International Journal of Basic and Applied Medical Sciences ISSN: 2277-2103 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jms.htm
2016 Vol. 6 (3) September-December, pp. 9-16/Wale et al.
Research Article

UROPATHOGENIC AND ANTIBIOTIC RESISTANCE OF *CANDIDA* SPECIES AMONG WOMEN VISITINGA TERTIARY CARE HOSPITAL IN GABORONE, BOTSWANA

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ABSTRACT
The present paper aims to determine the prevalence and antibiotic susceptibility of *Candida* uropathogens among women at a tertiary hospital in Gaborone, Botswana. A total of 180 urine samples were collected from females of different age groups with UTIs from Princess Marina Hospital, Gaborone and assayed for the presence of *Candida* species. Five species of *Candida*, namely *C. albicans*, *C. glabrata*, *C. krusei*, *C. dubliniensis* and *C.tropicalis* were tentatively identified and antibiotic suceptibility was carried out on chromogenic media using the Vitek 2 system. The identification of these species were confirmed by amplifying the internal transcribed spacer regions (ITS). To amplify the superseding 5.8S ribosomal Deoxyribonucleic Acid (rDNA) and the adjacent ITS1 and ITS2 regions, the ITS1-ITS4 primer pair was used. Candidal uropathogens accounted for 38.8 % of UTIs out of 180 urine samples. *Candida albicans* was the most predominant in the urine samples acounting for 45.7 % followed by *Candida glabrata* (17.1 %), while *C. krusei* and *C.dublineinsis* each acounted for 10 % and *Candida tropicalis* 5.5%. There was no statistically significant diferrence ($p<0.05$) between the rate of isolation of *Candida* spp. Although a high prevalence of candiduria (51.4%) was associated withfemales in the 26-35 age bracket, this effect was not statistically significant ($p<0.05$) among different age groups. Three non-*Candida* species (1.6%), *Cryptococcus neoformans*, *C. albius and Rhodotorula*sp were also detected from urine samples. This finding is alarming and demonstrates that these species may emerge as opportunistic pathogens in UTIs. *C. dublineinsis*was highly resistant to flucytosine (100%) and fluconazole (60%). The results of the present study highlight a growing problem of antifungal resistance among some *Candida* uropathogens and call for a need to establish stringent measures in monitoring the same.

Keywords: *Candida, Urinary Tract Infections (UTIs), Antibiotic Resistance, Internal Transcribed Spacer Region (ITS)*

INTRODUCTION
Urinary tract infections (UTIs) are the most common infections that afflict humans in the community and hospital settings, and often result in increased rates of morbidity and high economic costs associated with their treatment (Foxman, 2002; Shah *et al*., 2015; Preesthishree *et al*., 2016). Underlying host factors such as gender, age, urogenital dysfunction, immunosuppression, indwelling catheters and sexual behaviour have been shown to predispose individuals to urinary tract infections (Dwyer and O'Reilly, 2002).
Various studies (Stamm, 2001; Nimri and Batchoun, 2010) have shown that women are more likely to experience UTIs than men. This maily owes to the urethra of women being shorter and in closer proximity to the anus than in men, hence allowing pathogens to gain quicker access to the bladder. It is estimated that 20% of women experience urinary tract infections and the disease is especially more common among the eldery and pregnant women (Dwyer and O'Reilly, 2002).
Although *Escherichia coli* tends to be the most predominant microorganism associated with urinary tract infections (Orhue, 2014), *Candida* spp. have in the past few decades come to the fore as significant causes of UTIs (Brito *et al*., 2006; Kaplana *et al*., 2015). They are opportunistic microorganisms which are found as normal flora in the female genital area (Payam *et al.*, 2010), and have virulence factors such as secreted aspartyl proteinases (SAPs) and phospholipases which facilitate host tissue adhesion and invasion (Naglik *et al*., 2005, Birinci *et al*., 2006).

There are eight opportunistic *Candida* spp. namely; *C. albicans, C. glabrata, C. guilliermondii, C. krusei, C. lusitaniae, C. parapsilosis, C. dublineinsis* and *C.tropicalis* (Anaissie *et al.,* 2003). Urinary tract infections due to *Candida* species are known as candiduria and *C. albicans* has been found be the most prevalent cause of these infections, accounting for about 50% of the cases of candiduria (Alenzi, 2016).*Candida* infections are routinely treated with antifungals such as Itranacazole (De Backer *et al*., 2001) and Flucytosine (Sobel *et al*., 2003). However, a growing problem associated with clinical *Candida* isolates is antifungal resistance, where the species have been found to employ resistance mechanisms like biofilm formation (Douglas, 2003) and efflux pumps (Ramage *et al*., 2002).

