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Presumptive Identification Of Clinically Important *Candida Sps* From Oral Infections Of Diabetic Patients

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ABSTRACT

Purpose: The present study is aimed to determine the prevalence of *Candida* evolution, diversity, and their influence during infection as Diabetic subjects are very much prone to several secondary infections caused by microorganisms.

Materials and methods: 40 (30-70 age) patients were selected for the study who is regularly attending hospitals for medication, oral washings were collected regularly and cultured. The yeast like organisms were isolated and identified by various phenotypic and genotypic characterizations.

Results and conclusions: In diabetic patients, the frequency of *Candida* infection was found to be significantly higher than other infections, which was more prevalent and opportunistic infection. Albeit, *Candida albicans* was isolated predominantly in diabetic subjects, *Candida tropicalis*, *Candida ontarioensis*, *Dipodascus capitatus* are also involved in candidiosis. Interestingly it was observed a positive correlation in between glycemic control as well as the *Candida* colonization.

Key words: diabetic subjects, oral infections, Candida sps, CHROM agar.

INTRODUCTION:

Yeasts that exist as single cells are capable of reproducing quickly by budding; chains of buds, forming a short mycelium, referred to as pseudo mycelium, blastoconidia are produced by some species. *Candida* is yeast like fungi belonging to opportunistic pathogens that are frequently found in oral cavity and also capable of causing variety of diseases. *Candida albicans* and *non-albicans* are known to cause oral infections[1] . In immunocompramised patients, 90percent of the *Candida* sps are involving in nosocomial infections of the prolonged hospitalized patients. So far 100 species of Candida were identified however, some species (*C.albicans, C.tropicalis, C.parapsilosis,* and *C.ontareoensis*) were isolated from medical samples. *C.albicans* was frequently considered the part of normal flora, especially from mucocutaneous sources of the oral cavity.

Oral Candidiosis is an infection caused by several species of *Candida*. In general *Candida* is considered as normal flora at a frequency of 44-55% is supported by exofoliative cytology and also the predominancy shown in hospitalized patients [2]. The clinical manifestations and diagnosis of oral candidiosis relies on recognition of granular, erosive and pseudo membranous forms of infection, easily removable curd like plaques of oral cavity. However, substantial colonization can exists in the form of clinical lesions, the developed cultures have been confirmed usually by PAS (Periodic Acid Schiff)staining, culturing and also identified by germ tube technique etc.,

Diabetes is a multisystem metabolic disorder characterized by abnormal carbohydrate, protein and lipid metabolism due to decreased insulin secretions and

disturbed insulin activity[3]. Diabetes has been related to numerous oral complications such as periodontal diseases, decreased function of salivary glands (xerostomia) and burning mouth sensation. Subjective oral dryness is a frequent complaint among diabetic patients than the healthy subjects.

Another manifestation of diabetes is an oral sign of systemic immune suppression which enhances the opportunistic infections such as oral candidiosis. There are many reports suggested that *Candida albicans* is the commonest species, that harbors in the oral mucosa of the diabetic patients.

Our present study focuses on the biodiversity of the *Candid*a sps in oral infection of diabetic patients. Different sps had been involved and that influence the oral *Candida* infection. *Candida* sps are detected by various identification methods and distinguished by molecular genetic analysis. The *Candida* infections were believed to be more frequent in people with diabetes The *Candida* infections were believed to be more frequent in people with diabetes [4]. it has been suggested that the highest rate of colonization in diabetic patients with poor serum glucose controls. The main goal of the study is to determine the prevalence of *Candida* infection in diabetic patients and also controlling measures for predisposing factors.

