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Dipodascus capitatus: A rare and emerging yeast like fungal infection in immuno-compromised subjects

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ABSTRACT

Dipodascuscapitatus is a typical inhabitant of humans, especially on the skin, oral cavity, and respiratory tract. Fungi previously considered harmless colonizers (Opportunistic) are emerging as new fungal pathogens, particularly in an immune-compromised state. Infection caused by *Dipodascuscapitatus* is rare, and the treatment procedures are quite difficult. Here we reported the information on patients with chronic diabetic conditions. The fungus was isolated from oral washings, the phenotypic identification was based on mycological methods, the molecular marker was based on 18S r RNA sequencing, and the susceptibility test was conducted by micro-dilution technique. The present study mainly focused on quantifying virulence factors, and their activity is expressed in the form of hemolysin protease (1.812 mg/ml). Fungal infections in these patients are often severe, rapid progressive, and challenging to identify, including diagnostic and therapeutic modalities required to provide better patient care.

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1. Introduction

The rapid increase of immune-compromised patients resulted in a diagnosis of insidious mycotic infections that seems to be unusual yeasts.[1] In observation, the high incidence recorded by the non-albicans species, rather *Candida albicans*, would be alarming as they may be resistant to antifungal therapy by some antifungal agents.[2] Rhodotorula is one of the primary yeast-like fungi and belongs to the family of Sporidiobolaceae of the phylum Basidiomycota, a significant cause of fungemia caused by catheters in immune-compromised patients, especially patients with HIV infection/ different carcinoma and patients with undergone organ transplantation. The host's immune status would be a crucial determinant of patients at risk. Although very rare yeasts like fungi *Dipodascuscapitatus* are developing as opportunistic pathogens, especially in immune-suppressed cases, the diagnosis remains demanding, and treatment may be sub-standard. *Dipodascus* species is more uncommon than regular *Candidasps* and has been associated with dangerous infections in immune-competent hosts. The predisposing factors are highly associated with spectrum change in patients with risk management of therapeutics. For instance, those factors are very much intensive to alter the defense mechanism that results from immune suppressants, which disrupt the skin and mucosa interaction and interference of antibiotics. The patient population is increasing daily. Hence the, opportunistic pathogens are taking the chance to regulate the infestation in immuno-compromised subjects. In general, pathogens include *Aspergillussps, Candida sps, Cryptococcussps*, and *Mucoralesetc.*[3–5] The primary fungal etiology depends on an unusual and taxonomically diversified group of opportunists who

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belong to *Galactomyces and Magnusiomyces*. Usually, the morphological characteristics and the spore production vary from pathogen to pathogen.[6] The relative species of *Magnusiomyces and tricosporans* has progressed as a new pathogen in patients suffering from hematological problems.[7,8] These are all main clads of arthroconidia genes disseminated to different subgroups. Recent times, based on the taxonomical principles, names of the fungal pathogens have been replaced with another newly accepted names. Hence, It refers to molecular pathology which would be a key indicator for necessary identification of cultures. Majority of the characteristics of *dipodascus* seems to be similar to *Magnusiomyces*. But they frequently recover from blood-related infections that lack perfect diagnostic techniques, sufficient information for antifungal susceptibility, and no proper therapeutic regimen. Even then, the epidemiological data is very scanty, and the source of details is very few, although it has the potential to cause severe infections.[9]

Since then, we reported a *D.capitatus* a pleomorphic organism that mainly affects immuno compromised patients as opportunistic and creates invasive fungal infections. Conventional diagnostic methods are unable to detect this pathogen consistently. The current methodology systematically describes detecting the organism by isolation, identification, and molecular genetics methods. Using internal transcribed spacer sequences, the phylogenetic tree construction enables 100% identity with *Magnusiomyce capitatus*. The in-vitro fungal testing conducted with several antifungal agents (Fluconazole, Amphotericin B, Ketoconazole, and Itraconazole) found the following minimum inhibitory concentration. To the best of our knowledge, this is the first organism reported in our lab from oral washings of diabetic subjects suffering from an immune-compromised state. It is to be emphasized the importance and close observation for a timely and perfect diagnosis during glycemic state and to resolve the proper treatment with antibiotics for patient care.