Advanced molecular identification approaches of yeast species are based on polymerase chain reaction (PCR), that is, PCR-RFLP, real-time PCR which overcome the limitations of phenotypic methods of yeast identification (Hierro *et al.,* 2004). Between coding regions of the 18S, 5.8S, and 28S nuclear rRNA genes are the internal transcribed spacer 1 and 2 regions (ITS1 and ITS2, respectively) which evolve more rapidly and may therefore, vary among different species within a genus (Chen *et al.,* 2000). In this regard, PCR amplification of ITS region DNA and sequencing them stands out to be the most appropriate method used for identifying fungal species.

In Botswana, *Candida* spp. have not only been isolated from different food products such as vegetables and biltong (Manani *et al.,* 2006; Matsheka *et al*., 2014) but have also been found to account for up to 49% of pathogens in women with vaginal discharge (Paz-Bailey *et al*., 2006). Urinary tract infections (UTIs) and increasing antibiotic resistance of candida uropathogens are a global concern paticularly in immunocompromised patients. However, no study has been done in Botswana addressing the role of *Candida*sp. in urinary tract infections in women, and their identification using molecular tools. Hence, the objective of the present study was to identify and determine the prevalence and antibiotic resistance of *Candida* uropathogens among women at a tertiary hospital in Gaborone, Botswana.

MATERIALS AND METHODS

Sample Collection and Processing

Early morning mid-stream urine samples were collected in sterile containers from 180 women diagnosed as suffering from urinary tract infections from November 2013 to April 2014 at Princess Marina Hospital, a tertiary hospital in Gaborone Botswana. Upon collection, the color of the urine was noted and then samples transported aseptically in a cooler box to the Microbiology laboratory of the Department of Biological Sciences, University of Botswana.

Screening and Culturing of Candida Isolates

On arrival at the laboratory, the Combur 10 Test® (Roche Diagnostics Gmbh, Mannheim, Germany) was used to detect leukocytes in the urine which served as an indirect indicator of urinary tract infections. Isolates that tested positive for leukocytes were spun at medium speed in a centrifuge (Z 233 M-2, Hermle Labortechnik GmbH, Germany). The supernatant was discarded and a wet mount prepared and examined microscopically. Candiduria was confirmed by isolation of one or two *Candida* sp. with less than 10^5 colony forming units per milliliter and presence of pyuria(>6 pus cells per high power field). Using a calibrated loop of 3mm diameter, appropriately labeled CHROMagar Candida (CHROMagar Company, Paris, France) plates were streaked aseptically with 0.01 ml urine sample. The plates were then incubated at 37°C for 24 hours and thereafter observed for characteristic colors displayed by different *Candida* species.

Identification and Antibiotic Resistance Testing of Yeast Isolates

Identification and antibiotic resistance testing of the tentative *Candida* isolates on chromogenic media was carried out using the Vitek 2 system (BioMerieuxVitek. Inc. Hazelwood, MO) following the manufacturer's instructions. The antifungals evaluated were amphotericin B, caspofungin, fluconazole, flucytosine, and voriconazole.

DNA Extraction and Amplification of the Internal Transcribed Spacer Region

Candida isolates were sub cultured on Sabouraud dextrose agar (Merck KGaA, Darmstadt, Germany) and CHROMagar Candida (CHROMagar Company, Paris, France) and incubated at 30°C for 48 h. Genomic

DNA of each fresh colony was extracted using ZRFungal/Bacterial DNA Miniprep™ (Zymo Research., Irvine, CA, USA) according to the manufacturer's instructions.
The ITS gene region was amplified in a GeneAMP PCR system (Applied Biosystems, Carlifornia, USA). The primer pair used in the amplification of the ITS region, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), have been described previously (White *et al*., 1990). Each PCR reaction mixture consisted of 12.5µl of 2X Master Mix (New England Biolabs, Ipswich, MA, USA), 1µl each of reverse and forward primers, 2µl of genomic DNA and the mixture made up to 25µl with sterile nuclease-free water.
The PCR was run with the following thermocycling conditions: initial denaturation at 94°C for 8 min; followed by 35 cycles for 1 min at 94°C, 55°C for 1 min and 2 min at 72°C and final extension at 72°C for 12min.
Three micro liter of the PCR products were resolved on 1.2% agarose gel (Sigma Aldrich, Missouri, USA) in TBE buffer (Tris 90mM, Boric acid 90mM, EDTA 2mM), stained with 0.5 µg/ml of ethidium bromide.
The gels were run for 45 minutes at 80 volts and gels were then visualized on a gel documentation system (Bio-Rad, Carlifornia, USA).

