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Isolation, characterization and antifungal susceptibility of candida species in suspected cases of oral candidiasis with special reference to oral microbiome at a tertiary care hospital

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Abstract: Candidiasis is the commonest fungal infection infecting human mucosa, skin, nails and internal organs. Oral candidiasis is one of the most common clinical manifestations of candidiasis. In immuno-compromised patients, oral candidiasis progresses to severe systemic infections. Oral candidiasis is caused by different species of Candida. There is a difference in anti-fungal susceptibility among different species of candida. The present study is undertaken to identify different species of Candida causing oral candidiasis and their susceptibility to various anti-fungal antimicrobials by both conventional and automated methods. Oral microbiome has been linked to oral and systemic diseases. The microbial flora at different sites of human body are widely recognized for their role in protecting, initiating, and facilitating disease pathogenesis and oral cavity is relatively understudied in this regard. Therefore, knowledge on how colonization occurs, how oral microbiome co-evolves with host, how they interact with each other may be important to understand etiology and progression of candidiasis The present study will also study the constituents of oral microbiome in presence of oral candidiasis by targeted metagenomics method (Illumina sequencing).

Keywords: Candida, Oral candidiasis, Oral microbiome, targeted metagenomics, VITEK

Introduction

The term Candida is derived from Latin word candid meaning white. Candidiasis is the commonest fungal infection infecting human mucosa, skin, nails and internal organs [1]. Oral candidiasis, as recognized throughout the history, is the most common clinical manifestations of candidiasis. In recent years, there has been an increased interest in infections caused by opportunistic pathogen, Candida. The growing interest in Candida is also due to emergence of HIV and other

immuno-compromised conditions. Oral candidiasis is a HIV-defining condition [2].

Candida albicans (C. albicans), C. glabatra, C. tropicalis, C. krusei, and other species of Candida have been isolated from suspected cases of oral candidiasis [3]. In the past few years an increase in isolation of non-C. albicans species is seen in suspected cases of oral candidiasis [4, 5]. There is a difference in anti-fungal susceptibility among different species of Candida. Resistance to common antifungal drugs like fluconazole, ketoconazole are very common [4, 6]. Therefore, antifungal sensitivity of Candida spp. is important for the proper treatment of oral candidiasis.

Microorganisms found in human oral cavity have been referred to as oral microflora, oral microbiota or oral microbiome.

The oral microbiome is crucial in maintaining oral and systemic health [7]. The microbial flora at different sites of human body are widely recognized for their role in protecting, initiating, and facilitating disease pathogenesis [8, 9] and oral cavity is relatively understudied in this regard.

The present study is undertaken to identify different species of Candida causing oral candidiasis and their susceptibility to various anti-fungal antimicrobials by both conventional and automated methods. The present study will also study the constituents of oral microbiome in presence of oral candidiasis by targeted metagenomics method (Illumina sequencing).

Methods

The study was started after approval of institutional ethical committee of the institution Two hundred oral sabs were collected from suspected cases of oral candidiasis from March 2020 to August 2021 from various departments of our hospital. Swabs from lesion sites were collected by cotton swabs by gently rubbing them over the oral cavity lesions. After collection of specimens mycological tests were done.

For targeted metagenomics study of oral microbiome, thirty salivary samples were collected. Subjects were told not to eat, drink or smoke 30 minutes before giving their salivary sample. Fifteen samples were from oral candidiasis patients and fifteen from healthy individuals.

Microscopy

It was done by using 10% KOH and Gram's staining.

Culture

Culture was done on SDA (Sabouraud's Dextrose Agar) with and without antibiotics (chloramphenicol and cycloheximide). The inoculated SDA were labelled with patient's name and lab ID and incubated at 25ºC and 37ºC. It was observed on daily basis for fungal growth. The growth and colony characteristics were recorded. Identification of Candida species was done following growth of fungi.

Gram's stain from growth

If Gram positive oval budding yeast like cell were observed then it was identified as Candida species.

The colony of Candida species was further identified up to species level by following tests.

1. germ tube test

Germ tube test was done and observed for tube-like extension from parent cell half the width and three to four times the length. This was done by picking one single colony from SDA and incubating it with 0.5 ml of human serum at 37 ºC for 2 – 3 hours.

2. Growth on chrome agar

Candida species were inoculated in Hichrom Candida differential agar. Then it was incubated at 25-30°C for 48 hours. After two days of incubation colour of colony was recorded.

