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Original Research Article

Prevalence of Oral Candidiasis in HIV positive patients: speciation by phenotypic methods and correlation with CD4+ count in Southern Rajasthan

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Abstract

Objective: *Oral Candidiasis* or *Oral thrush* is the most common mouth lesions occur in HIV positive patients caused by opportunistic yeast *Candida* which living as a commensal in healthy individuals. The clinical severity of *candida infection* prevalence may be a reflection of decreased CD4 count in HIV patient. The aim of the study to determine the prevalence of *Oral Candidiasis* in HIV seropositive patients, their correlation with CD4+ and speciation of *Candida* isolates using different phenotypic methods. **Methods**: A total of 100 oropharyngeal swabs were taken from HIV seropositive patients with or without clinical evidence of Oral candidiasis in this study at the ART centre of RNT Medical College. The speciation of isolated Candida was done using 4 phenotypic test - germ tube test, sugar fermentation test, inoculation on Hi Candida CHROM agar and morphology on starchy media such as corn meal agar. The correlation between Oral Candidiasis and CD4+ count was determined by Pearson's Correlation test. **Results**: In our study 36 samples were found positive for *oral candidiasis* out of the 100 samples taken from HIV seropositive patients. 6 different species of *Candida* were found along with *Candida albicans* (61%) were the most common *Candida species*, followed *by Candida dubliniensis* (16%), *Candida tropilasis* (5%), *Candida krusei* (5%), *Candida parapsilosis* (5%), *and Candida glabrata* (5%). Oral *Candidiasis* was found to be significant correlated with decreased CD4+ counts (negative value of R). **Conclusion**: Human in immunocompromised states such as HIV AIDS are readily infected with opportunistic pathogen such yeast *Candida.* Speciation of *Candida* is necessary because the prevalence of NCA species increasing in last few decades and the prevalence of opportunistic *Candida* infection can be strongly correlate with decrease CD4 count.

Keywords: *Candida spp., Oral candidiasis, Non-Candida albicans,* HIV infection, *CHROM agar, Corn Meal Agar*.

Introduction:

Candida is most common opportunistic unicellular fungus caused various infection of mucous membrane in immunocompromised patients. *Candida species* are known colonizers of humans and other warm-blooded animals. *Candida* actually commensal, but the decrease of secretory Immunoglobulin A (IgA) and also the decrease of T lymphocyte cells become pathogen[14]. The primary site of colonization is the GI tract. They may also be found as commensals in the vagina and urethra, on skin and under the fingernails and toes. All *Candida* species exist as oval yeast like forms that produce buds or *blastoconidia*. Species of *Candida* other than *C. glabrata* also produce *pseudohyphae* and true hyphae[1]. HIV infection reduces the number and functionality of CD4 helper lymphocytes that direct, and coordinate acquired immunity against most pathogens[13]. *Oral candidiasis* affects mainly the infants, elders, users of removable prostheses, patients with long term use of antibiotics and steroids, chemotherapy or radiotherapy for cancer, individuals with xerostomia and those with immunocompromised conditions such as AIDS[15]. *Oral Candidiasis* is the most common HIV related oral lesion and most patients were infected with a strain originally present as a commensal of the oral cavity. The absolute low CD4+ T lymphocyte count has traditionally been cited as the greatest risk factor for the development of *oropharyngeal candidiasis[2]*. Oral manifestations are among the earliest and most important indicator of HIV infection[14]. *C. albicans* isolation has been reported to be 45% in neonates, 30-45% in healthy individuals, 90% in patients with *acute leukaemia* under chemotherapy and 95% of patients with HIV infection[16]. Although more than 100 species of *Candida* have been described, only few have been implicated in in clinical infection[1]. Five *Candida* species are responsible for a majority (>90%) of the invasive infections – *C. albicans, C. tropicalis, C. glabrata, C. krusei, C. parapsilosis.* In addition, the number of new *Candida species* isolated from clinical samples increasing continuously every year[3]. Recently, during Covid-19 pandemic *Candida auris* has been growing concern regarding its drug resistance difficulty in identification, as well as problems with eradication in several Arab country including Lebanon[4].