2. Materials and Methods

2.1. Isolation & Identification

Diabetic patients selected for the present work are the RIMS out patients (Rajiv Gandhi Institute of Medical Sciences), Kadapa, Andhra Pradesh, India. As a standard procedure selected Patients will be provided a consent form and the samples were collected (Oral washings) in a clean & sterile bottles and brought to the laboratory for further analysis.

2.2. Physiological parameters

2.2.1. Carbohydrate assimilation:

The carbohydrate utilization ability of an organism was tested by the carbohydrate-impregnated discs method in the presence of oxygen. The filter paper discs soaked in basal medium (1% peptone and 2% agar) are selected as carbohydrate medium. Twelve different carbohydrate components were used in the present study, i.e. Glucose, Maltose, Sucrose, Lactose, Galactose, Melibiose, Inositol, Xylose, Cellobiose, Raffinose, Trehalose, and Dulcitol. A carbohydrate-free basal medium was used as a control, and results were recorded. The growth of the test organism usually indicates positive assimilation.

2.2.2. Carbohydrate Fermentation:

Six different carbohydrates viz glucose, lactose, sucrose, galactose, maltose, and trehalose were used to measure the carbohydrate fermentation efficacy according to Ochei & Kolhatkar.[10] The medium contains 1% peptone and 2% agar and is supplemented with individual carbohydrates at 0.1% and Bromo cresol purple as an indicator. After inoculation with test organisms, the medium was incubated at 25°C-35°C for 24 days, while the result must be noted every 2-3 days. Positive results were recorded with acid and gas formation in tubes.

2.3. Evaluation of virulence factors

We isolated Candida sps., screened for various hydrolytic enzymes secreted extracellular such as Coagulase, Haemolysin, Phospholipase, and Protease, also analyzed for the intense formation of bio-film and cell surface hydrophobicity that promotes pathogenicity.

2.3.1. Cell surface hydrophobicity:

Hydrophobicity index (HI) of cell surface hydrophobicity is measured by using the modified tube method Hazen and Hazen.[11] The actively progressed cells were collected and further suspended in PBS with an optical density (OD) of 0.5 at 660nm. 2.5ml of the above suspension was mixed with 1ml of xylene & shake vigorously for 2 min, left it aside at room temperature for 20 min, and measured the turbidity of the aqueous phase at 660nm. Candida species were further classified based on the low and high CSH nature based on HI index. (For example, HI<30% shows low CSH, and HI >70% indicates high CSH, which is bound to their hydrophilic and hydrophobic nature). The following formula is used for the calculation of CSH.

HI = (A660nm control-A660nm test) × 100/A660nm control.

Here HI= hydrophobicity index, A660nm control = O.D of the samples before xylene treatment, and A660nm test = O.D of samples after xylene treatment.

2.3.2. Bio-film formation

The bio-film formation ability of different Candida sps was determined by using the cone-shaped tubes described by Yigit[12] with a bit of modification and appropriate controls. All the Candida isolates are to be tested for bio-film formation by inoculating in saline and then incubated at

37^0 C for 24h. After incubation, 1.5 ml of saline suspension was placed in screw cap polystyrene tubes containing 5 ml of SD broth supplemented with dextrose and incubated for 24 h at 37°C in static conditions. Immediately after the incubation, the broth was aspirated gently and washed the tube with PBS (pH 7.2) thrice and stained. After 15 min of incubation, the excess stain was decanted and the tubes were again washed with PBS. The film's visibility on the wall or the bottom of the tube indicates the bio-film formation by the test organism. The formation of the ring at the liquid interface was not considered a bio-film.

