women. The age of the patients varied between 36 and 85, with an average age of 64. More subjects, 22/58(37.9%) were in the 56 to 65 year age group (Table 1). All subjects were diagnosed with stage 3 and 4 HNC. Site wise distribution of the disease is described in Table 2. All subjects were histopathologically confirmed squamous cell carcinoma, except three whose histology were verrucous, sarcomatoid and mucoepidermoid carcinoma. Grade 2 and 3 mucositis were recorded in 29(50%) and 28(49%) patients respectively, and one patient had developed grade 4 mucositis.

Candida albicans was the predominant species, 39(67.2%) and NAC was 19(32.8%). No statistical significance (P = 0.055) was observed between the number of *Candida* infections among patients who received radiation alone

Table 1. Demography of head and neck cancer patients

Characteristics	Frequency	Percentage
Gender		
Women	11	19.0
Men	47	81.0
Age(Mean age-64±12)		
36-45 years	4	6.9
46-55 years	8	13.8
56-65 years	22	37.9
66-75 years	15	25.9
76-85 years	9	15.5

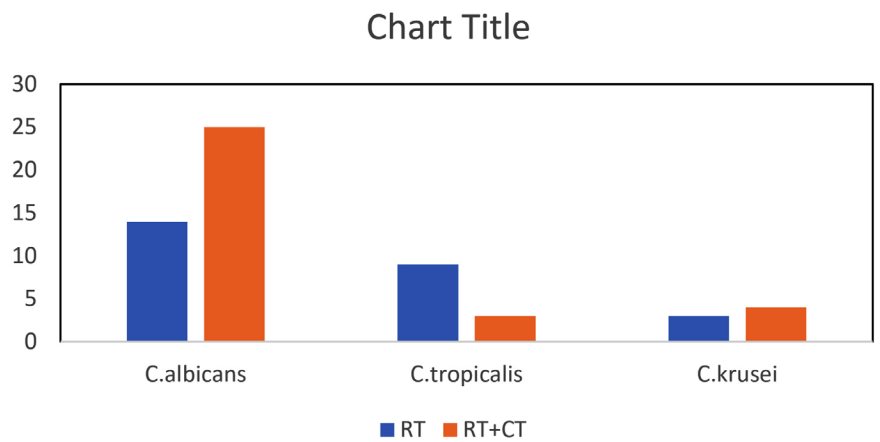

Figure 1. Distribution of *Candida* species causing infection among patients undergoing radiation therapy and radiation therapy with chemotherapy. p-value- 0.055

and radiation with chemotherapy (Figure 1). The adherence of *C. albicans* was more than about double the mean adherence of NAC species, which was statistically significant (P=0.026) (Table 3). However, there was no notable distinction observed in the adherence between *C. tropicalis* and *C. krusei*. (Figure 2).

A notable reduction in the lymphocyte count was observed at the time of *C. albicans* infection when compared with the count at the start of the treatment. Absolute neutrophil count (ANC), absolute lymphocyte count (ALC) and platelet count at the time of infection showed a negative correlation (p>0.05) with adherence, whereas total RBC count and haemoglobin level at the time of infection showed a positive correlation (p>0.05) (Table 4). The hemogram at the start of treatment and at the time of infection of the patients who had *C. albicans* infection showed a statistically significant decrease in the absolute lymphocyte count (p-value 0.0001), RBC count (p-value0.004) and haemoglobin level (p-value 0.003) (Table 5). No statistical significance was observed in the hemogram of patients with NAC species infection (Table 5). The average platelet count at the start of treatment of the patients who had *C. albicans* infection was 276±91.9 and 259.9±121.1, in patients who had NAC, which came down to 246.2±88.3 and 229.0±101.7 respectively when there was *Candida* infection. Less adherence was seen with NAC infection when compared to *C. albicans* (Odds 0.894,95%CI -0.818-0.977), significant at 5% (0.013).

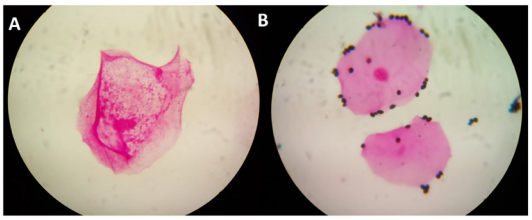

Figure 2. *In vitro* adherence test using human buccal epithelial cell with Gram's stain (x40). A) Washed buccal epithelial cell and B) *Candida* adherence

DISCUSSION

The average age of the subjects was 64 (range 36 to 85) with more subjects between the ages of 56-75. Men were more than women, which is similar to that described in other studies.[19,20] Age, gender and tobacco use are important known risk factors for HNC and reports confirm the highest rates of HNC in older males who have chronic smoking history. HNC are known to be a disease of

