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Antifungal susceptibility and hemolytic activity of *Candida albicans* isolated from urine of dog and cat sharing common household

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Abstract
Candida albicans is an emerging multi-drug resistant fungal pathogen which causes urogenital infections in animals and humans. There is very limited data about the antifungal resistance and virulence activity of hemolysin from companion animals. The aim of the present study was to investigate the *in vitro* antifungal susceptibility and hemolytic activity of *C. albicans* isolates from urine of dog and cat which shared a common household. Standard mycological technique was used for isolating and identifying the yeast. Sequencing with Internal transcribed spacer of ribosomal RNA revealed more than 99% similarity between the isolates. Antifungal resistance was seen for Fluconazole, Amphotericin-B and Itraconazole for both the isolates. Both the isolates showed high susceptibility to Micafungin, Voriconazole, Anidulafungin and Caspofungin and presented moderate hemolysin activity. The pathogen being zoonotic can cause a significant public health concern, thus care should be taken in avoiding rampant and irrational use of antifungals.

Keywords: Antifungal resistance, *Candida albicans*, hemolysin, pseudohyphae

Introduction
Candida albicans is a major opportunistic fungus which has recently transitioned from harmless colonizer to virulent pathogen [1]. It can cause disease in animals which include urinary tract infection, peritonitis, keratitis, arthritis and disseminated candidiasis [2]. Significant increase in candiduria continues to be noticed as a result of increase in patients with a compromised immune system [2].
The drug of choice for candiduria due to *C. albicans* is fluconazole, a member of azole class of antifungals [3]. However, resistance to azole antifungals has been reported due to its irrational use leading to challenges in clinical success [4]. During the course of infection, *C. albicans* causes manipulation of host immune response by changing cell shape, altering cell wall components and secreting virulence factors like phospolipase and proteinases [5]. Also it can occur in three phases: budding yeast, hyphae and pseudohyphae which is a determinant factor for drug resistance, invading host tissues and escaping during phagocytosis [6]. Virulence factor like hemolysin, enzymes related to iron acquisition and diminishing the immune response is not well evaluated in the context of *C. albicans* from animal isolates.
The aim of this study was thus to evaluate the antifungal susceptibility and hemolytic activity of *C. albicans* isolates obtained from urine of dog and cat which shared a common household.

2. Materials and methods
2.1. Isolates
Urine samples (10 ml) were collected in a sterile urine container (HiMedia®, India) from a dog and cat which were clinically suspected of candiduria. Microscopic examination of urine was done and samples were cultured onto Sabouraud's Dextrose Agar (SDA) with 0.05% chloramphenicol (HiMedia®, India) at 27 ºC for 7 days with few modifications [7]. Isolates were further confirmed through sequencing of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) and gene sequences have been submitted to GenBank database under accession number MT316125 and MT319079.

2.2. Antifungal susceptibility testing (AFST)

AFST was performed by using a Broth Micro-dilution Assay method recommended by Clinical Laboratory Standard Institute (CLSI) approved standard M27-A2 against seven commonly used antifungal drugs namely Fluconazole, Itraconazole, Voriconazole, Amphotericin-B, Caspofungin, Micafungin and Anidulafungin (all procured from Sigma-Aldrich®) [8]. All the agents were dissolved using 100% dimethyl sulfoxide (DMSO) except Fluconazole (in sterile water) to the strength of 1mg/ml which were diluted in Roswell Park Memorial Institute (*RPMI*) *1640* Medium with l-glutamine but without sodium bicarbonate and buffered at pH 7.0 with morpholinepropanesulfonic acid. Final concentrations ranged from 32.0 to 0.06 µg/ml for all the drugs except Fluconazole which was 64 to 0.13 µg/ml.