2.3.3. Hemolysin:

Production of Hemolysin was demonstrated by the modified assay as described by Manns[13] where 10 μl of test suspension was inoculated in triplicates on a sheep blood agar medium enriched with 3% sugar and incubated at 370C in a CO2 incubator for five days. The transparent zone appeared at the site of inoculation, judged as positive for hemolysin activity. EAI (HI) units measured the enzyme activity.

2.3.4. Coagulase

Coagulase is one of the prominent virulence factor, determined by the tube method, according to Isenberg.[14] Approximately 100 μl of each test culture suspension was inoculated to separate tubes containing filter-sterilized human plasma & incubated. Then the tubes were monitored for clot formation within 24 hrs along with controls. After gentle shaking, non-suspended clots were treated as positive.

2.3.5. Phospholipase

The production of Phospholipase was determined by inoculating on egg yolk agar medium by precipitation method with slight modification of Price[15] method. The composition of the egg yolk medium contains 65 g of SDA (Sabouraud Dextrose Agar), 55.3 g of NaCl, 5.5 g of CaCl2, and 10% sterile egg yolk. 10 μl culture (Approximately 106 cells/ml) was inoculated as spots and incubated for seven days at 37 °C & the enzymatic activity was measured.

2.3.6. Protease

Production of extracellular Protease (Prz) was measured by inoculating the culture filtrate on the fungal medium, which is supplemented with Bovine Serum Albumin (BSA) at 0.2% by slight modifications of the Staib[16] method. The culture medium was sterilized by filtration after adjusting the pH to 5.0. 10 μl of 48 h old fungal cells were inoculated on the plate to make the spots and incubated at 37 0 C. After 5 days, the proteolytic activity was measured by a zone of clearance of the concentric colony.

2.3.7. Calculation of Extracellular enzyme activity index

Screened isolates were tested for the production of extracellular enzymes Vig, Phospholipase [Pz], Protease [Prz], Hemolysin [Hz] and Coagulase using the standard protocols. The activity index of Phospholipase was calculated by following the ratio of colony diameter to colony diameter plus the diameter of sediment (in mm). The enzymatic activity was scored into four categories: Pz of 1.0 indicated no enzymatic activity; Pz between 0.99 and 0.90 revealed weak enzymatic activity; Pz between 0.89 and 0.70 corresponded to moderate activity; then the low Pz values $\leq$0.69 meant strong enzymatic activity. Similarly, the protease and hemolysin activities, assessed with the diameter of the translucent zone, were used instead of the sediment zone. The suspended clot after 24hr of incubation with gentle shaking treated as positive.

2.4. Invitro antifungal susceptibility testing

2.4.1. Antifungal susceptibility testing

Antifungal susceptibility of test cultures was examined using the HiComb MIC test (HiMedia Laboratories Pvt. Ltd., Mumbai, India) as per the instructions provided. The antifungal tested in the present study was fluconazole, amphotericin B, ketoconazole and itraconazole at different concentrations. 3-4 colonies of the Candida isolate were inoculated in saline to prepare inoculums, and suspension turbidity was set to match 0.5 McFarland standards. Then the culture was inoculated on MH agar supplemented with 2% glucose and methylene blue. The strip was placed on the agar surface with the help of forceps after inoculums were utterly dried and incubated at 35^0 C for 24–48 h. The results were interpreted as sensitive (S), susceptible dose-dependent sensitive (SDD), and resistant (R). The Clinical and Laboratory Standard Institute (CLSI) prescribes interpretive criteria for azoles. Due to the lack of defined breakpoints for amphotericin B, their arbitrary values based on other studies have been considered.

2.4.2. Plant extracts preparation

Dried clove buds (*Syzygiumaromaticum*) and bark of cinnamon (*Cinnamomum verum*) collected from the local market and thoroughly sterilized and mixed with distilled water, air dried, and then ground into a fine powder separately.