Confirmation of infections by *Candida* species requires isolation and identification. Various phenotypic and molecular techniques are used by research and clinical microbiology labs around the world for the identification of *Candida spp*[3]. The phenotypic method of speciation become highly valuable because of emergence of new species at faster rate and the molecular methods are too expensive to be used at clinical level.

4 main phenotypic methods are mainly used for candida speciation – 1) Germ tube test (differentiate *C. albicans from NCAs spp*.), 2) sugar fermentation test, 3) morphology on corn meal agar, 4) culture on *Candida* HiCHROM agar.

Methods and Materials:

This prospective study was conducted at the Microbiology department of RNT Medical College in Udaipur, Rajasthan, India over a period of six months from February 2021 to July 2021. Ethical permission for the study was obtained from the institutional ethical committee. 100 oropharyngeal swabs from HIV seropositive patients with or without clinical symptoms of oral candidiasis attending Anti-Retroviral therapy centre for routine check-up have been enrolled in this study. Demographic data have been taken

for each patient including age, sex and CD4 count. Samples have been collected from seventy males and thirty females who attending ART centre.

Following universal precautions, 2 Oropharyngeal swabs were collected from each patient. For sample collection autoclaved cotton swabs were used. After collecting samples, all the swabs were immediately transfer to the routine Microbiology lab. One swab was used to culture on *sabouraud dextrose agar* (SDA) for isolation and second swab was used to performed gram's stain to look for yeast cells. Four phenotypic tests (sugar fermentation test, germ tube test, Corn meal agar medium and Hi CHROM medium) were used to identify the 36 *Candida* isolates to species level.

Correlation between Oral candidiasis and CD4+ count was determined using Pearson's correlation test with $p<0.05$.

Figure 1: Showing the colonies of *Candida* on SDA slants

The four phenotypic tests are described below:

1. Sugar Fermentation test-

Sugar fermentation test was performed by purple broth containing 1% peptone, 0.5% sodium chloride with Bromo cresol purple. The broth was poured into test tubes containing Durham's tube and sterilized by autoclave. The commercial purchased sugar disc was put into the broth. The sugars used were *sucrose, xylose, maltose, lactose, dextrose*. A set of these 5 sugars were used for the identification of each *Candida* isolate. Each tube was inoculated with heavy inoculum of each isolate. The tubes were incubated at 35 degree C for 24 hr to 72 hrs and examined for the production of acid (pink colour solution turn yellow in colour) and gas (bubbles in Durham's tube). Production of gas interpret as fermentation positive while only acid production interprets as carbohydrate assimilation[5].

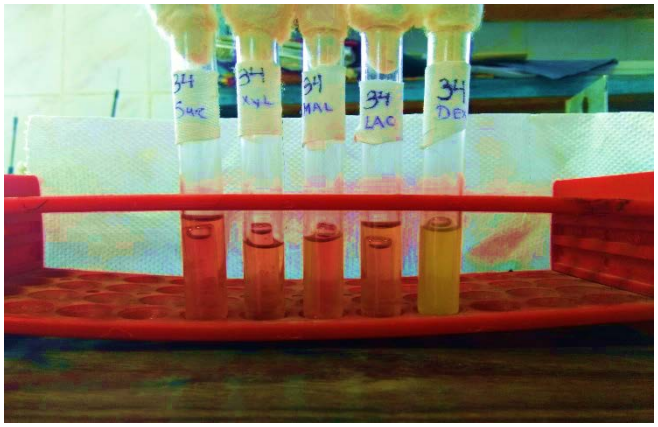

Figure 2: Showing the result of sugar fermentation test

2. Candida CHROM agar Medium-

The *Candida* CHROM agar is a deferential and selective chromogenic medium that is used for the identification of various *Candida* species. The principle behind this medium is the direct detection of specific enzymatic activities by adding multiple chemical dyes i.e., substrates of fluorochromes to the media. Due to the chromogenic substrates added in the medium and different *Candida species* having different enzyme, the *Candida* colonies of various species produce different colour hence allowing us to directly detect of these *Candida species* on the isolation plate[3]. This media also contains antibiotics like chloramphenicol and cyclohexenamide to suppress the growth of bacteria. The media was purchased commercially and prepared by instructions given by the manufacturer. The molten agar media was poured into sterile petri plate and allowed to settle at room temperature. Colonies from the various *Candida* isolates were inoculated onto the CHROM agar medium and incubated at 37degree for 24 to 48 hrs after which the colours were noticed.