C. albicans isolates were sub-cultured on Potato dextrose agar (PDA) for 24 hour at 35 °C. Inoculum was prepared by picking five colonies (~1mm) and suspending them in 5 ml of sterile normal saline (0.145 mol/L). The suspension was then transferred to a sterile tube, vortexed for 15 seconds, cell density adjusted to transmittance of 70% and diluted with RPMI 1640 medium to acquire the final inoculum concentration of approximately 1 to 5 × 10^6 cells/ml. Sterile micro dilution plates (Tarsons®) were taken and Column 1 was filled with 200 µL of inoculums to serve as a positive growth control. Columns 2 to 12 were filled with both inoculum and serially diluted antifungal agent (100 µL each). The micro dilution plates were then incubated at 35 °C and MIC's were interpreted after 48 hours of incubation [8].

2.3. Hemolysin evaluation

The hemolytic activity was evaluated in a commercial sheep blood agar plate (HiMedia®, India). The colony diameter (a) and the diameter of the colony plus the precipitation zone (b) were measured by a graduated ruler, and the enzymatic activities were expressed as the P_z value (a/b). The P_z value was scored into four categories: P_z equal to 1.0 indicated no enzymatic activity; P_z between 0.999 and 0.700 indicated weak (low) enzymatic production; P_z between 0.699 and 0.400 corresponded to good (moderate) enzymatic production; and a P_z lower than 0.399 meant excellent (high) enzymatic production [9].

3. Results and discussion

Microscopic examination of urine specimens showed pus cells, budding yeast cells and hyphae (Figure 1). The colonies on SDA were white to creamy with smooth and glistening surface (Figure 2A). Fluorescent staining with calcofluor white (CFW) revealed oval, elongated budding yeast cells and pseudohyphae (Figure 2B). Macroscopic and microscopic examination confirmed *C. albicans*. ITS sequencing of rRNA gene of both the isolates revealed a similarity of more than 99% in their gene sequence. In the past few years, the number of pet animals especially dogs living with humans in India have increased [10]. The strict relationship between pet animals and their owners raises the attention to the pathogens that can be zoonotically transmitted, such as *C. albicans* [11]. Situation is further aggravated with the emergence of antifungal resistance among the isolates which do not respond to the therapeutic options available. AFST of both the isolates showed similar patterns of susceptibility against a particular antifungal agent tested. MIC values for Fluconazole, Itraconazole and Amphotericin-B were 64, 32 and 4µg/mL respectively while that for Voriconazole was 0.5µg/mL. MIC values for Caspofungin, Micafungin and Anidulafungin were 0.13µg/mL. For the treatment of candiduria, the drug of choice is fluconazole or amphotericin-B [12], but in the present study the isolates were highly resistant for both the antifungals along with itraconazole. Widespread use of these antifungals for prophylaxis in animals has triggered this emergence of resistance. This development of resistance in *C. albicans* is a way to adapt to environmental stress and/or within animal host [13].

Both the isolates produced moderate hemolysin activity (Figure 3). The pathogenesis of Candiduria is associated, among other factors, with the secretion of enzymes that degrade the components of the infected tissue. The spectrum of enzymes secreted by *C. albicans* is broad, and the intensity of the enzymatic production differs between the isolates [6, 14]. A better understanding of the mechanisms underlying an infection can be the rationale for a future development of therapeutic and prophylactic strategies. During infection the host immune system secretes macrophages and mast cells into affected area as an immunological reaction [15]. In the present study, both the isolates presented moderate hemolysin activity which could help them in destruction of these cells which is a well known phenomenon in bacteria and few funguses [9, 16, 17]. The isolates also produced hyphae and pseudohyphae which helps the pathogen to increase its virulence and cause tissue damage by invading epithelial cells [6].

