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Original Article

In vitro identification and Antifungal susceptibility of different Candida Species isolated from patients with or without Diabetes having Chronic Periodontitis

Abstract:

Background: Candida albicans is the commonly associated species with oral lesions, but other Candida spp., such as C. glabrata, C. tropicalis, C. parapsilosis, C. krusei, and C. dubliniensis have also been isolated from the human saliva, even irrespective to the oral candidiasis. Candidal colonization has also been documented from subgingival plaque of adults with periodontitis. **Objective:** Nowadays there is a great demand of natural herbal medicines. For a Diabetic person having Periodontitis, the use antifungal drugs are must. Therefore the antifungal capacity of the commonly used drug and herbs has been evaluated. **Methods:** For the study, the patients were screened and categorised into two study and one control group, having 20 patients in each group, on the basis of their respective Blood Glucose level and Dental status using standard clinical parameters. Oral rinse of 60 patients including both gender were selected from the Out Patient Department of Periodontitis, Rungta College of Dental Sciences and Research, Chhattisgarh. Chrome Candida Agar Media was used to identify Candida albicans and NAC. In vitro tests of the effectiveness of selected antimycotic agents against test candidal isolates recovered from the patient were performed to check the efficacy of the antifungal agents by the Kirby-Bauer Disc Diffusion method. **Result:** We found that by the use of this chromogenic media most of the commonly recovered NAC can be easily distinguished as each species produced the widest range of colours and morphologies. The herbal extracts Aloe Vera and Garlic failed to inhibit the growth of the candidal species but Neem, Meetha Neem, Triphala, Tulsi showed sensitivity to varying degree. Now a day there are much popularity of using herbal products, having its extracts incorporated in the cosmetics and other products used by humans. **Conclusion:** We conducted the sensitivity check of the candidal isolates against the dental dentrifices and found that however all the toothpastes contained herbal extracts capable of destroying bacteria but it did not showed good results against candidal species except the Patanjali Toothpaste. This may be due the fact that Patanjali Dant Kanti Dental Cream has the combination of all the herbal extract mixed altogether.

Key Words: Diabetic; Periodontitis; Antifungal agent; Herbal dentrifices

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Introduction

Candida being the most prevalent fungus amongst diabetic group and the status if the patient gets worsens, if Periodontitis is associated with it. Some species of fungi are commonly reported to be colonized in the dorsal surface of the tongue, palate and buccal mucosa. In this regard, since last few decades time to time various Candida species have been identified. Candida species have evolved as the most important opportunistic pathogens in immuno-compromised hosts and also playing an important role in life threatening infections [1].

Candida albicans is the species commonly associated with oral lesions, but other Candida spp., such as C. glabrata, C. tropicalis, C. parapsilosis, C. krusei, and C. dubliniensis have also been isolated from the human saliva, even irrespective to the oral candidiasis [2]. Numerous studies have already revealed that with respect to the accession of broad-spectrum antibiotics and increase in the incidence of consuming immunosuppressive corticosteroids and antitumor agents the prevalence of opportunistic pathogens have increased many folds [1,3,4,5].

Thus, the need of the time is now to recognize perfectly C. albicans and other Candida species in routine clinical microbiology laboratory before starting any clinical measure against any subject. The routine and the conventional methods of identifying these pathogenic yeasts based on their respective morphological, biochemical and physiological characteristics. The most distinguished feature is their respective growth characteristics and carbon source assimilation or fermentation, ability to form germ tubes (GT) or chlamydospores etc. Furthermore, many of such yeasts also represent different characteristics viz., approximately 5% of the strains of C. albicans often reveal germ-tube negative whereas others produce germ tube-like structures e.g., pseudohyphae[1,6].