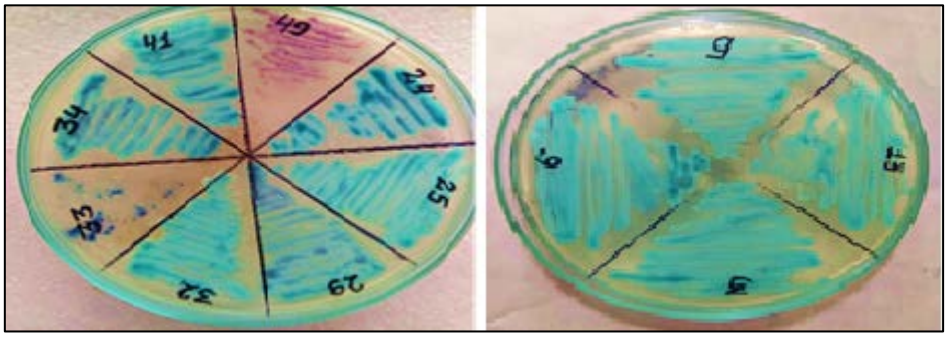

Figure 3: *Candida CHROM agar* plates showing different spp.

3. Morphology on Corn Meal Agar:

Morphology examination of yeast isolated under the microscope is essential to avoid errors in identification of organisms with identical biochemical profiles. Growth in microaerophilic conditions on cornmeal or other starchy media, such as rice agar, stimulates the formation of *hyphae, pseudohyphae, anthrospores and chlamydospores* in those species able to produce them.

The surface of the medium (corn meal agar) is inoculated across the centre of a plate using a wire loop. A sterile coverslip placed over part of the inoculum and the plate incubated at 30 degree C for least 48 hrs. At intervals, for a period of up to one week, the lid of the plate is removed, and the growth under the cover slip examined under the low power objective of a microscope. *Chlamydospores* are indicative of *Candida albicans* and *Candida dubliniensis* but *Candida africana* does not produce these, which can be a useful distinguishing feature.