3. Corn meal agar [Dalmau culture method]

Colony of Candida species was streaked on Corn meal agar by Dalmau technique and was incubated at 25°C for 24-72 hours. The presence of budding cells, hyphae, blastospores, and chlamydospores were examined under low power magnification. Examination was done daily for up to four days.

4. Sugar fermentation test

A set of these 4 sugars (Maltose, Glucose, Sucrose, Lactose) were used for the identification of each *Candida i*solate. The liquid media used consisted of 1% peptone, 0.5 NaCl and Andrade's indicator. Filter sterilized sugars are added at 2% to the medium. The solution was then poured to test tubes containing Durham's tube. Each tube was inoculated with 0.1 ml yeast inoculum from SDA. The tubes were incubated at 25º C up to one week and examined at every 24 hours interval for the production of pink colour for acid and gas in Durham's tube

5. Sugar assimilation test (disc impregnation technique (pour plate auxo graphic method)

For sugar assimilation test 12 sugars were used. (Glucose, Maltose, Sucrose, Lactose, Galactose, Mellibiose, Cellobiose, Inositol, Xylose, Raffinose, Trehalose and Dulcitol). First Yeast nitrogen base (YNB) was prepared. In 2ml YNB, a heavy inoculum from 24 -48 hours old culture was mixed to make a suspension. This was mixed with 18 ml of molten agar and poured into a petri plate. Disc impregnated with carbohydrate were placed onto surface of this plate it was incubated at 37ºC for 3 -4 days. The presence of growth around the disc was considered positive

6. Vitek-2

VITEK 2 was performed directly from culture from SDA by using VITEK 2 machine. Results was taken out in printed format. Both isolation of species and anti-fungal sensitivity results were recorded.

Antifungal sensitivity test-disc diffussion method

It was done using Mueller-Hinton Agar plus 2% Glucose and 0.5 µg/mL methylene blue Dye medium. Antifungal susceptibility testing by disk diffusion method was performed according to CLSI guidelines.

Targeted metagenomics

a) DNA extraction

The DNA from salivary samples was extracted using the RTP Pathogen Kit, following the manufacturer's instructions.

b) 16S Metagenomic Sequencing Library Preparation and Data Analysis

One of the important steps in genomic sequencing is Library preparation.

It was done using Illumina sequencing by following 16S Metagenomic sequencing library preparation protocol. It helps the DNA to adhere to the flow cell of sequencer and helps in sample identification. It consists of following steps like amplicon PCR, Index PCR, PCR clean up, Library quantification and pooling and sample loading. After this procedure bioinformatics was done for data analysis. It is the method of subjecting the DNA sequence to wide range of analytical methods to understand its features, functions, structure or evolution. We screened reads for high quality bases and trimmed low quality bases. In the present study it was done to detect oral microbiome in healthy people and oral candidiasis patients using DADA2 pipeline and microbiome analyst software.

Statistical analysis

The data were statistically analysed by using Microsoft Excel, t test and microbiome analyst software.

Results

Out of 200 suspected cases 120 (60%) were females and 80 (40%) were males. The culture was also more positive in females compared to males. In all, 64% of samples collected from female patients showed growth of Candida. Out of 200 suspected cases, 36 samples recorded growth upon culture indicating the prevalence to be 18%. Out of total cases of oral candidiasis, 41.66% had uncontrolled diabetic mellitus, 13.88% was due to dentures and corticosteroids, 11.11% patients were exposed to higher antibiotics and other immuno suppressive drugs. HIV was a risk factor in 11.11% patients. Smoking and usage of tobacco constituted 8.33%.

Out of 36 Candida species isolated**,** 15 were C. albicans, 5 were C. tropicalis, C. krusei, and C. parapsilosis. C. dubliniensis and C. glabrata constituted 3 each out of total by conventional methods. Out of 36 culture grown, 24 (66.66%) showed yeast cells on direct Gram's staining

Vitek 2 identification

Out of 36 Candida grown, C. dubliniensis, C. tropicalis, and C. glabrata were identified same compared to conventional method. C. ciferri was not identified by using conventional methods. Statistical analysis carried out for conventional and automated methods, shows almost perfect agreement of 88.88% (95% CI) in identification of 32 out of 36 Candida isolates.

Antifungal sensitivity by disc diffusion and VITEK 2: All Candida species were sensitive to amphotericin. C. dubliniensis, C. parapsilosis, and C. ciferii showed 100 percent sensitivity to all 3 anti-fungal drugs by both the methods. C. albicans showed 80% and 84.61% sensitivity to fluconazole by both disc diffusion and VITEK- 2 susceptibility testing respectively. It was 100% sensitive to voriconazole by disc diffusion whereas 7.7 % showed resistance by VITEK- 2.

