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Virulence Markers and Antifungal Susceptibility Profile of *Candida glabrata*: An Emerging Pathogen

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Authors' contributions

This work was carried out in collaboration between all authors. Author SD designed the study, managed the literature searches, wrote the protocol, and wrote the first draft of the manuscript. Author SS managed the analyses of the study. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To study virulence factors and antifungal susceptibility profile of *C. glabrata* isolated from various clinical specimens.
Study Design: A total of 175 *C. glabrata* spp. isolated from various clinical specimens were included in the study.
Place and Duration of Study: Department of Microbiology, Rural Medical College, Pravara Institute of Medical Sciences (PIMS), Loni, Maharashtra, India, between March 2008 to March 2013.
Methodology: *C. glabrata* was identified by sugar assimilation and fermentation tests and colony color on Hichrome Candida agar. HiCandida identification kit supplemented the identification of the isolates. The virulence markers studied were production of extracellular hydrolytic enzymes (phospholipase, proteinase and coagulase), haemolytic activity and biofilm formation. The antifungal susceptibility profile of *C. glabrata* isolates was determined by Hicomb minimum inhibitory concentration (MIC) test. The antifungal agents used were amphotericin B (range 0.002-32 µg), fluconazole (range 0.016-256 µg), itraconazole (range 0.002-32 µg) and ketoconazole (range 0.002-32 µg).
Results: Maximum number of isolates were obtained from blood culture (36%) followed by urine sample (29.7%). ICU stay followed by HIV infection were the main predisposing factors found to be associated with *C. glabrata* infection. A total of 53 (30.2%) *C. glabrata*

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isolates showed phospholipase activity. Proteinase production was seen in 56 (32%) isolates. 48 (27.4%) isolates were coagulase positive. Haemolytic activity was noted in 43 (24.5%) isolates. Most of *C. glabrata* produced β- type of haemolysis on sheep blood SDA agar. Biofilm forming ability was noted in 68 (38.8%) isolates. Maximum isolates were resistant to fluconazole (46.8%) and ketoconazole (46.8%) followed by itraconazole (45.7%). Amphotericin B resistance was seen in 58 (33.1%) isolates.

Conclusion: Once considered as a non pathogenic human commensal, *C. glabrata* has emerged as an important pathogen in various clinical types of candidiasis. *C. glabrata* is innately resistant to antifungal drugs and various antifungal mechanisms of the body. Present research data available is not satisfactory to understand the pathogenic and other mechanisms involved in the transition of *C. glabrata* from nonpathogenic commensal to a potential pathogen. Therefore more research studies are needed to explain pathogenesis, host-pathogen interaction and other survival properties of this emerging pathogen.

Keywords: Candida glabrata; emerging pathogen; fluconazole; virulence factors.

1. INTRODUCTION

The incidence of candidiasis has increased dramatically over the past three decades. Increased therapeutic applications of immunosuppressive drugs, the use of broad spectrum antibiotics and the emergence of HIV/AIDS are important predisposing factors [1].

Candida is capable of causing infections in both immunocompetent as well as immunocompromised hosts but the incidence of candidiasis is more in immunocompromised individuals. Therefore the candidiasis can be rightly called as “disease of diseased”.

During the past decade, infections due to *Candida* species other than *C. albicans* have increased. Among these non *albicans Candida* (NAC) spp, *C. glabrata* has emerged as an important opportunistic pathogen [2]. In recent years, *C. glabrata* is found to be responsible for increasing number of systemic infections like candidemia.

In contrast to other species of *Candida*, *C. glabrata* is a haploid yeast. It is incapable of forming pseudohyphae and exists as blastoconidia in both pathogenic and commensal state [3]. The increase in the incidence of systemic infections due to *C. glabrata* is of great concern because the organism is either intrinsically resistant to antifungal agents or may acquire resistance after exposure [4].

Mechanisms like modification of ergosterol biosynthesis, efflux of antifungal drugs and alteration in the affinity of the drug target are suggested for resistance to azole group of antifungal drugs [5]. Surprisingly, despite the high mortality rate, *C. glabrata* demonstrates low virulence in experimental animals [6].

