MATERIALS AND METHODS

Study design
A total 385 patients having different types of oropharyngeal cancers will be selected for this study having received different types of chemotherapy / radiotherapy prescribed by attending physicians. Oral candidiasis will be clinically diagnosed by clinician.

Diagnosis will be based on lesions clinically recognized as creamy, whitish, curd-like plaques or pseudomembranes involving the oropharyngeal mucosa and the tongue.

Type of study: Prospective study

Sample size calculation
Sample size has been calculated in order to control type I & type II error. Assuming a minimum power 80% and 95% significance level the sample size has been calculated using this formula:

We assume the incidence of oral cancer 0.84 in India.

We accepted the allowable error to be 10 % using the formula:

\[ Z_{\text{power}} = \frac{p_1 - p_2}{2 \times S. E. \text{of difference}} - Z_{\alpha/2} \]

Formula for sample size calculation

\[ \text{sample size } n = \frac{2pq(Z_\beta + Z_{\alpha/2})^2}{d^2} \]

\[ q = 1 - p \]

\[ d = p1 - p2\] is the difference which we want to detect at a specified power & level of confidence.

\[ Z_\beta \] – power of statistical test we want to be minimum 80% for which is \( Z_\beta \) is 0.84.

\[ Z_{\alpha/2} \] – is the level of confidence we have chosen 95% confidence in this \( Z_{\alpha/2} \) = 1.96.

Solving the above equation the sample size for oral cancer comes out 384 rounding off; we can safely assume that sample size of 385.

Duration of study: Two years.
Place of study: Rama Medical College, Hospital & Research Centre, Kanpur, U.P.
Inclusion Criteria:
1. Patient suffering from oral cancer.
2. Patient suffering from oral cancer with diabetes.
3. Criteria decided by clinician.

Exclusion Criteria:
1. Oral cancer patients not suffering from oral candidiasis.
2. Patients suffering from other cancers.
3. Criteria decided by clinician.

Procedure:

Laboratory diagnosis
Laboratory diagnosis of candidiasis depends upon the nature of the different clinical manifestations. Laboratory diagnosis will be in reference to the two groups of clinical manifestations.

Mucocutaneous candidiasis
Laboratory diagnosis of the different clinical forms of mucocutaneous candidiasis involves two steps:
1. Direct examination of pathological specimen to demonstrate fungal presence
2. Isolation of the fungus in culture and its identification.

Direct examination
Specimens from patients with oral infections will be collected by swabs or scraping and kept, preferably, in transport medium before being processed in the laboratory. Both wet and fixed mounts will be done. Wet mounts can be unstained, prepared in normal saline or KOH, or stained with lactophenolcotton blue or calcofluor white. Fixed mounts will be stained by Gram, methylene blue and Giemsa stain. Microscopic examination of specimen will demonstrate the presence of budding yeasts, pseudohyphae, and/or hyphae.

Direct examination in KOH wet mount or normal saline preparation and gram staining will be performed to see the presence of yeast and pseudohyphae of Candida spp.

Culture
Isolation of Candida
The routine medium used for isolation of fungi in culture [152] from mucocutaneous infections is Sabouraud dextrose agar (SDA) supplemented with antibiotics (chlorphenicol, cyclohexidide) to prevent bacterial overgrowth. Cultures can be incubated at 37°C, and Candida colonies will be apparent within 2–3 days; in some cases growth will be noted already after 24 hours, but may take in others more than 3 days.

Phenotypic characterization
The CHROM agar system is based on the reaction between specific enzymes of the different species and chromogenic substrates, which results in formation of differently colored colonies. This system recommended by the manufacture for isolation and identification, permits, based on the color of the colonies, fast presumptive identification of C. albicans, C. krusei, C. tropicalis and others.
Genus identification as well as speciation the CMA (cornmeal agar) plate (Dalmau plate) supplemented with Tween 80 or rice agar will be used. Sub cultures on CMA plates, made from the SDA isolation medium by furrowing the CMA plates, and incubated for 2–5 days at 28°C, will reveal, upon direct mount microscopic examination, the presence of pseudo- and true hyphae.