One hundred grams each of cloves and cinnamon powder was soaked in 400 ml of acetone or methanol with constant agitation in a bio-shaker with temperature control overnight at 20°C. The layers were separated using sterilized cheesecloth and filtered through Whatman paper (No. 2). A rotary vacuum evaporator then concentrated the extracts at 40°C. After that, the concentrated extracts were diluted to the desired concentration with 10 % DMSO, further filter sterilized by (0.45μm), and stored at −20°C until use.

2.4.3. In-vitro screening of plant extracts for antifungal property

The obtained plant extracts therefore dissolved in different gradients of 1, 2.5 and 5 mg/ml concentrations. The NCCLS proposed method (M27-P) broth micro dilution test was modified.[17] To the 400 μl of 24 h-old Candida cultures, 4ml of sterile saline was added approximately to get the desired dilution of the test organism equal to that of a 0.5 McFarland tube. From the prepared stock culture of Candida, a 1:1000 dilution was made, and the same 100 μl was added to each well of a 96-well micro plate. 25 μl of each organic solvent extract was mixed with 175 μl broth and diluted. Controls were made in the wells with broth plus fungal strains, with no extract, and serial dilutions of Amphotericin B with the fungi at the recommended concentrations for inhibitory action. The results were recorded at 630 nm in an ELISA reader, then covered with parafilm and incubated at 370C overnight.

2.5. Statistical analysis

Statistical analysis was made for all results using one-way analysis of variance (ANOVA) along with SE and SD to compare EAI levels between different Candida species where the $P < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation, identification and phylogenic evaluation

Over 70 different fungal strains were isolated from 125 oral washings of diabetic subjects, of which 67 samples were collected from males, and 58 samples were from females. Depending on the morphological and cultural characteristics on the CHROM agar medium, among the total strains isolated, 31.4% showed homology with *Candida albicans*, and the remaining 68.5% strains with high homology to non-albicans Candia strains. The predominantly isolated non-albicans *Candia strains* are composed of 23% of Candida tropicalis 10% of C. ontarioensis, 8% of *C.parapsilosis*, and 7% belonging to *Dipodascuscapitatus*. In this study, the *Dipodascus* capitatus, which can cause fatal disease in immunocompromised subjects. We used it for further analysis of significant virulence factors qualitatively under *in-vitro* conditions. The phenotypic characteristics of *Dipodascuscapitatus* are mentioned in Figure 1.

All the isolated cultures were further confirmed by 18S rRNA sequencing and also by using PCR-based markers like RAPD and ISSRs. The 18S rRNA sequence of *Dipodascuscapitatus* was deposited in the GenBank database with the accession number MW435403. Phylogenetic analysis is used to find evolutionary similarities and rational relationships between ancestral lines and their descendants. From Fig. 2 it was clear that the test organism (*Dipodascus*) showed 90% similarity to the 18s rRNA partial gene sequence of *Magnusiomycescapitatus*. Partial sequence of strain mandori and another strain CBS 162.80 with rRNA partial sequence, and small subunit ribosomal RNA and has a very close gene distance between CBS strain 197.35. The maximum parsimony distance between all these strains is about 0.001 i.e $p< 0.001$.

3.2. Evaluation of physiological parameters and extracellular hydrolytic enzymes as virulence factors

The test results revealed that the test organism showed the positive assimilation with the glucose, sucrose, galactose, xylose, and trehalose and the remaining carbohydrates i.e., fructose, lactose, melibiose, inositol, cellobiose, raffinose, and dulcitol represented the negative results. In the case of carbohydrate fermentation, *Dipodascuscapitatus* noticed positive results i.e., both acid and gas formation with glucose, maltose, and sucrose. In contrast, partial fermentation i.e, only acid production, was observed with galactose and trehalose and observed negative results with lactose (Figure 3). Furthermore, the test organism also showed adverse effects with pseudohyphae and germ tube test (Figure 4).