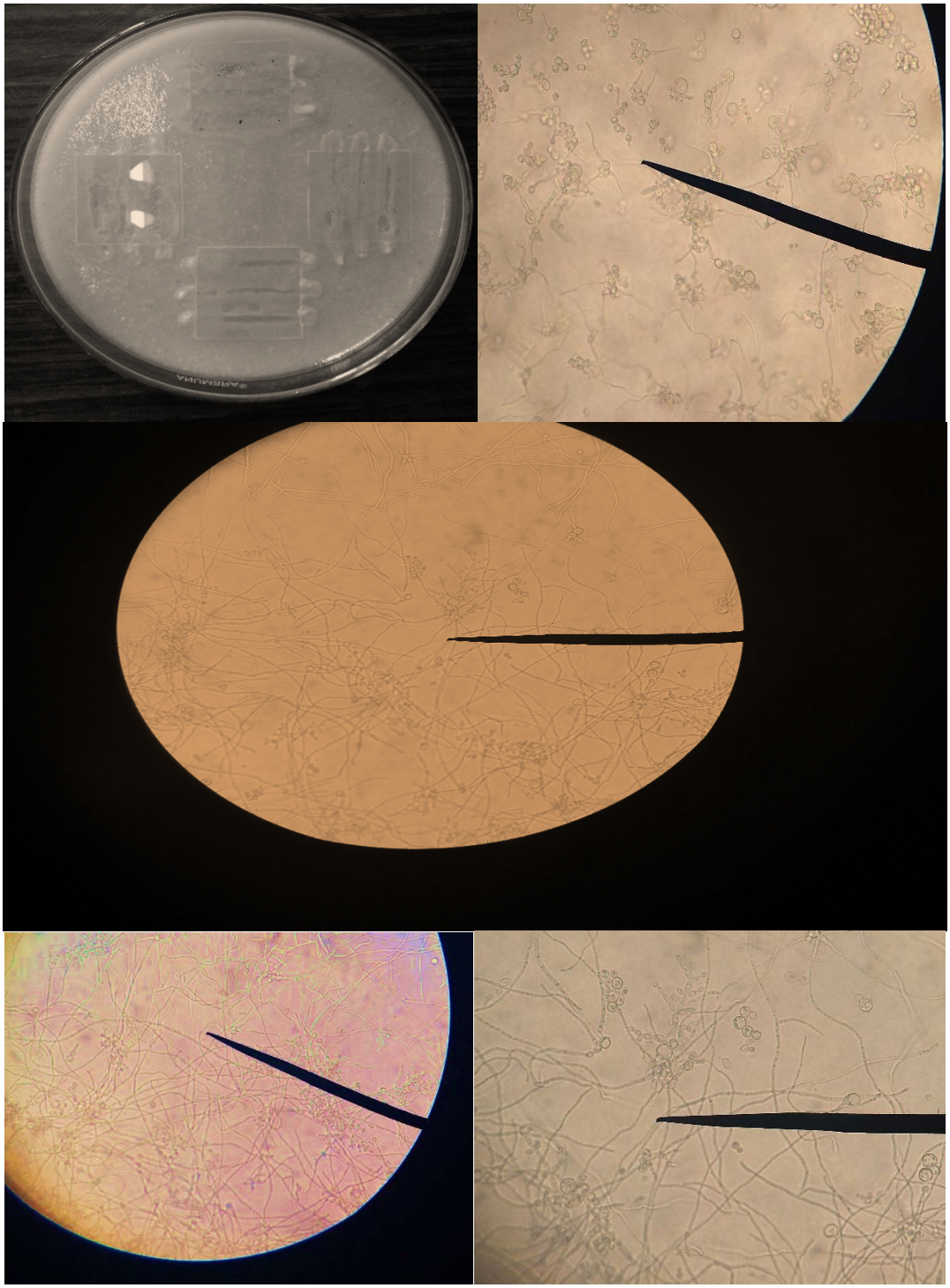

Figure 4-7: showing Dalmau's plate culture and morphology on corn meal agar

4. Germ tube test:

The Germ tube test allows a rapid identification *of Candida albicans, C. dubliniensis, C. africana* from other *Candida* species using either the original isolation plate or a purified culture. The test consists in taking a light loopful of inoculum from a culture plate, suspending it in 0.5 of sterile horse serum and incubating at 37 degree C for 2-3hrs. A drop of the suspension is then placed on a microscope slide, a coverslip is added, and the preparation examined under a microscope. The isolate under test is *C. albicans, C. dubliniensis or C. africana* if the cells have produced short hyphae and there is no constriction at the junction between the parent cell and the hyphae.

Over inoculation of the serum can result in inhibition of germ tube formation and too short an incubation period can lead to false negative results[5].

Positive Test: A short hyphae extension arising laterally from a yeast cell, with no constriction at the point of origin. Germ tube is half the width and 2 to 3 times length of the yeast cell and with no nucleus.

Negative test: No hyphal extension arising from a yeast cell or a short hyphal extension constriction at the point of origin. E.g., *Candida* other than *C. albicans.*

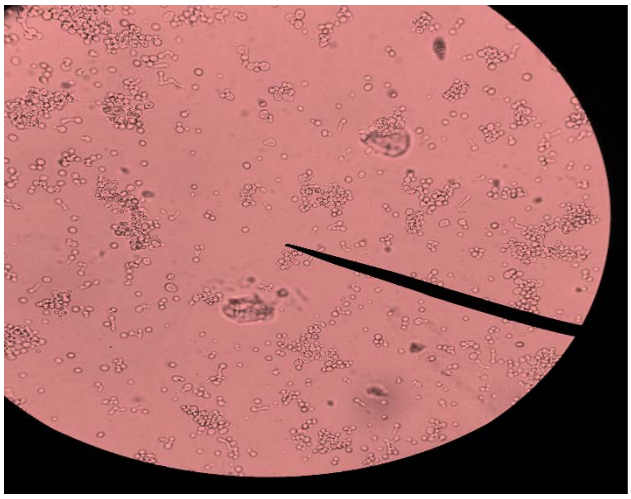

Figure 8: showing the formation of germ tubes

Result:

In this study, out of 100 oropharyngeal swabs taken from HIV seropositive patients, 36 samples were found to be positive for *oral candidiasis.* Majority (56%) of the patients were in the age group of 21 to 40 years. 70 males and 30 females were included in the study. Males (37.1) accounted for the higher prevalence as compared to females (33.3). No significant difference was found in CD4+ counts of males and females.