Due to drug resistance, treatment failure and high mortality rate, *C. glabrata* has gained the attention of clinical Mycologists and clinicians. However, there are only few studies available on this emerging opportunistic pathogen. Keeping this in mind the present study was designed at rural tertiary care hospital of Maharashtra, India with an aim to study virulence factors and antifungal susceptibility profile of *C. glabrata* isolated from various clinical specimens.

2. MATERIALS AND METHODS

The present study is a part of PhD thesis and was approved by Institutional Ethics Committee. A total of 175 *C. glabrata* spp. isolated from various clinical specimens were included in the study. *C. glabrata* was identified by sugar assimilation and fermentation tests and colony color on Hichrome Candida agar (Himedia Laboratories Pvt. Ltd Mumbai, India). HiCandida identification kit (Himedia Laboratories Pvt. Ltd Mumbai, India) supplemented the identification of the isolates.

2.1 Virulence Factors

The virulence markers studied were production of extracellular hydrolytic enzymes (phospholipase, proteinase and coagulase), haemolytic activity and biofilm formation.

2.1.1 Phospholipase production

The phospholipase activity of *C. glabrata* isolates was screened by measuring the precipitation zone on egg yolk agar [7]. The egg yolk consisted of 13.0 g of Sabouraud dextrose agar (SDA), 11.7 g Nacl, 0.11 g $CaCl_2$ and 10% sterile egg yolk. Egg yolk was centrifuged at 500 g for 10 min at room temperature and 20 ml of supernatant was added to the sterilized medium.

5 µl of standard inoculum of test strain (*C. glabrata*) and control strain (*C. albicans* ATCC 10231) containing approximately 10^8 *Candida* cells/ml was aseptically deposited onto the egg yolk agar. The plates were dried at room temperature and then incubated at 37ºC for 48 h. After incubation the plates were examined for the presence of precipitation zone (indication of phospholipase production) around the *Candida* colony (Fig. 1).

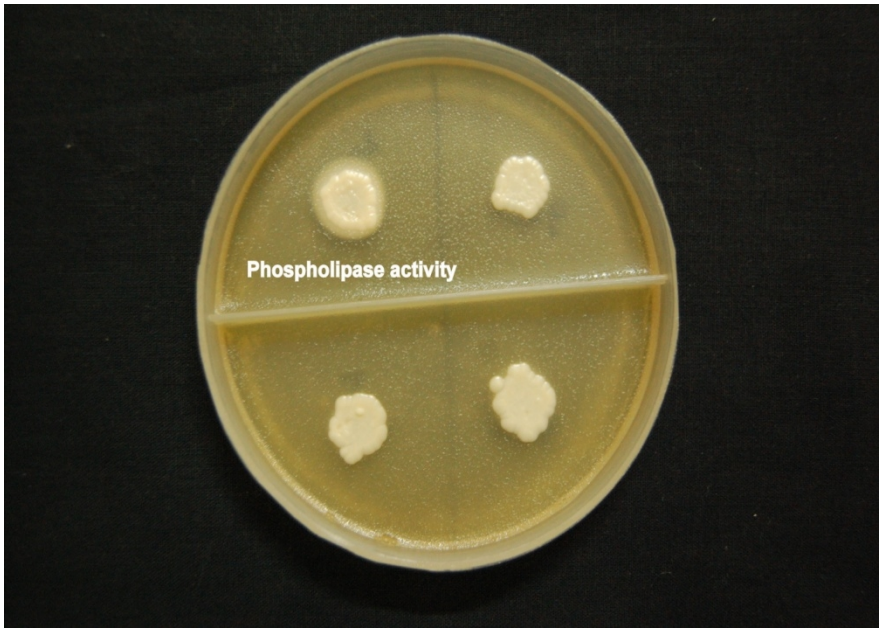

Fig. 1. Zone of precipitation around the colony indicating phospholipase activity

The phospholipase index (P_z) was measured by the method described by Price et al. [8]. It was define as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone. A P_z value of 1 denoted no phospholipase activity, P_z<1 indicated phospholipase activity. The lower the P_z value, the higher the phospholipase activity.

2.1.2 Proteinase activity

C. glabrata isolates were screened for the production of proteinase in terms of bovine serum albumin (BSA) degradation [9]. The BSA medium contained dextrose 2%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, agar 2% and BSA solution 1%.