Extracellular hydrolytic enzymes act as virulence factors, and presently the test organism expressed the maximum levels of hemolytic and Protease enzymes (Figure 5A, B). The expression of Protease was analyzed by spectrophotometric method at two different temperatures (25°C and 37°C) and three different pH (4, 7 and 8). Then we found that 37^0C and PH 7.0 (1.812 mg/ml) were the optimum conditions for the maximum production of the enzyme. The mean values were recorded as 0.4232 and 0.4215, respectively, with $P < 0.05$, which is statistical significance. The moderate activity of the phospholipase enzyme (Pz 0.82) was recorded for the test organism. The overall mean of Pz value for this isolate was statistically significant ($P<0.05$). The test organism was analyzed for the efficacy of coagulase enzyme production and recorded a moderately high (0.61) activity. Abiotic factors like cell surface hydrophobicity and bio-film formation (Figure 6) were also evaluated and recorded results considerably as 78% and 0.21, respectively.

3.3. Antifungal susceptibility

3.3.1. Chemotherapeutic drugs

The antifungal activity of Nystatin, Amphotericin B, Ketoconazole & Fluconazole used to evaluate efficacy at different concentrations (10, 20, 30 & 40 μg/ml). The results are shown in Fig.7A. Test organism showed remarkable significance with one combination of antifungal drugs i.e. Ketoconazole versus Fluconazole with correlation coefficient which is statistically significant and showed in table.1

3.3.2. Plant extracts

Apart from the chemotherapeutic drugs, we also tried to evaluate the antifungal efficacy of crude plant extracts of clove and cinnamon with varied concentrations (1, 2.5, and 5mg/ml). The susceptibility pattern of *dipodascuscapitatus* for tested with plant extracts was depicted in Fig 7B. The maximum susceptibility range of the test organism was observed at 5mg/ml concentration. Clove methanol versus Cinnamon acetone, Cinnamon acetone versus Cinnamon methanol obtained with P<0.05 significance level (r=0.9942), but Clove methanol versus Cinnamon methanol showed P<0.01 (r=0.9998).

Table: 1 Coefficient correlation of different combinations of drugs for *Dipodascus capitatus*.

Table 1: Coefficientcorrelation of different combinations of drugs for *Dipodascus capitatus*.

S.No.	Combination of drugs	r value
1.	Nystatin versus Amphotericin-B	0.9898
2.	Nystatin versus Ketoconazole	0.9918
3.	Nystatin versus Fluconazole	0.9959
4.	Amphotericin-B versus Ketoconazole	0.9657
5	Amphotericin-B versus Fluconazole	0.9771
6.	Ketoconazole versus Fluconazole	0.9902

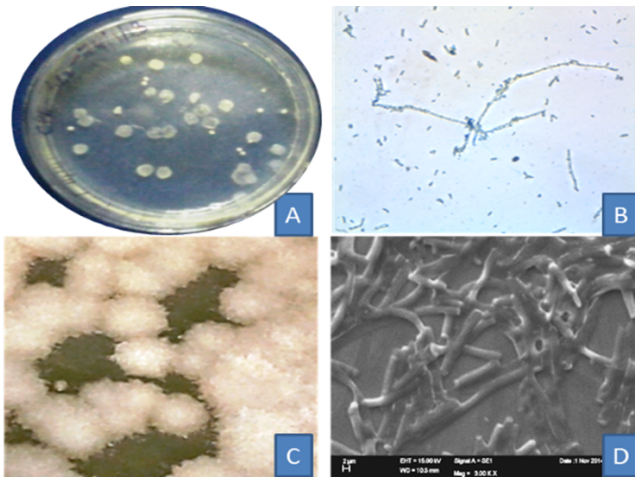

Fig. 1: Phenotypic characterization of *Dipodascus capitatus*; **A**: Growth on Sabouraud dextrose agar medium, **B:** Microscopic observation by simple staining; **C:** Colony morphology on CHROM agar; **D:** Scanning electron microscopic image.