MATERIALS AND METHODS:

Oral washings were collected from the diabetic subjects who is attending clinics under treatment have been chosen for the present study. Based on various signs and symptoms of oral infections selected 40 patients and also equal controls were examined. All the patients were asked to sign on consent form in order to understand the data (not for commercial purpose). The details of patient information in a demographic form and health status data was shown in **(Table 1)** Oral washings were collected and then inoculated on sabourad dextrose agar and incubated at 37°c for 24hr **(Fig 1&2).**

Isolated yeast cells were inoculated on the plasma fluid and then incubated at 37°c for 3hr. After incubation the aliquots were subjected for microscopic observation. Germ tube was considered as a slender tube with straight walls without septum and constriction at the junction between the cells. But most of the *Candida* sps have been differentiated by germ tube test. (**Fig 3)**

Carbohydrate assimilation is the utilization of a carbon source in the presence of oxygen, a positive assimilation reaction is usually indicated by the presence of growth and using carbohydrate impregnated discs is a convenient method and commonly used. Carbohydrate utilization profiles are used to distinguish the organism's up to species level and also their characteristics. A carbohydrate free basal medium was used for carbohydrate assimilation test and results were recorded in **(Table 1)**

Carbohydrate fermentation test was performed aerobically by yeast to produce ethanol and carbon dioxide, the fermentation process was confirmed by acids as well as gas production. The *Candida sps* can also be differentiated by fermentation test and six different carbohydrate components like glucose, lactose, sucrose, galactose, maltose, and trehalose and were incubated at 25°C-35°C for 24 days. The results were

noted for every 2-3 days. **(Table 1)**

Urease test was conducted for identifying the medically important yeast like fungi within the genera of *Candida, Cryptococcus, rhodotorula, trichosporon*. Urea hydrolysis was performed by using Christensen's urea agar medium. The slants were prepared and then cultures inoculated on to the slants and incubated for five days at 30°C (**Table 1).**

Tween 80 agar test was used for the differentiation of *Candida sps*. The fresh overnight cultures were inoculated on the tween 80 agar and incubated for 7 days **(Fig 4).**

CHROM agar medium is a selective and differential medium for the isolation of dimorphic fungi with the inclusion of chromogenic substrates in the medium , the colonies of *Candida sps* produce different colors thus allowing the direct detection of organisms on the culture plate usually *Candida albicans* appear light green in colour , *Candida tropicalis* appear bluish green and light pink in colour, creamy etc other yeast may develop either natural color or appear rose or light to dark , main advantage of this medium is for differentiation and for easy detection of mixed cultures of yeast **(Fig 5).**

The Candida sps differentiated on chrome agar were analyzed by various molecular methods i.e. by amplifying fragment of 18S region by using PCR and application of several bioinformatics tools for the identification of different species of the *Candida*, based on the nucleotide homology and phylogenetic analysis the *Candida sps* were differentiated. In our present study there are four different species which were differentiated genetically and sequenced**(Fig 6).**

Results:

The *Candida albicans* is the predominant species causing the oral infections of diabetic subjects, present investigation showed that there were several *Candida sps have been* isolated and identified was playing an important role in the disease manifestation (i.e. non albican species). As per the data shown in the **(Table 2),** 50.2% of the diabetic patients were affected by *C.albicans,* 30% by *C.tropicalis, 15*% by *C.ontarioensis,* 10% by *Dipodascus capitatus.* Clinically significant or pathogenic *Candida sps* was usually confirmed by germ tube test which is one of most predominant and preliminary test for the detection of the *C.albicans* and *C.tropicalis.* There are various biochemical tests were performed for the identification and confirmation of *Candida sps* carbohydrate assimilation test is one of the most prominent test widely used for *Candida sps* differentiation, it can be differentiated by the growth around the carbohydrate impregnated discs. The pattern of carbohydrate assimilation is considered a reliable test and is generally used for the correct identification of yeasts of clinical interest, (*C. albicans, C. tropicalis, C. krusie, C. parapsilosis*) the present investigation showed the results of *C.ontarioensis*(non-albicans) and non *Candida sps* (*Dipodascus capitatus*)chosen for the present study were tabulated in(**Table 1**). Carbohydrate fermentation tests conducted for all the isolated *Candida sps (both albicans and non-albicans)* resulted the acid and gas formation that represents the specific carbohydrate utilization that differentiates the