In all, 80% of C. tropicalis were susceptible to fluconazole by both the methods and showed 100% and 80% sensitivity to voriconazole by disc diffusion and VITEK- 2, respectively. C. krusei and C. glabrata showed 100% resistance to fluconazole and voriconazole and 100% sensitivity to amphotericin by both the methods. All species were sensitive to amphotericin by both the methods.

Oral microbiome

In the present study, a comparison was made among 20 most abundant genera in oral microbiome between oral candidiasis and healthy Individuals.

It was seen that there was an increase in Veillonella and Allo Prevotella and a decrease in Rothia, Gamella, Fusobacterium and Corynebacterium in oral candidiasis patients compared to healthy individuals.

By using t test as statistical method alpha diversity graph was plotted by microbiome analyst software based on comparison of oral candidiasis patients and healthy individuals. P value was 0.29, which was not statistically significant.

Discussion

Age and Gender distribution

In the present study oral candidiasis patients were divided into 4 different age groups: 1-10 years, 11-30 years, 31-50 years and above 50. Out of 36 growths seen 18 belonged to people above 50 years of age and 23 were females.

Jayachandran et al from Tamil Nadu reported that highest prevalence (58%) of oral candidiasis was seen in above 40 years of age [10]. Magare et al [11] also reported highest prevalence of Candida species in the age group 40 and above in their study. Singh et al stated

that C. albicans was isolated in 60% of all people more than 60 years in their study conducted in Uttar Pradesh; India[3]. Their study reported an increase in rate of carriage with increasing age. The present study is comparable to most of the studies conducted in India.

A probably explanation, for elderly being more affected by oral candidiasis, is due to the presence of risk factors in these age groups. The worsening of overall state of health, poor oral status, and the side effects of treatments taken may be some contributing risk factors. They may be more affected due to increase exposure to medications that reduce the number of beneficial bacteria in our body like antibiotics and steroids. In a similar study in Brazil

by Araujo et al., women were affected more than men by Oral candidiasis [12]. The authors attributed that this predilection to women was due to greater number of dental prostheses used by them. Gaspar to also reported more female to male ratio in their study conducted in Brazil [13]. Caldeira et al concluded that women have a higher perception of the impact of rehabilitation on the quality-of-life indexes, are more concerned with oral health and are more often looking for oral care for function rehabilitation and aesthetics.[14]

Prevalence

The prevalence of oral candidiasis in the present study was comparable with the studies done by Mubarak et al, Shafi et al, and Datta et al[15, 16, 17].

Risk factors

Diabetes mellitus was a major risk factor involved in oral candidiasis as per a study conducted in Karnataka, India by Shafi et al [16]. It was followed by other factors like COPD, cancer, and exposure to drugs like corticosteroids. Datta et al reported diabetic mellitus as highest risk factor for oral candidiasis (42.2%), followed by HIV and exposure to immunosuppressive drugs [17]. Tarcin et al stated in his study that growth of Candida in saliva is enhanced by glucose despite the presence of nutrient competing bacteria in salivary flora. He added that glucose favors Candi dial adhesion and biofilm formation [18]. Oral candidiasis is an AIDS defining condition. There are many studies which show HIV as a major risk factor for oral candidiasis [19, 20]. In the present study, it is only 11.11%. From the time of introduction of HAART, the prevalence of oral candidiasis in HIV patients are showing a decreasing trend [21].

Species distribution

In present study NCA's [Non-Candida albicans] (58.34%) were more isolated than C. albicans (41.66%).

A study was conducted in Assam (2008-2009) also observed an increase in non-C. albicans species in their study [17]. In a study conducted in Maharashtra (2014) to evaluate the prevalence of candida species in oral cavity of immuno-compromised patients, Candida albicans was most isolated, followed by C. tropicalis, and C. krusei [11]. Baradkar et al conducted a study in Mumbai (2009) to identify Candida isolates from oral lesions in HIV infected patients. C. albicans was the commonest isolate (70%) followed C. parapsilosis (15%), C. glabrata (7.5%) and C. tropicalis (5%), respectively [22].

Even though the present study differs from all the above-mentioned studies in species spectrum and percentage of isolation, there is an observable increasing trend of NCA species isolated in different parts of world.