The standard inoculum containing approximately 10^6 *Candida* cells/ml was prepared from both test strain (*C. glabrata*) and positive control strain (*C. albicans* ATCC 10231). 10 µl of standard inoculum was aseptically inoculated on 1% BSA plate. The plate was incubated at 37ºC for 5 days.

After incubation, the plate was fixed with 20% trichloracetic acid and stained with 1.25% amidoblack. Opaqueness of the agar corresponding to a zone of proteolysis surrounding the colony that could not be stained with amidoblack indicated degradation of the protein. The value of proteinase activity (Pr_z) was calculated in the terms of the ratio of the colony to the diameter of proteolytic unstained zone. A Pr_z value of 1 indicated no proteinase production, Pr_z<1 indicated proteinase activity. The lower the Pr_z value, the higher the proteinase activity.

2.1.3 Coagulase production

C. glabrata isolates were screened for coagulase production by classical test tube method [10]. 0.1 ml of an overnight culture of *C. glabrata* was aseptically added to tube containing 500 µl of EDTA rabbit plasma. The tubes were incubated at 35^0 C for 4 h.

The presence of a clot that could not be resuspended by gentle shaking indicated positive coagulase test. If no clot formed, the tube was reincubated and reexamined after 24h. *Staphylococcus aureus* ATCC 25923 and *S. epidermidis* ATCC 14990 were used as positive and negative controls.

2.1.4 Haemolytic activity

The *C. glabrata* isolates were screened for haemolysin production by the method described by Manns et al. [11]. For this test, the media was prepared by adding 7 ml aseptically collected sheep blood to 100 ml SDA supplemented with glucose at a final concentration of 3% (w/v). The standard inoculum containing approximately 10^8 *Candida* cells/ml was prepared from test strain (*C. glabrata*) and control strain (*C. albicans* ATCC 90028). 10 µl of this standard inoculum was deposited onto the medium. The culture plates were incubated at 37^0 C for 48 h.

After incubation the plates were examined for the presence of zone of haemolysis around the colony (indication of haemolytic activity) (Fig. 2).

Haemolytic activity (H_z) was determined by calculating the ratio of diameter of the colony to that of the translucent zone of haemolysis (in mm). *Streptococcus pyogenes* (Lancefield group A) and *Streptococcus sangius* was used as positive control for beta and alpha haemolysis respectively.

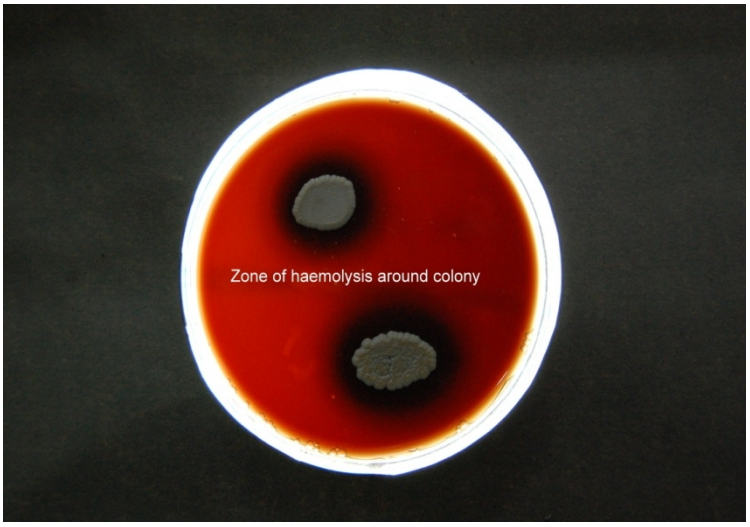

Fig. 2. Zone of haemolysis around the colony indicating haemolytic activity

2.1.5 Biofilm formation

Biofilm forming ability of *C. glabrata* was assessed by tube method. Colonies of *C. glabrata* from SDA plate were inoculated in saline and incubated at 37ºC for 24 h. About 0.5 ml of this culture broth was added into screw capped conical polystyrene tube containing 5 ml of Sabouraud dextrose broth supplemented with glucose (final concentration 8%). The tubes were incubated at 37ºC for 48 h without agitation.

After incubation, the culture broth from the test tubes was gently aspirated by Pasteur pipette. The tubes were washed twice with distilled water and stained with 2% crystal violet for 10 min.