Candidal colonization has also been documented from subgingival plaque of adults with periodontitis. The virulence factor of the Candida not only helps in its colonization but also in proliferation inside the mucosal layer of oral cavity and periodontal pockets too. Candida species also found to co-aggregates with bacteria in dental biofilm, attaches to epithelial cells and causes to annihilation in oral diseases as it has the capacity to invade gingival conjunctive tissue. To add to its virulence Candida sp. also produces enzymes such as the collagenases and proteinases which in turn degrade extracellular matrix proteins and immunoglobulins[2,7]. It has also been established that an elevated blood glucose concentration increases hemolysin activity among C. albicans isolates in diabetic patients [7]. The presence of anaerobic environments, i.e., in root canal systems and periodontal pockets etc., also lead to poly-microbial infections. The presence of a large amount of carbohydrate in the oral cavity influences several virulence factors of Candida sp. Incubation in sucrose, glucose, fructose, or maltose promotes adhesion of C. albicans, C. tropicalis, and C. krusei to epithelial cells, which in turn increases acid production, and lowers pH, with consequent activation of acid proteinases and extracellular phospholipases – factors involved in yeast adhesion.

Although bacteria plays a major role in the pathogenesis of periodontal disease, the yeast Candida albicans has also been isolated from periodontal pockets[8]. Patients with reduced immunity show increased periodontal colonization by Candida sp. The cases of severe and chronic periodontal infections are seen mostly in immuno-compromised patients or individuals under antimicrobial therapy for long periods.

In the present study we checked the prevalence of Candida , albicans and NAC in different group of patients using Chrome candida Agar and checked its sensitivity against herbal tooth-pastes commonly available in Indian market.

Methods

The study was carried out following the proper guidelines of the ethical committee of the Institute. Total 60 patients of age group 25 – 60 yrs including both genders were evaluated for the presence of Candida sp. A written consent was taken by the proposities explaining the procedure and its outcome.

Source of Data:

For the study, 60 patients of both the genders were selected from the Out Patient Department of Periodontitis, Rungta College of Dental Sciences and Research, Kohka – Kurud Road, Bhilai, and Chhattisgarh.

Screening of the Propositas:

The patients were screened and categorised into two study and one control group, having 20 patients in each group, on the basis of their respective Blood Glucose level and Dental status using standard clinical parameters.

Group 1- Diabetic patients suffering from Periodontitis

Group 2 - Non-Diabetic patients suffering from Periodontitis

Group 3 - Non- Diabetic patients not suffering from Periodontitis (Control & Healthy Persons)

Collection of sample:

The porosities were asked to rinse their mouth for at least 30 seconds with 10 ml of phosphate buffer saline. The samples thus collected were concentrated by centrifuging at 3000 rpm for 5 minutes each. The supernatant were discarded and deposits were used as inoculums [9].

Cultivation of sample:

we used CHROM Candida agar, HiMedia. Indeed 'CHROM Candida agar' is a medium used to differentiate Candida albicans from non-albicans Candida (NAC) species.

Identification of Candida sps.

A sterilised swab was dipped in the tube having deposits and streaked onto the surface of chrome Candida agar plates, prepared by sterilising the media in the water bath till boiling (as per instructions on the pack) and plating in a sterilised petriplates. The inoculated plates were subjected to incubation at 370C for 2 days.

Anti-mycotic Sensitivity Tests:

By using Hexa anti myco-01 HiMedia

In vitro tests of the effectiveness of selected anti-mycotic agents against test candidal isolates recovered from the patient were performed to check the efficacy of the antifungal agents by the Kirby-Bauer Disc Diffusion method.

Inoculum Preparation:

The inoculum density of fungal isolate to be tested was standardized with 0.5 McFarland turbidity standards at 625 nm. For the fungal suspension to have a final inoculum of 1 x 108 cfu /ml, 1ml of fungal suspension was added to Sabourad's Dextrose Broth and incubated at 37oC overnight. The inoculum was taken out and adjusted as per 0.5 McFarland standards by adding Sabourad's Dextrose Broth. By the help of swab the inoculums were spread on to the agar plate and kept for 30 minutes.