In this study we found that the prevalence of Oral candidiasis increases as the CD4+ count of the HIV patients decreases. Pearson's Correlation test showed that there was a negative correlation between OC and CD4+ with the value R= –0.967 and p value 0.006.

A total of 6 different *Candida* species were found in our study. *Candida albicans* was found as majorly isolated species with 59%. The 6 different species found in our study were: *C. albicans (59%), C. dubliniensis (16%), C. krusei (5%), C. glabrata (5%), C. tropicalis (5%), C. parapsilosis (5%).* 4 phenotypic tests (sugar fermentation, germ tube test, corn meal agar, *candida* CHROM agar) were used for speciation of Candida isolates. The amount of time taken by different phenotypic methods for Candida speciation was found different with *Candida* CHROM agar has been fastest within 24 hrs of inoculation. However, the time taken by sugar fermentation test varied

from 48-96 hrs. The germ tube test was able to differentiate *C. albicans* from NCAs with higher sensitivity within 2-4 hrs. Morphology on corn meal agar was observed with 24-72 of inoculation.

Table1: Distribution of candida albicans and non- candida albicans (NCA) species in clinical isolates (n=36)

NAME	NUMBER	PERCENTAGE
Candida albicans	22	59%
Non-Candida albicans (NCA)	14	41%

Table 2: Showing prevalence of OC in males and females

SEX	No. of patients (n=100)	No. of patients with oral candidiasis (n=36)	% Of positive patients
MALE	70	26	37.4%
FEMALE	30	10	33.3%

Table 3: Showing the CD4+ count and No. of positive patients of OC

CD4+ Count	No. of positive patients of OC (male+female)
<100	11
100 -200	9
200 – 300	6
300 - 400	6
400 -500	3
>500	1

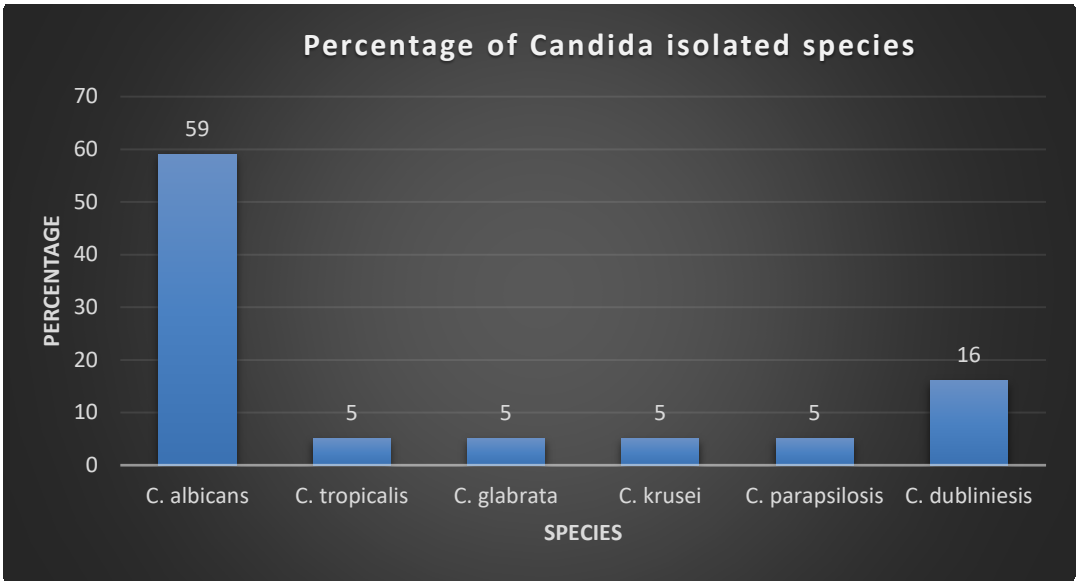

Figure 9: showing the percentage of *Candida* isolated species

Discussion:

Candidiasis or Oral thrush is an opportunistic fungal infection caused by several different species from genus *Candida*, amongst which *C. albicans* is the most common causative species[12]. Species of candida genus may be normal

components of the oral flora in 30% to 50% of the population with no evidence of infection[6].