Comparison with VITEK- 2 identification

Statistical analysis carried out for conventional and automated methods, shows almost perfect agreement of 88.88% in identification of 32 out of 36 Candida

isolates. A similar study conducted in India by Prasanna et al showed substantial agreement of 80% (95% CI of 69 – 91%) in identification of 40 out of 50 isolates. [23] Study conducted at New Delhi by Kaur et al showed more than 94 % agreement between both methods.[24] The present study is comparable with most of all studies mentioned above, suggesting VITEK-2 system an alternative method for speciation of Candida species infections.

Anti-fungal sensitivity testing by disc diffusion and VITEK- 2

The present study showed similar patterns of antifungal sensitivity by both disc diffusion method and VITEK- 2, with more resistance towards fluconazole and voriconazole and 100% sensitivity to amphotericin. Antifungal sensitivity pattern comparison between conventional and VITEK- 2 have shown similar results in other studies also [24, 25, 26]. This makes VITEK- 2 an alternative method for finding the antifungal susceptibility pattern for Candida species. The present study has documented that NCA showed a higher percentage of resistance compared with Candida albicans.

A similar antifungal sensitivity pattern was found in another study [27]. They determined antifungal susceptibility pattern of 171 Candida isolates. Nystatin (83.6%) was the most sensitive drug, followed by amphotericin (76%) The most resistant antifungal drugs were ketoconazole (29.2%), followed by fluconazole (24.4%). Jayachandran et al conducted a study in south India which showed the following findings [10]. The overall resistance percentage for fluconazole and Itraconazole was 14% and14.8%, respectively. Fluconazole showed a resistance of 5.8% and 12% for C. albicans and C. tropicalis, respectively. None of the isolates of C. krusei was susceptible to fluconazole similar to the present study. Amphotericin sensitivity pattern is also comparable with the present study. This study also documented that NAC showed a higher percentage of resistance than C. albicans. These results are in concurrence with the results of other studies like Barry et al and Ruhnke et al [28, 29].

Inappropriate usage of azoles for the empirical treatment of Candi dial infection, has led to a rise in the incidence of non- albicans Candida like C. krusei with reduced susceptibility to azole antifungal agents [30]. Such drug resistant strains can invade underlying mucosa and enter blood stream causing invasive infections.

Oral microbiome

Oral microbiome is constantly changing with the host condition and other factors. Microbiome of oral candidiasis remains poorly understood. There are only a very few studies associated with this regard.

In the present study, a comparison was made among twenty most abundant genera in oral microbiome between oral candidiasis and healthy Individuals. It was seen that there was an increase in Veillonella and Allo Prevotella and a decrease in Rothia, Gamella, Fusobacterium and Corynebacterium oral candidiasis.

Veillonella species have been associated with the development of many oral diseases such as oral caries, endodontic infections and periodontitis [31, 32]. These diseases are important risk factors associated with oral candidiasis. Allo Prevotella is also known to cause similar conditions [33].

A similar study was conducted by Lyu et al at Beijing in 2021 where they compared microbiome of oral candidiasis and healthy individuals [34]. They found an increase in Rothia and Gamella genera in contrary to the present study. Using 454 pyrosequencing, Kraneveld et

al from Netherlands (2012) revealed that oral Candida load correlated negatively with the diversity of the salivary microbiome and positively with the relative abundance of Streptococci in older adults [35]. In the present study, percentage of Streptococci was same in case of both healthy and oral candidiasis patients.

From above observations, it is well understood that more studies are required in this field to know more about importance of oral microbiome and oral candidiasis.

Conclusion

To conclude the present study highlights the incidence of oral candidiasis in a tertiary care hospital. The knowledge of risk factors like over usage of antibiotics and corticosteroids can be prevented by appropriate implementation of antibiotic stewardship program. Complications of other risk factors like diabetes mellitus and HIV can also be reduced by appropriate treatment. Thus assessment of risk factors associated with oral candidiasis is very important in each and every case.

The resistance pattern of Candida species varies from species to species. Hence identification and determining anti-fungal sensitivity pattern are very important for the physician for proper treatment. The present study also shows importance of VITEK- 2 as an alternative technique for identification and determining antifungal sensitivity of Candida species.

More studies are required for microbiome detection to know about colonization, how oral microbiome co-evolves with the host, how they interact with each other which in turn helps to understand etiology and progression of candidiasis.