Data Analysis

Graphpad Prism 7 (GraphPad Software Company, LaJolla, CA, USA) was used to analyze the statistical significance of the data.
One-way ANOVA was employed to separate the means of occurrence of *Candida* spp. in different urine samples investigated in this study. Fisher's exact tests were used to analyze the frequency of *Candida* spp. in urine samples.

RESULTS AND DISCUSSION

Results

Out of the 180 urine samples, a total of 60 (38.8 %) tested positive for *Candida* spp. The most predominant *Candida* spp. in the samples isolated was *Candida albicans* with a prevalance of 45.7 % (Figure 2).
The next most prevalent was *Candida glabrata* followed by *Candida krusei*,*Candida dublineinsis* and *Candida tropicalis* with a prevalence of 17.1%, 14.2%, 10% and 10 % respectively. Three (3) isolates (1.6%) were of non-*Candida* species namely; *Cryptococcus albius, Cryptococcus neoformans* and *Rhodotorula* species. There was no statistically significant diferrence ($p<0.05$) on the isolation rate of *Candida* spp. from urine samples.
A comparison of the occurrence of different *Candida* spp species in urine samples showed that their rate of occurrence was not statistically significant ($p<0.05$).
PCR amplification using universal fungal primers (ITS1/ITS4) yielded fragments of about 450-850 bp long (Figure 1). The sizes of the PCR products were nearly equal in size for some species; *C. tropicalis* 530, *C. albicans* 550 and *C. krusie* 510.
However, all yeast isolates showed species-specific differences in the sizes of the PCR products amplified. *C. glubrata* had the largest fragment size of about 780 bp.
Figure 1 shows the distribution of *Candida* spp. and candiduria among different age groups of women. *C. albicans* was found to be the most dominant cause of candiduria in all age groups whereas other *Candida* spp. were mostly associated with females in the age bracket 26-35.
C. glabrata and *C. krusei* were equally the second most common cause of candiduria in females in age group 26-35. *C. glabrata, C. dubliniensis* and *C. tropicalis* were the second most common cause of candiduria in females in the age group 19-25.
A high rate of *Candida* infection was recorded among the age group 26-35 years (51.4%), the age group 46 and above had the least incidence of *Candida* uropathogens (2.9%). The age group 19-25 and 36-45 had an incidence of 34.2 % and 11.4 % respectively. There was no statistical significance ($p<0.05$) in the prevalence of *Candida* spp. among all age groups.

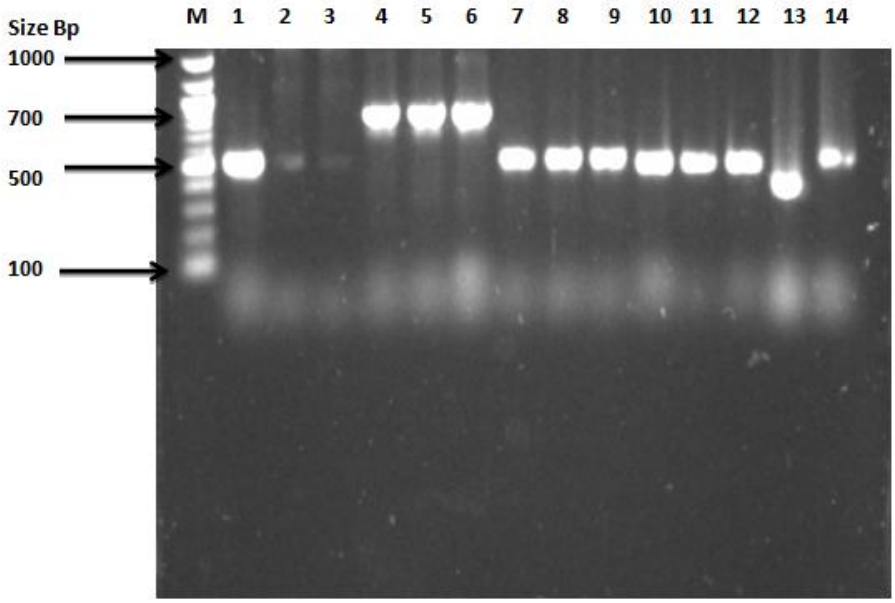

Figure 1: Gel Electrophoresis of the Amplified rDNA Internal Transcribed Sequence (ITS) Region of *Candida* Isolates with ITS1 and ITS4 Primers Pairs; (M) 100 bp DNA Ladder (New England Biolabs).*Candida Tropicalis* (Lanes 1-3), *C. Glabrata* (Lanes 4-6), *C. Albicans* (Lanes 7-9), and *C.krusei* (Lanes 10-12); *C. Lusitaniae* ATCC 34449 (Lane 13) and *C. Albicans* ATCC 10231 (Lane 14) Served as Positive Controls