Table 2. Distribution of head and neck tumors in patients who had *Candida* infection during treatment

Primary site	Frequency	Percentage	Primary site	Frequency	Percentage
Base of tongue	3	5.2	Retromolar trigone	3	5.2
Buccalmucosa	3	5.2	Alveolus	1	1.7
Hypopharynx	4	6.9	Secondary neck node	1	1.7
Larynx	3	5.2	Sino nasal	1	1.7
Nasal cavity	1	1.7	Supraglottis	1	1.7
Nasopharynx	1	1.7	Tongue	16	27.6
Oropharynx	14	24.1	Tonsil	1	1.7
Parotid	1	1.7	Upper lip	1	1.7

Table 3. Risk of *Candida* with adherence

C.species	Mean adherence	95% Confidence Interval for Mean	Kruskellwallis test (p-value)
C. albicans (n=39)	13.5 ± 10.1	10.2-16.8	
C. tropicalis (n=12)	6.3 ± 4.0	3.7-8.9	0.026
C. krusei (n=7)	7.2 ± 5.0	2.6-11.9	

Table 4. Correlation between adherence and hemogram at the time of infection

Correlation between hemogram at the time of infection and Adherence	Correlation coefficient	p-value
[a]ANC and Adherence	-0.215	0.182
[b]ALC and Adherence	-0.202	0.212
[c]RBC and Adherence	0.116	0.487
[d]HB and Adherence	0.235	0.145
Platelets and Adherence	-0.045	0.784

[a]ANC-Absolute neutrophil count; [b]ALC-Absolute lymphocyte count; [c]RBC-red blood cells; [d]Hb-Hemoglobin

the old and middle age group, but recent reports are suggesting an increase in the incidence in younger individuals, especially women.[21,22] In our study, the majority of the subjects were in the fifth, sixth or seventh decade of life.

In the present study, a total of 58(35.3%) patients developed *Candida* infection during the course of treatment. Previous studies have reported a low prevalence rate(25%) to a high prevalence rate (53.5%) of infection.[11,12-15] Saito *et al.*[12] in a study on 153 patients on concurrent CRT containing Cisplatin has reported a prevalence of 37% and Kermani *et al.*[13] in their study on patients on RT for HNC has reported a prevalence of 35.59%. Bonar-Alvarez[14] has reported a *Candida* prevalence of 47% among HNC patients on RT. In a retrospective study by Kawashita *et al.* 75/300(25%) patients were having candidiasis.[11] Imjai Chitapanarux *et al.* in their study on patients undergoing RT has reported a high incidence rate of oropharyngeal candidiasis (53.5%) throughout the course of treatment.[15] This difference in prevalence may be due to the difficulty in differentiating oral mucositis from candidiasis, as candidiasis may be presented with variable symptoms, from no symptoms to burning sensitivity and pain, which may be the same as for mucositis also. Microbiological culture positivity alone is not diagnostic of infection as this may represent the carrier state of these yeasts, which is seen in about 90% of these patients. In the present study, we correlated the clinical diagnosis of Candidiasis with microbiological confirmation by taking swabs from the visible lesions and thereby confirming these as true candidial infections.

In this prospective study, *C. albicans* was the predominant organism causing oropharyngeal candidiasis (67.2%) and NAC isolated was 32.8%. In the studies on *Candida* by Kermani*et al.*[13] Chitapanarux *et al.*[15] and Makinen*et al.*[23] have reported *C. albicans* as the predominant species followed by *Candida tropicalis* and *Candida glabrata*.In the present study, the NAC species isolated were *Candida tropicalis* and *Candida krusei*. No statistical significance was observed in the rate of candidiasis among two treatment modalities, namely RT and CRT. Makinen *et al.* also reported similar findings in subjects with squamous cell carcinoma.[23] Three patients in this study had their histopathology as verrucous, sarcomatoid and mucoepidermoid carcinomas. Recently there has been an increase in the incidence of NAC species being reported from patients who are undergoing RT for HNC.[24] In a study by Martinez *et al.*[25] on candidiasis in patients during the post-radiotherapy period, 35.9% of the isolates were NAC, out of which only 12.8% and 5.1% were *C. tropicalis* and *C. krusei,* respectively. In a recent study conducted in eastern India, researchers identified a notable presence of non-albicans *Candida* species causing oral infections, demonstrating a significant variance in virulence compared to *C. albicans*.[26] Out of the total 19 NAC isolated in our study, 12% were *C. krusei*, which is inherently resistant to fluconazole. Fluconazole is the recommended first line of drug used to treat fungal infections in head and neck cancer so is particularly challenging to choose the prophylactic antifungal drug.