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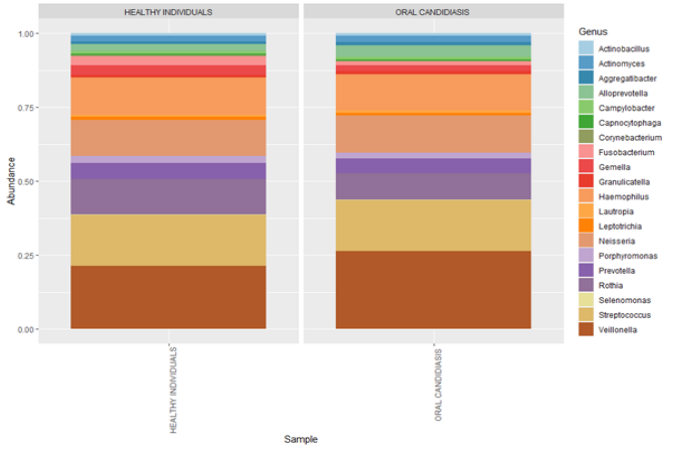

Fig 1: Comparison of top 20 Genera isolated from healthy people and oral candidiasis

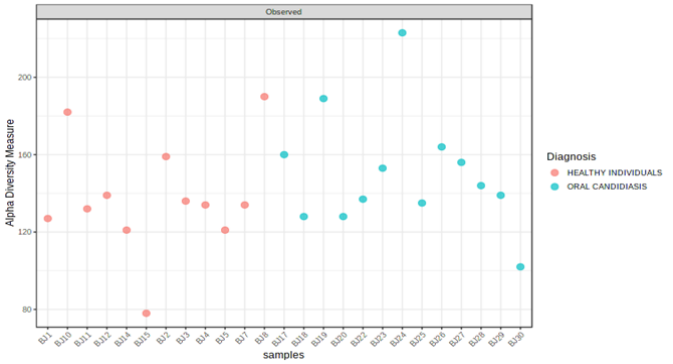

Fig 2: Alpha diversity graph (p-value: 0.29182)

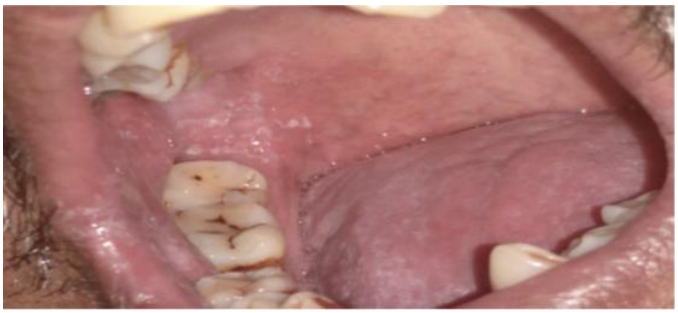

Fig 3: oral candidiasis.

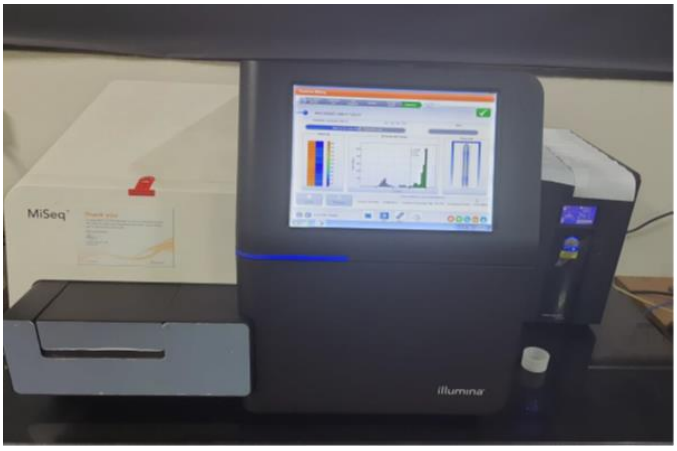

Fig 4: Illumina machine

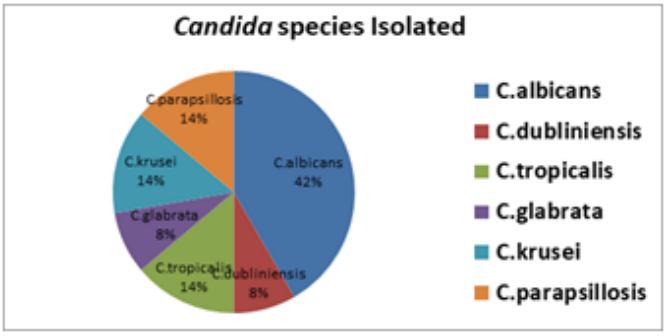

Fig 5: Candida species isolated by conventional methods.