4. Discussion

Magnusiomyces is a genus of arthroconidia yeasts belonging to dipodascaceae, a phylogenetic distance from ascomycetous producing bipedal asci containing spores with gelatinous sheath. It's an opportunistic pathogen causing systemic infection as fungemia and endocarditis, particularly in patients with hematological disorders; the

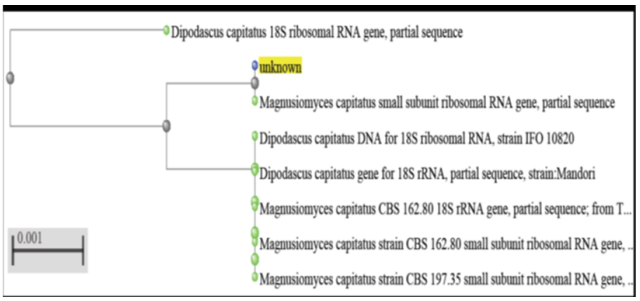

Fig. 2: Phylogenic tree analysis

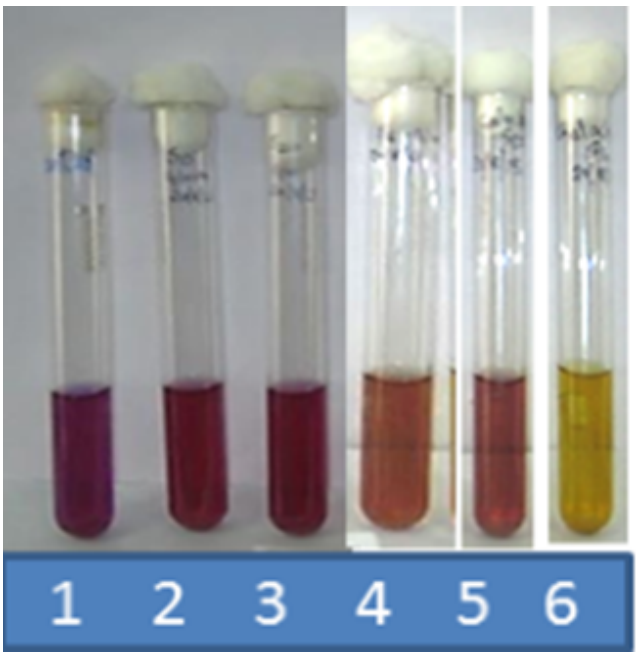

Fig. 3: Carbohydrate fermentation results. 1,2,3 Acid and gas production with glucose maltose and sucrose, 4 and 5 showing only acid production with galactose and trehalose and negative results with lactose.

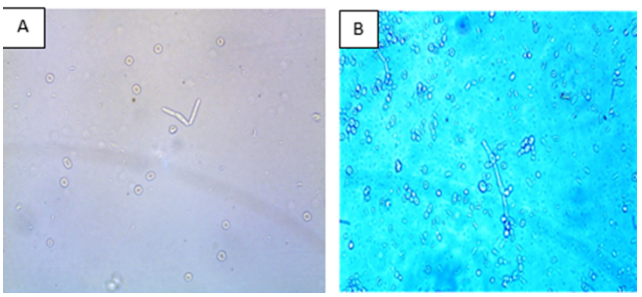

Fig. 4: A: *D. capitatus* showing negative results for germ tube; **B:** *C. albicans* showing positive results for germ tube.

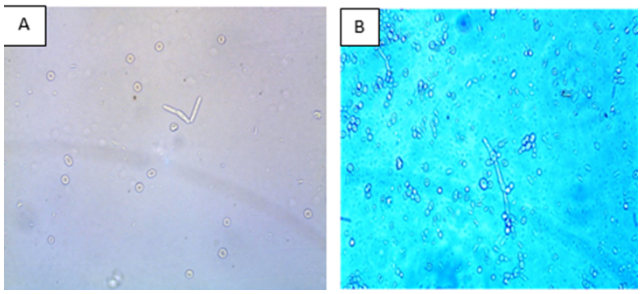

Fig. 5: Extracellular hydrolytic enzyme production by *D.capitatus;* **A:** Haemolytic enzyme activity. **B:** Protease enzyme activity.