Candida colonization patterns and their results were recorded in **(Table 2)**. *C.ontarioensis* and. *Dipodascus capitatus*(non *Candida sps*) were grown effectively on selective medium with dark gray and pink -white color mixed margins were the close representative features of newly detected sps showed clinical importance into the manifestation of the virulencity .Generally most of *Candida sps* (C. krusie) produced the optimum levels of urease enzyme, which hydrolyses the urea to ammonia & carbon dioxide by changing the medium colour from yellow to red / pink after five days of incubation, which indicates the positive result. In these lines we conducted the urease hydrolysis test for the isolated *Candida sps*(both albicans and non-albicans), no considerable results were obtained from our study **(Table 1).** Similarly the tween 80 agar test was conducted for the differentiation and evaluation of the lypolityc activity of the strains of *Candida sps* especially *C. albicans & C. dubliniensis* from other species. After the considerable incubation period , hallow growth was obtained that is centrally opaque and fried egg appearance would observed at the site of inoculation, due to the production of esterase enzyme , this hallow site represents the positive result for the *Candida sps*. Further, all the isolated *Candida* sps from SDA were selected and re-inoculated on the CHROM agar medium which is a selective and differential medium used for species differentiation. After the Incubation at 37°C for 24hrs, all the Candida sps were effectively grown and were observed in different colour after 48hrs of incubation. 40isolates were chosen for present study and very interestingly, almost all the species were showed magic in the colour formation that is green color by *C.albicans,* the distinctive dark blue-gray color shown by C.tropicalis, only dark gray colour by C.ontarioensis and pink-white mixed margins by Dipodascus capitatus **(Table 2).**The species which were differentiated by the CHROM agar were subjected to PCR and electrophoresis, based on the formation of bands the differentiation of the species were selected for further sequencing process. **(Fig 6)**.The 18S region was sequenced (ABI 3730*1 genetic analyzer) and later performed the BLAST analysis with NCBI Database of gene bank. Based on the maximum identity score all the species were named and their Gene Bank accession numbers were recorded. ***Candida tropicalis*** the Gene Bank accession number: **JQ008834.1.** It was showing 99% of similarity with the *Candida tropicalis* original sps of gene bank, *Candida tropicalis* showing the pink color colonies and the blue color colonies on the CHROM agar. ***Candida ontarioensis*** the Gene Bank accession number: **JN820129.1.** It was showing 99% of similarity with the *Candida ontarioensis* original sps of gene bank *Candida ontarioensis* showing the grayish purple on the CHROM agar. ***Candida albicans*** the Gene Bank accession number: **HQ876034.1.** It was showing 99% of similarity with the *Candida albicans* original sps of gene bank, *Candida albicans* showing the green color colonies on the CHROM agar ***Dipododascus capitatus.*** The Gene Bank accession number: **ab083080.1. I**t was showing 99% of similarity with the *Dipododascus capitatus* original sps of the gene bank Dipododascus *capitatus* showing the pink colonies with the white borders on the CHROM agar.

Table-1: Based on different biochemical tests the Candida sps variation is observed.

	Assimilations												Fermentations						Other Reactions			
	Glucose	Maltose	Sucrose	Lactose	Galactose	Melibiose	Inositol	Xylose	Cellobiose	Raffinose	Trehalose	Dulcitol	Glucose	Maltose	Sucrose	Lactose	Galactose	Trehalose	Urease	Pseudohyphae	Growth of 37° C	Germ tube
Candida albicans	+	+	+	-	+	-	-	+	-	-	+	-	AG	AG	A	-	AG Or A	AGOrA	-	+	+	+
Candida parapsilosis	+	+	+	-	+	-	-	+	-	-	+	-	AG	-	-	-	AG Or A	AG Or A	-	+	+	-
Candida tropicalis	+	+	+	-	+	-	-	+	+	-	+	-	AG	AG	AG	-	AG	AG	-	+	+	+
Candida krusie	+	+	-	-	-	-	-	-	-	-	-	-	AG	-	-	-	-	-	+	-	-	-
Candida ontarioensis	+	+	+	+	-	-	-	-	+	-	+	-	AG	A	AG	AG	A	A	-	-	+	-
Dipodascus capitatus	+	-	+	-	+	-	-	+	-	-	+	-	AG	AG	AG	-	A	A	-	-	+	-