In the context of morphology, as a basis for species identification, the so-called ‘germ tube test’ should be mentioned. The principle of this test is the ability of C. albicans or C. dubliniensis blastospores to produce, under defined conditions (generally, in serum incubated at 37°C for 2 hours) germ tubes. The formation of germ tubes and chlamydospores is indicative of these two species and therefore offers reliable and easily performed tests for routine diagnosis.

The Candida spp. can utilize carbohydrates both oxidatively (assimilation) and anaerobically (fermentation). Yeasts possessing the ability to ferment a given carbohydrate do also assimilate that substance, but not necessarily vice versa.

Thus, biochemical identification of Candida spp. is based primarily on assimilation and fermentation tests. This technique employs minimal media agar plates on which paper disks impregnated with different carbohydrates are placed, and the growth ability of the yeast around a specific disk is an indication of the yeast’s ability to assimilate that carbohydrate.
Biofilms were formed on commercially available, presterilized, polystyrene, flat-bottom 96-well microtiter plates. Biofilms were formed by pipetting 90μl of Sabouraud’s dextrose broth supplemented with 8% glucose and 10μl of standardized cell suspensions (prepared as above) into wells of the microtiter plate and incubating them for 48 hours at 37°C as described above. After biofilm formation, the medium was aspirated, and the planktonic cells were removed by thoroughly washing the biofilms three times in 0.9% sterile saline. Sterile paper towels were used to remove the residual saline from the microtiter plate, and the tubes were stained with 2% safranin for 10 min. Excess of stain was removed by rinsing with distilled water and the tubes were examined for the presence of adherent layer. The isolate was considered positive for biofilm formation when a visible film was seen on the wall and bottom of the tube. The formation of ring at the liquid interface was not considered as an indication of biofilm production. Following which the antifungal agents were added.

**Molecular characterization**

Randomly amplified polymorphic DNA technique will be used for molecular characterization of *Candida* species. The *SAPI* primer (SAPIF-TCAATCAATTTACTCTTCCATTTCTAACA) and (SAPIR-CCAGTAGCATTAACAGGAGTTTTAATGACA) were used for the real-time PCR assay.

**Antifungal Susceptibility testing**

Antifungal susceptibility tests will be performed according to the Clinical and Laboratory Standards Institute (CLSI) guideline 2014 using 2% Glucose methylene blue Mueller- Hinton (GM-MH) agar diffusion method.

**Procedure**

Inoculum will be prepared by picking five distinct colonies of approximately 1mm from 24 hours old culture grown on Sabouraud's dextrose agar (SDA). Colonies will be suspended in 5ml of sterile 0.85% saline. This suspension will be vortexed to adjust the turbidity yielding 1x10^6-5x10^6 cells/ml and streaked on the entire surface of GM-MH agar. The antifungal disc will be placed 24mm apart from each other. The plates will be incubated at 37°C for 24 hours. If insufficient growth will be observed after 24 hours the plates were read after 48
hours. Zone diameters were interpreted as per the approved CLSI guidelines. The quality control test was performed by using C. parapsilosis (ATCC 22019), C. krusei (ATCC 6258), and C. albicans (ATCC 90028).

The minimum inhibitory concentration (MIC) of the isolates will be performed to the following mentioned drug using RPMI medium and MOPS buffer.

**Antifungal drugs**

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<tr>
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<th>Antimicrobial agent</th>
<th>Symbol</th>
<th>disc content</th>
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<tbody>
<tr>
<td>1</td>
<td>Amphotericin-B</td>
<td>AP</td>
<td>20 mcg</td>
</tr>
<tr>
<td>2</td>
<td>Clotrimazole</td>
<td>CC</td>
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</tr>
<tr>
<td>3</td>
<td>Fluconazole</td>
<td>FLC</td>
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<tr>
<td>4</td>
<td>Itraconazole</td>
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<tr>
<td>5</td>
<td>Ketoconazole</td>
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</tr>
<tr>
<td>6</td>
<td>Nystatin</td>
<td>NS</td>
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STUDY PLAN

Specimen

- Gram stain
- KOH wet mount
- Culture on SDA agar
- Yeast culture & isolation
  - +ve
  - India Ink Wet mount
  - -ve
  - Record

- Gram’s stain
  - Phenotypic identification
    - Antifungal susceptibility testing
      - Disc diffusion method
      - MIC
  - Exclude

- Genotypic identification