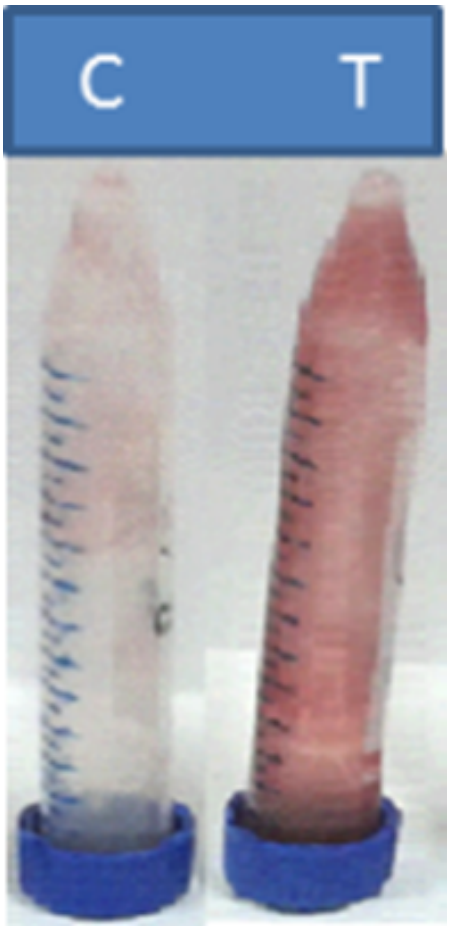

Fig. 6: Bio-film formation showed by a thin layer whichis stained attached to the polystyrene tube. C- Control, T- Test organism.

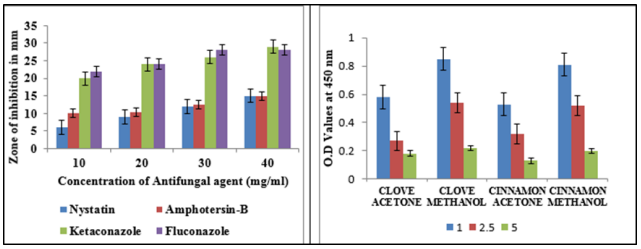

Fig. 7: Antifungal activity of different compounds on *Dipodascus capitatus;* **A:** chemotherapeutic drugs; **B:** plant extracts.

mortality rate may be the high speed of dissemination is relatively high. Invasive fungal infections are the most important causes of severe infections that may lead to morbidity and mortality in a specific group of subjects. It is reported that approximately 1.5 million deaths are encountered annually, and fungal infections increase the cost of health care.[18] *Dipodascuscapitatu*s, the teleomorph form of *Geotrichum capitatum*, cause life-threatening invasive infections with incredibly increased morbidity & mortality rate, particularly in critically ill patients and immune-compromised persons.[1,19] The homologous genus of Geotrichum has also been reported in Europe and in, the United States, and in the Mediterranean area[20] and recently reported from South East Asia[21] from woods, poultry feces and found in nature as a soil saprophyte. But *D. capitatus* is one of the normal floras of human skin and is recurrently isolated from healthy people's sputum and digestive tract. As with other opportunistic yeast infections, several complications predisposition by this species increased during the last two decades. Consequently, the rising of hosts presents factors that show predisposing of fungal infections like steroids, adverse chemotherapy, broad-spectrum antibiotic treatment, neutropenia, and invasive catheterization.[22,23]