Table 2: List and Details of the diabetic patients with their age, sex and representing the Candida species based on germ tube formation and colony colour formation on the chrom agar and the bio diversity of Candida species in oral infection of the diabetic patients.

s: no	name of the patient	Sex	age	Colour of the colony observed on the CHROM agar	Germ tube test	Organisms observed
1	Subject-1	M	30	Green	+ve	*Candida albicans*
2	Subject-2	F	60	Green	+ve	*Candida albicans*
3	Subject-3	M	50	Green	+ve	*Candida albicans*
4	Subject-4	M	60	Green	+ve	*Candida albicans*
5	Subject-5	M	60	Green	+ve	*Candida albicans*
6	Subject-6	F	55	Green	+ve	*Candida albicans*
7	Subject-7	M	70	Green	+ve	*Candida albicans*
8	Subject-8	F	41	Green	+ve	*Candida albicans*
9	Subject-9	F	56	Green	+ve	*Candida albicans*
10	Subject-10	M	68	Green	+ve	*Candida albicans*
11	Subject-11	M	40	Green	+ve	*Candida albicans*
12	Subject-12	M	35	Green	+ve	*Candida albicans*

13	Subject-13	F	44	Green	+ve	*Candida albicans*
14	Subject-14	F	50	Green	+ve	*Candida albicans*
15	Subject-15	M	43	Green	+ve	*Candida albicans*
16	Subject-16	M	43	Green	+ve	*Candida albicans*
17	Subject-17	M	55	Green	+ve	*Candida albicans*
18	Subject-18	F	60	Green	+ve	*Candida albicans*
19	Subject-19	M	70	Green	+ve	*Candida albicans*
20	Subject-20	M	60	Green	+ve	*Candida albicans*
21	Subject-21	M	75	Green	+ve	*Candida albicans*
22	Subject-22	F	46	Green	+ve	*Candida albicans*
23	Subject-23	F	53	Baby Pink	+ve	*Candida tropicalis*
24	Subject-24	M	56	Baby pink	+ve	*Candida tropicalis*
25	Subject-25	M	62	Baby pink	+ve	*Candida tropicalis*
26	Subject-26	M	43	Metallic blue	+ve	*Candida tropicalis*
27	Subject-27	M	60	Metallic blue	+ve	*Candida tropicalis*
28	Subject-28	F	60	Metallic blue	+ve	*Candida tropicalis*
29	Subject-29	M	50	Metallic blue	+ve	*Candida tropicalis*
30	Subject-30	M	42	Metallic blue	+ve	*Candida tropicalis*
31	Subject-31	M	46	Metallic blue	+ve	*Candida tropicalis*
32	Subject-32	M	55	Metallic blue	+ve	*Candida tropicalis*
33	Subject-33	M	58	Grayish purple	-ve	*Candida ontarioensis*
34	Subject-34	F	60	Grayish purple	-ve	*Candida ontarioensis*
35	Subject-35	M	39	Grayish purple	-ve	*Candida ontarioensis*
36	Subject-36	F	43	Grayish purple	-ve	*Candida ontarioensis*
37	Subject-37	M	46	Grayish purple	-ve	*Candida ontarioensis*
38	Subject-38	M	43	Pink with white borders	-ve	*Dipodascus capitatus*
39	Subject-39	F	66	Pink with white borders	-ve	*Dipodascus capitatus*
40	Subject-40	F	50	Pink with white borders	-ve	*Dipodascus capitatus*

Fig 1: Colony formation on the SDA plate of the samples isolated from the oral cavity of the diabetes.

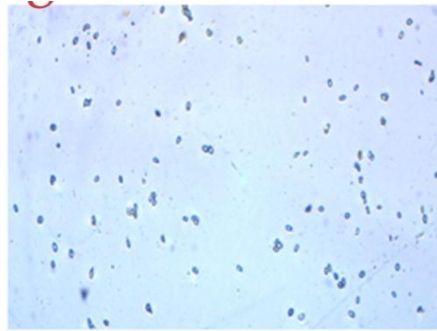

Fig 2: Microscopic observation of the yeast cells formed on the SDA plate at X40.