Biofilm formation was considered positive when a visible film was seen on the wall and at the bottom of the tube. Ring formation at the liquid interface was not considered as indication of biofilm formation.

Streptococcus epidermidis ATCC 35984 and non-biofilm producer *C. albicans* ATCC 10231 were used as positive and negative controls, respectively.

2.2 Antifungal Susceptibility Testing

The antifungal susceptibility profile of *C. glabrata* isolates was determined by Hicomb minimum inhibitory concentration (MIC) test (Himedia Laboratories Pvt. Ltd Mumbai, India). The antifungal agents used were amphotericin B (range 0.002-32 µg), fluconazole (range 0.016-256 µg), itraconazole (range 0.002-32 µg) and ketoconazole (range 0.002-32 µg). The test was carried out in accordance with manufacturer's instructions.

The inoculum was prepared by inoculating 2-3 colonies of *C. glabrata* in 0.85% saline. The turbidity of suspension was adjusted to 0.5 Mc Farland standard. The lawn culture of the suspension was done with help of tipped cotton swab on agar plate containing RPMI 1640

supplemented with 2% glucose. The antifungal strips were aseptically placed on the media with the help of forcep and the plates were incubated at 35ºC for 48 h.

Interpretative susceptibility criteria for fluconazole and itraconazole were those recommended by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) [12]. Due to the lack of defined breakpoints for amphotericin B and ketoconazole, arbitrary values based on the studies of other researchers were used.

Standard strains *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 were used for quality control. The antifungal susceptibility of the isolates were interpreted as sensitive (S), dose dependent-susceptible (DDS) and resistant (R) [2,13].

3. RESULTS AND DISCUSSION

The sample wise distribution of *C. glabrata* is shown in Fig. 3. Maximum number of isolates was obtained from blood culture followed by urine sample. As shown in Fig. 4 ICU stay followed by HIV infection were the main predisposing factors found to be associated with *C. glabrata* infection.