Placing the Antibiotic Disc:

The Antibiotic Disc, that are commercially available were chosen having the antibiotics that are commonly used by the Dentists and placed at the periphery of the petriplates to form a circle. After placing the paper disc containing specific concentration of antibiotics the plates are incubated at 37oC for 24 hours, prior to determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimetre with the help of Antibiotic Zone Reader Scale, HiMedia [13,14].

Antibiotics used :

Amphotericin B (AP) 100 units
Clotrimazole (CC) 10 mcg
Fluconazole (FLC) 25 mcg
Itraconazole (IT) 10 mcg
Ketoconazole (KT) 10 mcg
Nystatin (NS) 100 units

Results

Figure 1. Appearance of different species of candida and NAC on Chrome candida agar media

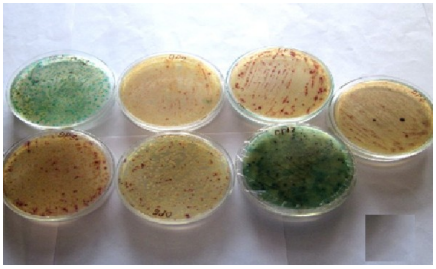


Figure 2. AST of Candida sps.

After 24 hours of incubation the plates showed following results (Table I).

Table I Appearance of Candida species on Chrome Candida Agar Media

Sr. No.	Colony Colour	Candida sp.
1	Pink to violet	Candida krusei
2	Turquoise blue	Candida tropicalis
3	Cream	Candida parapsilosis
4	White	Candida glabrata
5	Green	Candida albicans

Candida albicans (Figure 3, 4) showed smooth, greasy colonies with light green colouration on the chrome Candida agar. When subjected to AST test, it showed minimum sensitivity to amphotericin B and maximum sensitivity to other antifungal agents used with maximum sensitivity zone between 20 – 30 mm inhibition zones. This isolate showed resistant to all the herbal extracts. It showed maximum sensitivity zone to Neem active and Patanjali tooth paste whereas intermediate sensitive to Neem active and Meswak toothpaste but appeared resistant to Babool toothpaste.

Fig 3. C. albicans showing budding appearance

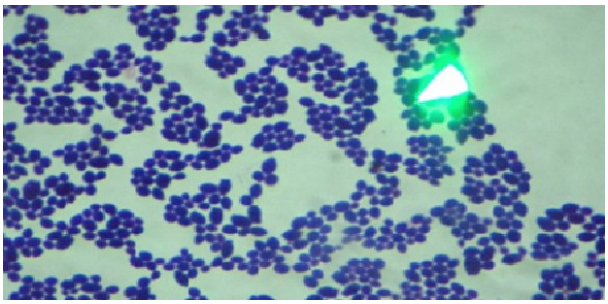
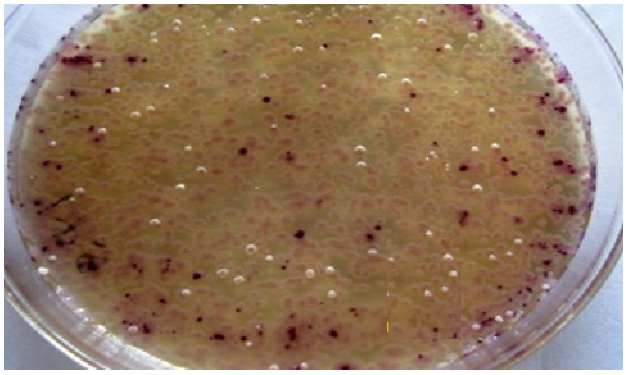


Fig 4. C. albicans germ tube

Candida tropicalis appeared as raised colonies having metallic blue shine. In its AST it showed resistant to all antifungal agents used. It was resistant to Aloevera extract but sensitive to all other herbal products used. When tested against toothpastes showed intermediate sensitivity to Neem active and Patanjali toothpaste and resistant to rest all dentrifices.