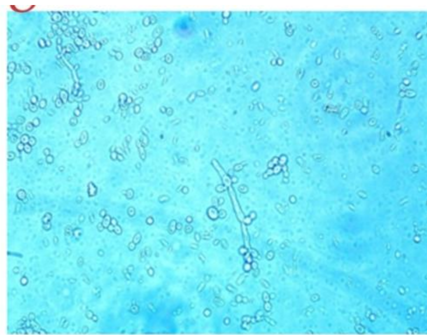

Fig 3: The preliminary identification of the *C.albicans, C.tropicalis* by the formation of the germ tube identification by germ tube technique.

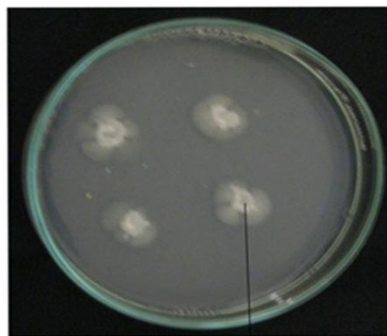

Fig 4: Identification of Halo growth at the site of inoculation on tween 80 Agar plates.

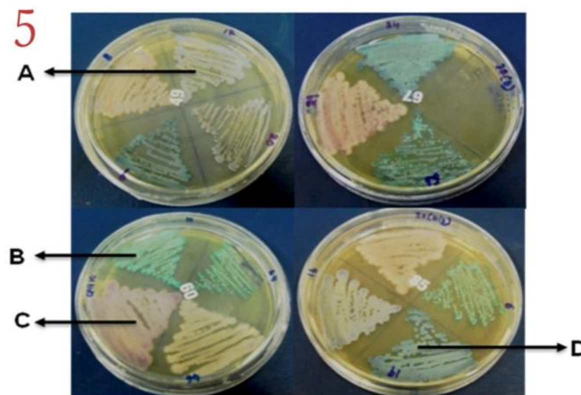

Fig 5: Colony color differentiation of different Candida sps namely (A) *C.ontarioensis* (B) *C.albicans* (C) *Dipodascus capitatus* (D) *C.tropicalis* on CHROM agar plates.

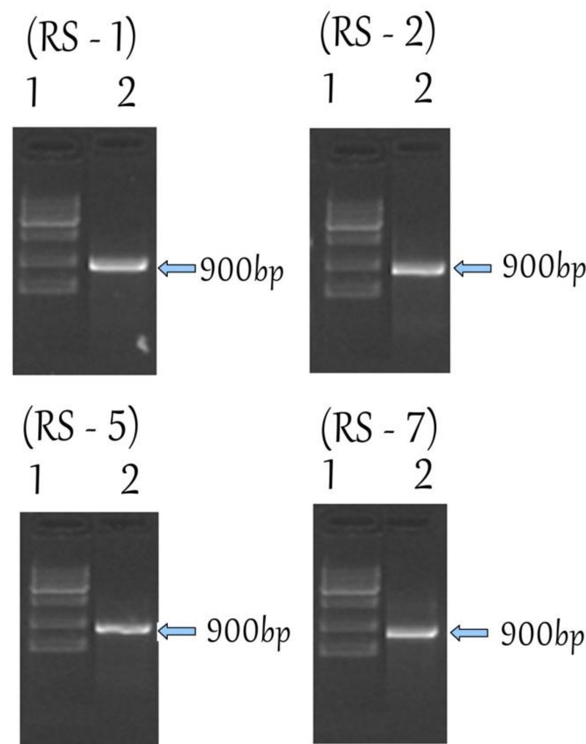

Fig 6: 1.2% Agarose gel showing single 900 bp and 18S rDNA amplicon of (RS-1) *C.tropicalis*, (RS-2) *C.ontarioensis,* (RS-5) *C.albicans* and (RS-7) *Dipodascus capitatus*. Lane 1 represents the DNA marker (1kb ladder) and Lane 2 represents the 18S rDNA amplicon.

