UROPATHOGENIC AND ANTIBIOTIC RESISTANCE OF CANDIDA SPECIES AMONG WOMEN VISITING A 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 susceptibility 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 accounting for 45.7% followed by Candida glabrata (17.1%), while C. krusei and C. dubliniensis each accounted for 10% and Candida tropicalis 5.5%. There was no statistically significant difference (p<0.05) between the rate of isolation of Candida spp. Although a high prevalence of candiduria (51.4%) was associated with females in the 25-35 age bracket, this effect was not statistically significant (p<0.05) among different age groups. Three non-Candida species (1.6%), Cryptococcus neoformans, C. albicans 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. dubliniensis was highly resistant to fluocitosine (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 mainly 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 elderly 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; Kaplan 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).
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There are eight opportunistic Candida spp. namely; C. albicans, C. glabrata, C. guilliermondii, C. krusei, C. lusitaniae, C. parapsilosis, C. dubliniensis and C.tropicalis (Anaissie et al., 2003). Urinary tract infections due to Candida species are known as candiduria and C. albicans has been found to 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 Itracanzole (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 particularly in immunocompromised patients. However, no study has been done in Botswana addressing the role of Candida spp. 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 Combir 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 Laborteknik 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 10^3 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, California, 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 12 min.

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, California, 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 prevalence of 45.7 % (Figure 2).

The next most prevalent was *Candida glabrata* followed by *Candida krusei*, *Candida dubliniensis* 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 difference (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. krusei* 510.

However, all yeast isolates showed species-specific differences in the sizes of the PCR products amplified. *C. glabrata* 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. dublineinis 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. dublineinis 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 the activities of the tested drugs (p<0.05).

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

<table>
<thead>
<tr>
<th>Candida Species</th>
<th>Number of Isolates</th>
<th>Flucytosine</th>
<th>Fluconazole</th>
<th>Voriconazole</th>
<th>Amphotericin B</th>
<th>Caspofugin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>32</td>
<td>6 (18.8%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. dublineinis</td>
<td>10</td>
<td>10 (100%)</td>
<td>6 (60%)</td>
<td>2 (20%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>16 (26.7%)</td>
<td>6 (10%)</td>
<td>2 (3.33%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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 ITS4 primer pairs. However, some non-Candida yeast species (Cryptococcus neoformans, Cryptococcus albicus and Rhodotorula sp) were also detected from urine samples. These species may be responsible for some cases of UTIs. Although this investigation focused on candiduria, this finding is alarming and demonstrates how other non-Candida yeast species may emerge as 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 Candida albicans 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, accounting for 54.5% prevalence 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 uncontrolled 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., 2004).

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, voriconazole, amphotericin B and caspofungin. 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 candidiasis in 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.

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