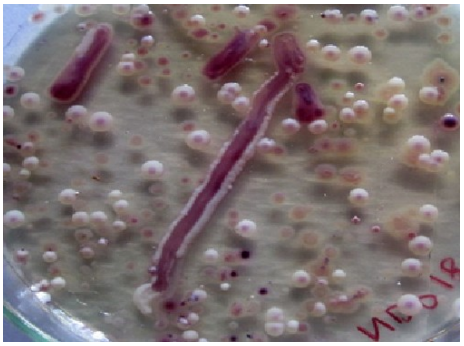
Candida glabrata (Figure 5) produced smooth cream to white colony on chrome Candida Agar. Against the antifungal agents clo-trimazole and flucanozole were not able to show sensitivity zone, intermediate sensitive zone to amphotericin B and ketoconazole, maximum inhibitory zone to nystatin and itraconazole. When tested against herbal products it showed resistant against Aloevera and Garlic. Against the tooth paste it showed resistant to Meswak while being sensitive to all other dentrifices.

Fig 5. Colony of C.glabrata on CAC



Candida krusei (Figure 6) appeared purple with fuzzy colonies on Chrome candida Agar plate. When subjected to AST against commonly used antifungal agents it showed resistant to amphotericin B, intermediate sensitive zone to clo-trimazole and flucanozole, maximum inhibitory zone to ketoconazole, nystatin and itraconazole. When tested against herbal products it showed resistant against Aloevera and Garlic. Against the tooth paste it showed resistant to Meswak while being sensitive to all other dentifrices.

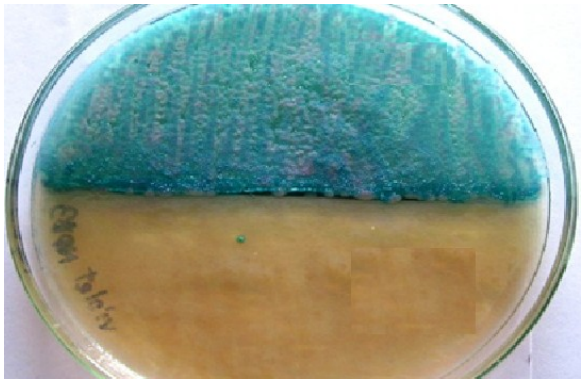
Fig 6. Colony of C.kruzei on CAC



Discussion

It is evident that there is enormous escalation in the increasing incidence of human diseases being produced by *Candida species* [5] (Figure 7). Rapid identification of NAC can assist the clinician in selecting appropriate antifungal therapy. Identification of *C. dubliniensis* and *C. glabrata* were also seen to be based on CaC [8,10]. Many studies show that Chrome candida agar is a potential screening medium that can rapidly identify potentially azole-resistant as well as amphotericin B-resistant species, including *C. krusei*, *C. glabrata*. *C. rugosa*, and *C. inconspicua, C. albicans*, *C.* Tropicalis [11,12]. We found, as previously reported, that by the use of this chromogenic media most of the commonly recovered NAC can be easily distinguished as each species produced the widest range of colours and morphologies, making it possible to identify using this medium. *Candida albicans* are said to be well connected with most serious nosocomial candidal infections[15].