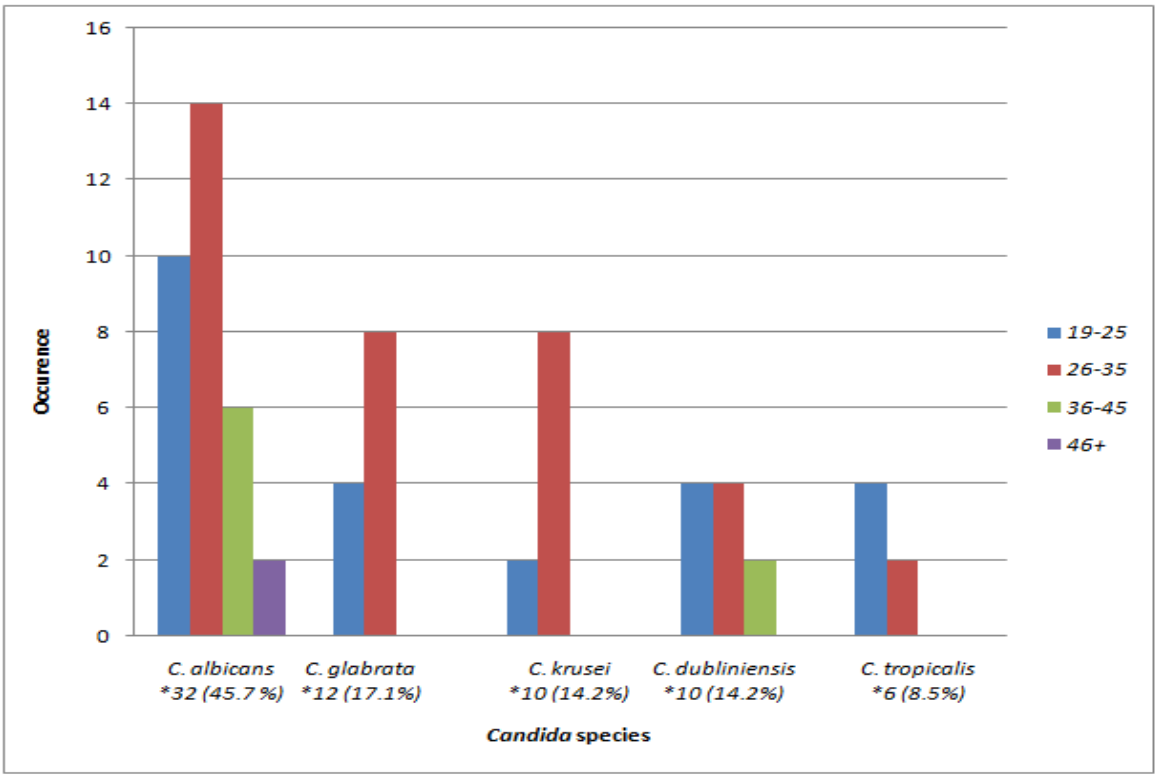

Figure 2: Distribution of Different *Candida* spp. within Different Age Groups of Women
*Total Number and percentage of *Candida* species isolated from urine samples.

Table 1 shows the resistance pattern of *Candida* spp.*C. albicans* and *C. dublineinsis* showed resistance of 18.8% and 100 % to flucytosine respectively. *C. glabrata* and *C. tropicalis*was susceptible to all antifungal agents tested against them. *C. dublineinsis* was resistant to three out five antifungal agents used in this study. Both antifungals amphotericin B and caspofugin each showed 100% of activity against all yeast tested in this study. There was no statistical significance between theactivities of the tested drugs ($p<0.05$).