In some studies, NCAs species specially *Candida tropicalis* has been regarded as the most prevalent Candida species[3,8]. However, the results of our present study show that *Candida albicans* were observed in higher frequency (59%).

Recently during the COVID-19 pandemic, the occurrence of opportunistic fungal infections is dramatically increased in COVID-19 patients with predisposing factors (e.g. diabetes mellitus, mechanical ventilation and cytokine storm). Interestingly, the fungi most commonly associated with COVID-19 infection have been those that commonly colonize the respiratory tract and oropharyngeal mucous membrane, including Aspergillus and candida[7].

According to *Ofonime M. et* al. CD4 cell count has been used extensively as a surrogate marker for HIV disease progression and is an excellent indicator of an HIV-infected patient's risk of development a specific opportunistic infection. The severity of fungal infection has also been shown to increase with fall in CD4 count[9].

A number of protective and very immunogenic vaccine formulations have been developed against Candida infections in the recent times. In one approach *C. albicans mannan* extracts encapsulated in liposomes were used previously to stimulate mice to produce antibiotics protective against candidiasis, in another approach, significant protective was achieved in murine models against vaginal *candidiasis* and disseminated candidiasis by a vaccine conjugated with a protein *laminarin* linked to a carrier protein diphtheria toxoid[12].

In our study 4 different phenotypic methods were used for the speciation of isolated Candida colonies. 4 different methods had different degree of sensitivity and specificity. Germ tube test was found sufficient to differentiate *C. albicans* from NCAs species. Fermentation test was found to be not 100% efficient to identified *Candida species*.

According to *vignesh kanna et al*. CHROM agar facilitates the detection and identification of *Candida* species from mixed culture and provides results in 24-48 hrs. the sensitivity and specificity of CHROM agar for *Candida albicans* were 96% and 100%, *C tropicalis* were 100% and 100%, *C. krusei* were 100% and 100%, *C. glabrata* were 100% and 100% and C. *dubliniensis* were 100% and 100%.

Direct inoculation of CMT agar can easily differentiate common Candida species microscopically on the basis of *chlamydospores, blastospores* and arrangement of *pseudohyphae.* However, identification of rare species from specimens containing two different species using CMT method is challenging[11].

According to *Sidhartha et al*. One major limitation of these 5 phenotypic methods is that they are not able to differentiate between *C. parapsilosis, C. orthosilosis, C. metasilosis. Candida parapsilosis* is a new emergence of infection in the immunocompromised patients. Molecular methods like PCR and RAPD are require differentiating between these three species. We are agreeing with this remark by the author[3].

In our study, we found that immunocompromised patients like HIV seropositives were readily infected by *Candida albicans* followed by NCAs at oral mucous membrane which are living as commensal flora in the healthy individuals, reduction in the CD4 is found prime factor for this. The 4 different phenotypic method used were found different in sensitivity and time consumption.

Limitation:

One major limitation of the study was the less no. of sample. In future any study with more no. of samples and involvement of

molecular techniques will be helpful in further validating the finding of this study.

Conclusion:

In our study, we detected a higher frequency of *oral candidiasis* in HIV seropositive patients. In recent times, fungal infections have increased prevalently in immunocompromised patients as a result of HIV infection, aggressive therapies for cancer, autoimmune disorders and organ transplant[13]. Early detection and speciation of *Candida species* is necessary to cure patients with *candidiasis* because each species is showing different antifungal sensitivity pattern. We also found a good correlation between 4 main phenotypic tests (sugar fermentation test, germ tube test, Hi CHROM agar and morphology on corn meal agar) used for speciation of *Candida.* However different test was differed in their sensitivity and time requirement.

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