Fig 7. Comparative colony characteristic of Diabetic and Non-diabetic



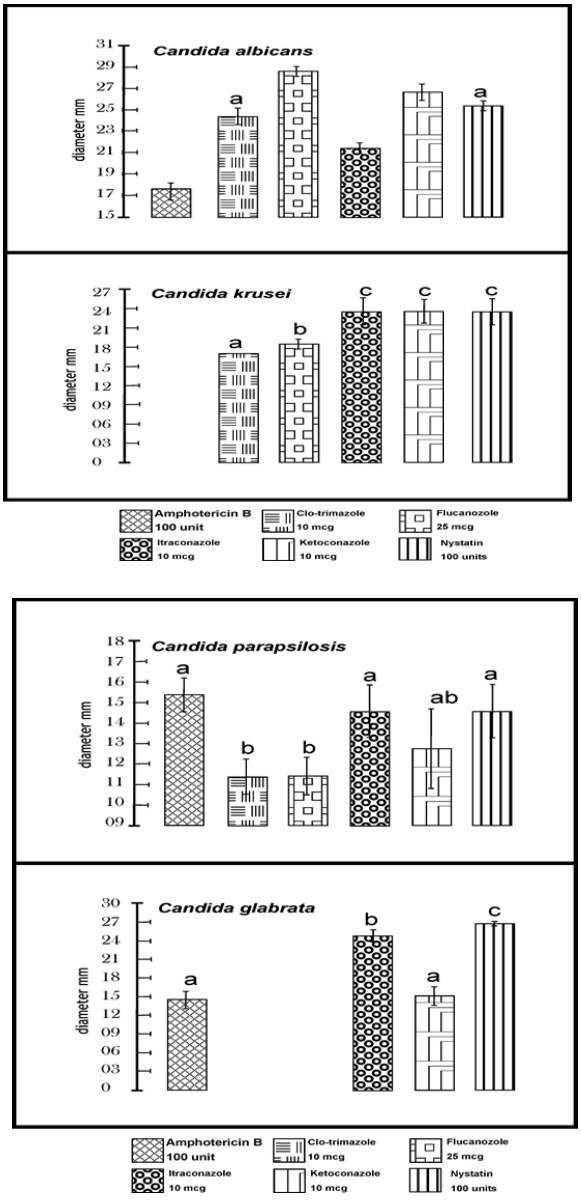
But at the same time it is well documented that in the last decade candidemia, due to C. albicans have declined [4] rather non-albicans Candida species (NAC) are being isolated in most of the cases of candidemias [5] (Table II). Most NAC infections are caused by C. glabrata, C. parapsilosis, or C. tropicalis[1]. The practice of using amphotericin B, fluconazole, and caspofungin to treat candidemia is approved by FDA and the most recent Infectious Diseases Society of America guidelines [11]. All these agents have been used effectively in clinical studies, which included mostly patients with C. albicans infections; thus their efficacy against NAC is not well known. In vitro study has shown decreased susceptibility to amphotericin B in isolates of C. krusei, C. tropicalis, C. glabrata and also C. albicans. The use of antifungal agents available tends to increase the shift of candidal infections to those caused by non-albicans species. Because of this, rapid, reliable identification of species and increased use of susceptibility

testing has become necessary to appropriately select which agent to use and make treatment choices. In vitro testing has revealed that there are clear differences among the various NAC in their susceptibility to specific drugs[16].

Table II Prevalence of Candida species amongst the test group

Sr. No.	Candida sps.	Healthy	Diab + Perio	Non-Diab + Perio
1	Candida krusei	Present ++	Present +++	Present +
2	Candida tropicalis	Absent	Present +++	Present +
3	Candida parapsilosis	Present ++	Present ++	Present +++
4	Candida glabrata	Absent	Present ++	Present +++
5	Candida albicans	Absent	Present +++	Present +

Fig 8. Statistical analysis of Different Candida species against Anti – fungal agents



Conclusion

Now adays there are much popularity of using herbal products, having its extracts incorporated in the cosmetics and other products used by humans. Taking this into consideration we conducted the sensitivity check of the candidal isolates against the dental dentifrices and found that however all the toothpastes contained herbal extracts capable of destroying bacteria[17] but it did not showed good results against candidal species except the Patanjali Toothpaste. This may be due the fact that Patanjali Dant Kanti Dental Cream has the combination of all the herbal extract mixed altogether.The herbal extracts AloeVera and Garlic failed to inhibit the growth of the candidal species but Neem, Meetha Neem, Triphala, Tulsi showed sensitivity to varying degree.

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