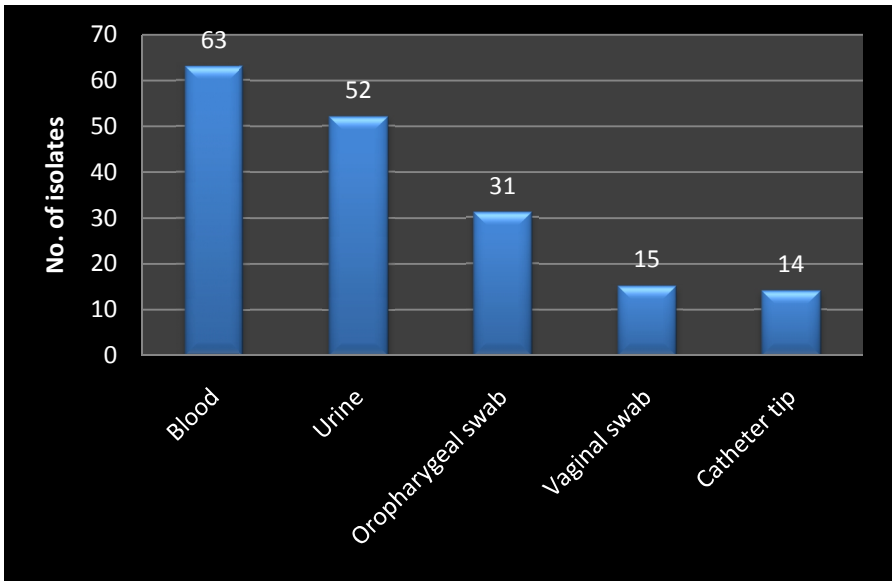

Fig. 3. Number of *C. glabrata* isolated from different clinical specimens

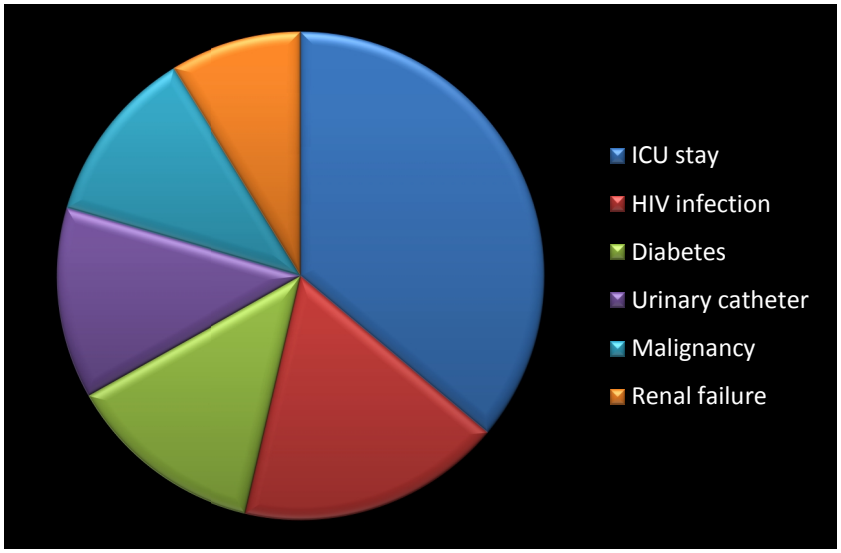

Fig. 4. Risk factors for *C. glabrata* infection

A total of 53 (30.2%) *C. glabrata* isolates showed phospholipase activity. Proteinase production was seen in 56 (32%) isolates. 48 (27.4%) isolates were coagulase positive. Haemolytic activity was noted in 43 (24.5%) isolates. Most of *C. glabrata* produced β- type of haemolysis on sheep blood SDA agar. Biofilm forming ability was noted in 68 (38.8%) isolates (Table 1).

As shown in Table 2, maximum isolates were resistant to fluconazole (46.8%) and ketoconazole (46.8%) followed by itraconazole (45.7%). Amphotericin B resistance was seen in 58 (33.1%) isolates.

Table 1. Production of virulence factors in *C. glabrata*

Virulence factor	No. of isolates (%)
Phopholipase activity	53 (30.2)
Proteinase activity	56 (32)
Coagulase production	48 (27.4)
Haemolytic activity	43 (24.5)
Biofilm formation	68 (38.8)

Table 2. Antifungal susceptibility profile of *C. glabrata*

Antifungal agent	Sensitive (%)	Dose-dependent sensitive (%)	Resistant
Fluconazole	51 (29.1)	42 (24)	82 (46.8)
Ketoconazole	49 (28)	44 (25.1)	82 (46.8)
Itraconazole	48 (27.4)	47 (26.8)	80 (45.7)
Amphotericin B	86 (49.1)	31 (17.7)	58 (33.1)

Since 1960s many new species of *Candida* have emerged as potential pathogens. The emergence of HIV/AIDS, increasing number of bone marrow and solid organ transplantations and increased use of commercial identification systems in clinical mycological services can be the probable reasons [14].

C. glabrata is the second or third most common *Candida* spp. found to be associated with different clinical types of candidiasis. Many studies have shown *C. glabrata* to be the most common cause of candidemia and Catheter associated urinary tract infection [3,15,16]. In the present study, maximum number of *C. glabrata* isolates was obtained from blood cultures. Trick et al. [17] also reported the increase isolation of *C. glabrata* from candidemia cases. *C. glabrata* is the second most common spp. of *Candida* responsible for fungaemia in United States. The incidence of *C. glabrata* blood stream infection (BSI) is more common in adults as compared to neonates and children [18]. The risk factors leading to *C. glabrata* BSI are similar to those of other *Candida* spp., but this species has emerged as a prominent pathogen among patients with haematological malignancies and bone marrow transplants [17].

In the present study 29.4% of *C. glabrata* isolates were obtained from urine sample. Approximately 50% of candiduria are due to NAC spp., among them *C. glabrata* is the most common isolate. *C. glabrata* is a frequent cause of polymicrobial urinary tract infection (UTI) [3]. The majority of *C. glabrata* UTI are asymptomatic. The incidence of candiduria due to *C. glabrata* is more in elderly debilitated, hospitalized and catheterized individuals on antibiotics [3]. Fluconazole use is a significant risk factor for *C. glabrata* candiduria.