C. albicans has a set of factors that are capable of transforming itinto a pathogen,the most important of which is adherence to the host mucosal epithelial surfaces, which is considered its primary virulence factor.[7,27] When we quantitated the adherence property of different *Candida* species isolated from the immunocompromised patients, we found that *C. albicans* had the highest adherence,which suggests that adherence might play an important, if not pivotal role in the initiation of infection. Similar findings have been reported in various other studies as well.[28,29] The non-albicans species, *C. tropicalis* and *C. krusei* which were causing infection in the patients and also demonstrated a significant adherence, though not as much as that shown by *C. albicans*.

Table 5. Difference in hemogram at the start of treatment and at the time of detection of infection

Variable	Time	*C. albicans* (n=39)		Non albicans *Candida* spp. (n=19)	
		Values	P-value (Paired t test)	Values	P-value (Paired t test)
WBC*10^3/µL	At the start of treatment	9.4± 2.3	0.155	8.9± 4.5	0.450
	At the time of infection	8.0± 4.1		8.0± 4.0	
Absolute neutrophil count/ µL	At the start of treatment	6641.3± 2256.7	0.902	6288.2± 3172.4	0.416
	At the time of infection	6750.4± 3964.6		6874.2± 3819.6	
Absolute lymphocyte count/ µL	At the start of treatment	1317.5± 474.6	0.0001	1225.5± 1001.8	0.072
	At the time of infection	729.8± 392.1		587.6± 288.5	
RBC*10^6/ µL	At the start of treatment	4.4± 0.5	0.004	3.9± 0.5	0.157
	At the time of infection	4.1± 0.6		3.8± 0.6	
Hb g/dL	At the start of treatment	12.8± 1.3	0.003	10.9± 1.0	0.590
	At the time of infection	12.1± 1.6		10.8± 1.3	
Platelets*10^3/µL	At the start of treatment	276.0± 91.9	0.1	259.9± 121.1	0.280
	At the time of infection	246.2± 88.3		229.0± 101.7	

Values= Mean ± SD

Almost 90% of HNC patients develop mucositis which may affect their quality of life,and treatment prognosis if a break in treatment occurs and also increase the cost of treatment. The course of development of these treatment factors are related to the radiation treatment modality used,its dose and frequency,chemotherapeutic agents used and patient factors like age, gender, smoking, nutrition status,oral hygiene, hemogram etc.[8,9] Low leucocyte/lymphocyte count and low haemoglobin levels are well established host factors in the development of severe oral mucositis.[10,11] Kawashita *et al.* in a retrospective study have revealed a significant association between low lymphocyte count and the severity of oral mucositis with the development of oral candidiasis.[11] In the present study, a significant reduction was there in the lymphocyte count,haemoglobin level and RBC count at the time of infection than at the beginning of the treatment. As with any infection, platelets play a major role in the repair process and promote wound healing. So we analysed the platelet levels at the start of treatment and at the time of *Candida* infection. Though it was not statistically significant, we found that there was a decrease in the platelet count. Nishii *et al.* in a retrospective multicentric study on oral and oropharyngeal carcinoma patients had concluded that a decrease in leucocyte count and grade 2 or higher oral mucositis are factors that contribute significantly to the risk for the development of *Candida* infection.[10] All the subjects in our study had grade 2 or 3 mucositis,and one had developed grade 4 mucositis.

The primary strength of our study is that patient recruitment was uniform with all patients receiving IMRT treatment in the head and neck area and the chemotherapeutic agent used was cisplatin. Also, we could compare the hemogram levels at the start of treatment with the levels when there was *Candida* infection. There were no studies that have compared this and correlated it with the adherence property of *Candida* causing infection in these immunocompromised patients. One of the drawbacks of our study is that female subjects were less and so we could not do a gender wise distribution analysis. In addition, species identification was done by conventional methods rather than molecular identification which will give a more detailed picture of the isolates and virulence genes present in these organisms.

CONCLUSION

C. albicans showed significant adherence, which indicates the severity of the infection.

The occurrence of oral candidiasis is strongly associated with a low lymphocyte count and the severity of mucositis. Absolute lymphocyte count needs to be monitored in HNC patients who are undergoing RT or CRT to detect candidiasis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

NB and CJR designed the study. NB collected the samples and analysed the data. NB, CS and CJR interpreted the data. CS supervised the overall study. All authors read and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

The study was approved by the Institutional Ethics Committee of Amala Institute of Medical Sciences, Thrissur (Ref No. 30/IEC/19/AIMS-11).

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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