Table 1: Number and Percentage of Candida Uropathogens Showing Antifungal Resistance Using Vitek 2 System

		Antifungal Agent				
***Candida* Species**	**Number of Isolates**	**Flucytosine**	**Fluconazole**	**Voriconazole**	**Amphotericin B**	**Caspofugin**
C. albicans	32	6 (18.8%)	0	0	0	0
C. glabrata	12	0	0	0	0	0
C. dublineinsis	10	10 (100%)	6 (60%)	2 (20%)	0	0
C. tropicalis	6	0	0	0	0	0
Total	60	16 (26.7%)	6 (10%)	2 (3.33%)	0	0

Discussion

The present study utilized the biochemically-based Vitek 2 identification system and ITS PCR to differentiate *Candida* species among women with urinary tract infections at a tertiary hospital in Botswana. PCR amplification with specific primers for the ITS region generated bands ranging from 450 to 780 base pairs (Figure 1), with clear differences in fragment sizes among different species of *Candida* spp. analyzed. These findings are consistent with other studies (Fujita *et al*., 2001; Ferrer *et al*., 2001) which found different fragment sizes of PCR products when using ITS1 and ITS 4 primer pairs. However, some non-*Candida* yeast species (*Cryptococcus neoformans*, *Cryptococcus albius* and *Rhodotorula* sp) were also detected from urine samples. These species may be resposible for some cases of UTIs. Although this investigation focused on candiduria, this finding is alarming and demostrates how other non-*Candida* yeast species may emergeas opportunistic pathogens creating increased healthcare burden especially in instances where HIV/AIDS continues unabated as is the case in Botswana.

A total of 60 (38.8 %) *Candida* spp. were isolated from urine samples among women diagnosed with UTIs, with *Candida albicans* being the most predominant species (45.7%). The prevalence rate of *Candidaalbicans* reported here is comparable to findings of 30% reported by Debora *et al.*, (2007) in Brazil. However, much lower rates of 10.2% and 8% were reported in Pakistan (Bagai *et al*., 2008) and Saudi Arabia (El Sheikh *et al*., 2000), respectively. Similarly, Tortorano *et al*., (2006) found more than half of cases of candiduria to be accounted for by *Candida albicans,* followed by *C. glabrata* (14%) and *C. krusei* comprising only 2%. In contrast to this study, *C. tropicalis* was found to be the most predominant isolate from clinical samples, acounting for 54.5% prevalance of all isolates (Yesudhason and Mohanra, 2015). The recent upsurge in UTIs due to *Candida* spp. is not completely understood but recent increases in such factors as prophylaxis by antifungal agents, catheterization, and uncotrolled use of antifungals have also seen corresponding increased cases of candiduria (Behzadi *et al*., 2010). This study did not aim to investigate the factors that predispose women to candiduria, with age being the only demographic taken for the particular study population.

The highest proportion of *Candida* urinary tract infections was detected among women in the age bracket 19-35, with those above the age of 36 comparatively lower incidence rates. It is important to note that women in the former age bracket are more sexually active, and sexual activity is a well established factor that predisposes individuals to urinary tract infections (Ramesh and Agarwal, 2012). Some of the women with candiduria in this study were also pregnant and pregnancy has also been shown to predispose women

to UTIs (Haider *et al*., 2010). These two predisposing factors may be responsible for higher prevalence of candiduria in the 19-35 age bracket. The unprecedented levels of candiduria among women in this age bracket may also be exacerbated by HIV/AIDS infections in Botswana, which the Botswana AIDS Impact Survey in 2013 (Statistics Botswana, 2014) estimated to be 18.5%. Other studies also showed a higher frequency of UTI among HIV patients (Ibadin *et al*., 2006).

The emergence of drug resistant *Candida* spp. which is largely attributed to use of prolonged and inappropriate empirical therapy, has further complicated patient management (Pfaller *et al*., 2004). Except for *Candida dublinensis*, all *Candida* species were uniformly susceptible to fluconazole, voricanozole, amphotericin B and caspofugin. The multi-drug resistance displayed by *C. dublinensis*in this study remains the most worrisome. *C. dublinensis* isolates showing multidrug resistance have been reported, especially associated with oral candidiasisin HIV/AIDS patients (Quindós *et al*., 2000). This further buttresses our notion that a significant portion of women in this study may have been HIV positive. *C. dublinensis*is very closely related to *C. albicans*. Antifungal resistance in both species has been linked to multidrug transporters encoded by *MDR1* and *CDR2* genes (Moran *et al*., 1998) as well as point mutations in the *FUR1* and *FCA1* genes encoding enzymes that play a role in pyrimidine salvage pathway (Hope *et al.*, 2004).

Our study reports on the prevalence of candiduria among women visiting a tertiary hospital in Gaborone, Botswana. The high prevalence of antifungal resistance, especially among *Candida* species other than *C.albicans* is a cause of significant public health concern. It is suggested that vigilance be increased among healthcare workers in revising antifungal susceptibility testing and prescription regimen in order to curb antifungal resistance in Botswana due to *Candida* species.

ACKNOWLEDGEMENT

The authors are thankful to the Department of Biological Sciences, University of Botswana for facilities and support.

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