In the present study 17.7% of *C. glabrata* were isolated from isolated from suspected cases of oropharyngeal candidiasis (OPC). Individuals receiving radiation for head and neck cancer, denture users and HIV infected patients are at risk for *C. glabrata* OPC [6]. *C. glabrata* possess higher tendency of adherence to denture acrylic surface [19]. This property of C. glabrata protects them from antifungal effect of saliva.

Vulvovaginal candidiasis (VVC) is the most common mycotic infection in healthy women [20]. Approximately 75% of females suffer at least one episode of VVC in their lifetime [21]. Pregnancy, diabetes mellitus, HIV infection and antibiotic therapy are the most common predisposing factors. *C. glabrata* VVC is common in women receiving long term maintenance low-dose of fluconazole [3]. In our study 8.5% of *C. glabrata* were isolated from vaginal swab.

One of the important factors contributing to the virulence of *Candida* is production of extracellular hydrolytic enzymes. The three most significant extracellular hydrolytic enzymes produced by *Candida* are phospholipases, proteinases and lipases [22]. Phospholipase enzyme hydrolyzes one or more ester linkage in glycero-phospholipids and aids the invasion of host tissues [22]. In our study phospholipase activity was noted in 30.2% of *C. glabrata* which is in contrast to the observation of Samaranayanke et al. [7], where no strains of *C. glabrata* showed phospholipase production.

Proteinase enzyme facilitates adherence and phenotypic switching of *Candida* by hydrolyzing the peptide bonds in proteins [23]. In the present study 32% of *C. glabrata* isolates showed proteinase production. Although very little is known about proteinase production in *C. glabrata*, few studies have shown the capability of *C. glabrata* to produce proteinase [2,24].

Coagulase and haemolysin production are among the least studied virulence properties of *Candida* spp [10]. The secretion of haemolysin followed by iron acquisition facilitates invasion of host tissue [11]. In the present study 43 (24.5%) *C. glabrata* showed haemolysis on sheep blood SDA agar. Coagulase production was seen in 24.5% of *C. glabrata* isolates. Our observation is in accordance with that of Yigit et al. [10].

Health-care associated infections (HAIs) are a major problem in any health care set up. The surmounting task for health care professional is to prevent HAIs in a dynamically evolving milieu of scientific advances, immunocompromised patients, complex interventions and increasing antibiotic resistance. Nearly about half of HAIs are associated with medical devices [25]. There are number of published data on biofilm forming ability of *C. albicans*, but this property is least studied in *C. glabrata* [26]. Biofilm formation was seen in 68 (38.8%) *C. glabrata* isolates, which is similar to the observation of Mohandas et al [27]. *C. glabrata* is emerging as an important nosocomial pathogen hence there is a need of more research on *C. glabrata* biofilms.

As compared to other species of *Candida*, *C. glabrata* demonstrate more resistance to antifungal agents. In our study significant percentage of *C. glabrata* isolates were resistant to azole antifungal agents (fluconazole (46.8%), ketoconazole (46.8%), itraconazole (45.7%)). Studies of Sanguinetti et al [5], Barchiesi et al [28] and Moran et al [29] have also reported significant percentage of azole resistance in *C. glabrata.* The emergence of azole resistance is of concern because either higher dose of azoles are needed to be administrated or other costly antifungal drugs such as echinocandins or polyenes are to be used for the treatment of *C. glabrata* infections [5].

In this study amphotericin B resistance was noted in 58 (33.1%) isolates. Amphotericin B in various formulations is used for the treatment of systemic candidiasis. The resistance to amphotericin B is of concern because it is the drug of choice for the treatment of *C. glabrata* despite the increased toxicity associated with it [14].

4. CONCLUSION

During the past few decades, an increase in the incidence of superficial and systemic candidiasis due to NAC spp. is noted. Once considered as a non pathogenic human commensal, *C. glabrata* has emerged as an important pathogen in various clinical types of candidiasis. *C. glabrata* is innately resistant to antifungal agents and various antifungal mechanisms of the body. Present research data available is not satisfactory to understand the pathogenic and other mechanisms involved in the transition of *C. glabrata* from nonpathogenic commensal to a potential pathogen. Therefore more research studies are needed to explain pathogenesis, host-pathogen interaction and other survival properties of this emerging pathogen.

ETHICAL APPROVAL

The present study was approved by Institutional Ethics Committee (Registration No. PIMS/PhD/RC/2013/24).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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