An immune-compromised state of any patient is the most dominant risk factor for most invasive fungal infections like *Geotrichum capitatum*[24,25] Similarly, a 74-year-old male patient suffering from squamous cell lung carcinoma six years ago and had a history of recurrent lower respiratory tract infections Prasad[2] reported that non-*Candida albicans* species are more prevalent than *Candida albicans* in diabetic subjects where they can experience the immune-compromised state. Similarly, we isolated 4.9% of *dipodascuscapitatus* from the oral washings of severely diabetic persons, which is considered as a prolonged glycemic state and neutropenia cases. S. capitata is a rare fungal pathogen, often reported in many malignancy cases.[26] Faciana et.al.,[27] isolated from the neonatal intensive care unit patients from 28 week gestation and deficient birth weight patients and A male term neonate with a prenatal diagnosis of left-sided congenital diaphragmatic hernia successful treatment done by micafungin. Hazar

et.al.,[24] isolated Geotricum capitatum from an 82-year-old patient who had undergone a kidney transplant recipient from the deep tracheal aspirate, the laparotomy wound, bile, and urine. Tanuskova et.al.,[28] isolated a 19-year-old woman with refractory cytopenia type of myelodysplastic syndrome (RCC/MDS) and was admitted for planned allergenic stem cell transplantation. A few reports in the literature have connected it with others risk factors, such as contaminated milk[29] polytrauma.[30]

Presently, we need more information about the virulence mechanisms of *dipodascuscapitatus*. The positive culture method remains the diagnostic test for this mycosis; no dependable investigative tests are accessible so far in the nonappearance of any specific symptoms. Hence it is complicated to find early diagnostics, which augmented the likelihood for the optimal timing of treatment at the earliest, hence difficult-to-control the stage of the infection. In several manifestations, systemic disease of this pathogen merely resembles the Candidiasis but is usually fatal in neutropenic and some other clinical settings, despite the administration of systemic therapy. In most cases, this infection emanates from a common source within the hospital environment (Nosocomical origin) and exhibits indistinguishable restriction profiles. The low dose of antifungal treatment with azole drugs has been described as a predisposing factor for systemic infections caused by D. capitatus. There have been reported many cases associated with diabetes mellitus, and this is probably because of the high serum glucose levels that stimulate the growth of yeasts such as Candida, Geotrichum, and Trichosporon.[31–33]

Appropriate therapeutic regimens and antifungal prophylaxis are essential tools to trim down the rate of complications for any rare fungal infections in susceptible hosts. However, both are set hurdles because many uncommon types of yeast are inbuilt resistant to one or more antifungal drugs. For example, *M. capitatus*, *Trichosporon*, and *Rhodotorula* are believed to be inherently resistant to Echinocandins, and *Rhodotorula* shows resistance to several azoles drugs.[34,35]

Several investigations have been conducted to evaluate the antifungal effect of some spices and their extracts; they can also use to inhibit the microorganisms in food. In parallel to our outcomes, clove extracts exhibit more antifungal activity among the clove and cinnamon water extracts than other extracts in in-vitro and in-vivo.[36] Similar to the above, in another study Kaung et.al.[37] proved that clove has a good range of susceptibility among the various powders tested. Ethanol extracts of clove showed substantial dissimilarity in exposure out come against different bacteria and fungi examined in the study.[37,38] Castellanos et.al.,[39] also reported that the essential oils and available extracts of clove and pepper can inhibit the Fusarium oxisporum and A.niger at low concentrations in tomatoes. Hiwandika et.al.,[40] reported the anti-bacterial and antifungal activity of clove extracts. Nevertheless, the present investigation has marked that the antifungal activity and susceptibility changes were recorded successfully using cinnamon and clove extracts.

5. Conclusions

This is one of the first report identified and analyzed extensively from our lab and also in the literature. We confirmed this non-albicans (*Dipodascuscapitatus*) strain, with clinical significance by their virulence factors.

The infectious rate of this pathogen is higher than other non-Candida species, which is associated with the immune-compromised state. Hence, this is reported as a potential virulent and interferes with enhancing hemolytic and protease activity. However, it can proliferate and show specific cross-reactivity with other closely related pathogens. However, the appropriate clinical finding ensures a suspension with regard to the progenitive state of diplodocus. In susceptible hosts, to reduce clinical complications with an appropriate antifungal prophylaxis, emphasis on diagnostic tools and treatment with a proper drug regimen would be the future challenge.

6. Conflicts of Interest

None.

7. Source of Funding

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