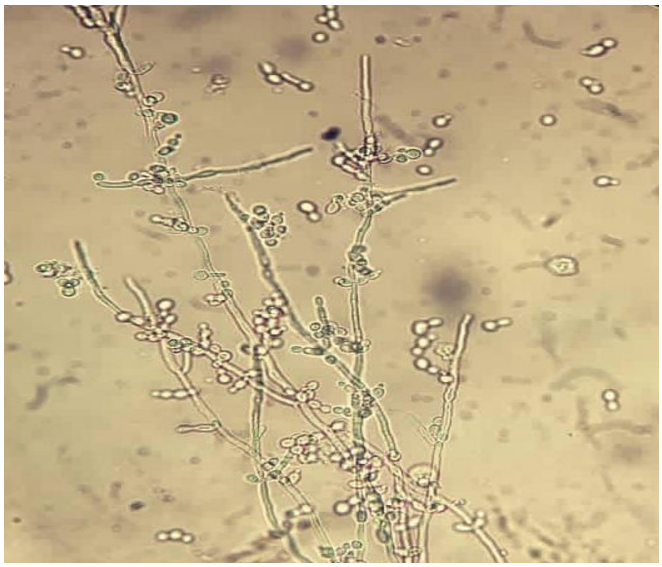

Fig 1: Microscopic examination of urine showing pus cells, budding yeast cells and hyphae (100X).

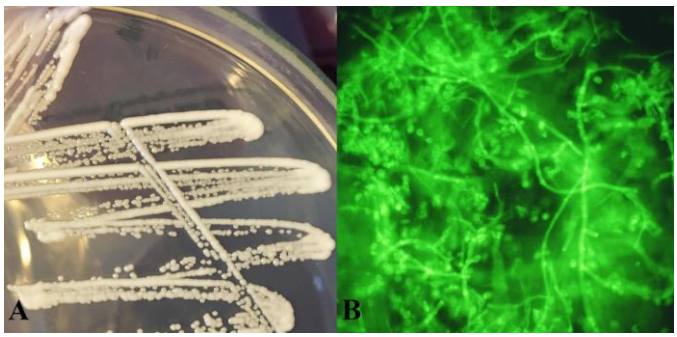

Fig 2: Obverse section of *Candida albicans* isolate on SDA medium (A) and Calcofluor white staining showing oval, elongated budding yeast cells and pseudohyphae under fluorescent microscope (B) (100X).

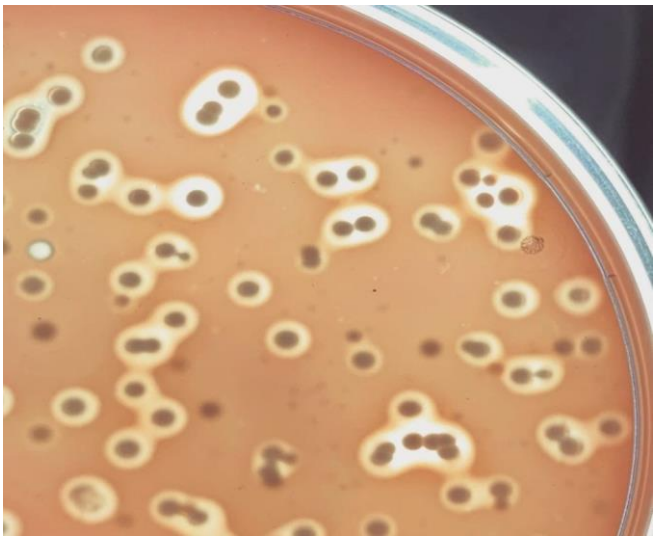

Fig 3: Hemolysis of sheep blood agar plate by *Candida albicans* isolate.

4. Conclusion

The fact that, *C. albicans* can be transferred from animals to humans makes pet owners an interesting potential source of the pathogen. The situation becomes a topic of concern particularly for the drug resistant isolates since resistance to even one class of antifungal drugs severely limits therapy and hampers patient management because of few antifungals available in the hand. Therefore antifungals should be carefully used and not rampantly in order to arrest and suppress development of resistance.

5. Acknowledgement

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6. Conflict of interest

The authors declare no conflict of interest.

7. References

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