Discussion:

Diabetes is the chronic disease throughout the world, frequently associated with many risk factors. It is a syndrome of abnormal carbohydrate, fat and protein metabolism due to decreased insulin secretion or disturbed insulin activity. Diabetes has been related to numerous oral complications, such as periodontal disease, decreased function of salivary glands, burning mouth sensation, oral dryness is more frequent complaint among diabetic patients [5]. Another manifestation of diabetes is systemic immunosuppressant which allows the opportunistic fungi like *Candida* cause oral Candida infection.*C. albicans*, the most commonly recovered species, is highly adapted for growth on mucosal surfaces and even minor disturbances in the host's immunity can lead to superficial infection[6]. Recent comparative genomic studies have identified a range of gene families in *C. albicans* with putative roles in adhesion and nutrient acquisition that may contribute to its greater success in vivo relative to other yeast species [7]. The current investigation is consistent with numerous previous investigations, which has shown that diabetes is major predisposing factor to symptomatic Candidiosis of oral or otherwise. The present study showed 50.2% oral infection by *C.albicans,* 30% by *C.tropicalis, 15*% by *C.ontarioensis,* 10% by *Dipodascus capitatus* was reported. As per the earlier report 60% of the oral infections in diabetes was due to the *C.albicans sps*,[8] the bio chemical tests like carbohydrate assimilation , fermentation and urease tests were conducted for all the isolated *Candida sps* from the oral cavity of diabetes subjects 90% positive reactions shown by *C.albicans*, while the other *Candida sps* mentioned in the present study viz., *C,tropicalis* , *C,ontarioensis*, and *Dipodascus capitatus* showed significantly for assimilations and fermentation reactions, but for the urease test the isolated species showed negative results, as per[9] only *C.krusie* was showing the positive result [9]. For the above biochemical tests *C.ontarioensis, Dipodascus capitatus were* showing the results positively **(Table 1)** which were newly studied having no evidence in the previous study. Rapid differentiation of *C.albicans* from other sps was made and conformed by germ tube test [10] , here in our study, by this test germ tube was formed not only by *C.albicans* but also by *C.tropicalis.* The lipolytic activity test was performed to differentiate *C.albicans* from other sps but it cannot differentiate *C.albicans* from *C.dubliniensis* [11] . CHROM agar is a prominent and presumptive selective medium for the identification of clinically important *Candida sps*.[12] CHROM agar test was performed to all the sps isolated from the oral cavity [13] the strains that develop green colour colonies on CHROM agar plate were identified as *C.albicans* and the colonies which were formed as dark blue to blue-gray as per were identified as *C.tropicalis* but in the present study pink colour colonies [14] were also formed by the same species, *C.ontarioensis* forms dark gray colour colonies, *Dipodascus capitatus* forms pink-white mixed margins colour colony formation on CHROM agar was newly identified in the current study. All the phenotypic methods used for the identification and differentiation of the *Candida sps* simple and inexpensive but they have their own limitations. These phenotypic methods are often unable to discriminate some *Candida sps, so* to overcome these problems molecular technique like PCR was used to differentiate the *Candida* strains [11] by this method the *Candida sps C.albicans, C.tropicalis, C.ontarioensis,*

Dipodascus capitatus were identified.

C.albicans is the normal microbiota isolated in the greatest frequency from the oral cavity in the human beings [15]. When the conditions are altered between the host and the microorganism *Candida* becomes pathogenous and oral candidiosis is manifested, as it occurs in diverse populations at risk among which the individuals are immunocompramised [16].

Conclusion:

Although considerable progress has been made in the understanding of oral infections involving *Candida* but diabetic patients suffering from candidiosis still presents a stiff challenge for the clinicians owing to multiple *Candida sps* involvement and therefore problem in recommending the correct drug regimen. In this context, much remains to be understood, the preinisiation of determination of virulence and response of host tissue is still unclear. Applications of molecular biology techniques to our findings showed that increasing incidence of oral infection is correlated to several predisposing factors like life style habits& age. The phenomena of phenotypic switching was one of the great feature that enhancing prevalence rate of oral *Candida* infection in diabetic subjects.

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