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Antifungal resistance among *Candida* species from patients with genitourinary tract infection isolated in Benin City, Edo state, Nigeria

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Even though there are abundant documented works on the prevalence of *Candida* species affecting the genitourinary tracts in Nigeria, yet data on their susceptibilities to antifungal agents are lacking. To determine the antifungal resistance among *Candida* species from the genitourinary tracts, 439 urine and high vaginal swab (HVS) samples collected from April to September, 2008 from female patients clinically diagnosed of genitourinary tract infection were inoculated onto Sabouraud dextrose agar (SDA). Isolates from SDA were plated on CHROMagar to ensure detection of mixed cultures. Germ tube and carbohydrate assimilation tests performed were necessary for isolate identification. Susceptibility testing was carried on the isolates using broth dilution method. The occurrence rate of *Candida* species were as follows: *Candida albicans* 138(63.9%), *Candida glabrata* 68(31.5%), *Candida krusei* 6(2.9%) and *Candida tropicalis* 4(1.9%). The rate of occurrence of *Candida* species in high vaginal swab (82.9%) was significantly higher than that of urine (17.7%) using Chi-square test for statistical analysis. Distribution of *Candida* species among different age groups showed the highest incidence in age brackets 26 - 35, followed by 16 - 25, while the ages of 46 and above had the least. High rate of susceptibility was observed for each isolate against fluconazole (97.2%) and ketoconazole (94.9%). The resistance rate was low for fluconazole (2.8%) and ketoconazole (5.6%). These results incriminated *C. albicans* as the most common *Candida* species causing genitourinary tract infection in women. This surveillance study has established fluconazole and ketoconazole as very effective antifungal agents for the treatment of genitourinanry tract infections caused by *Candida* species.

Key words: Genitourinary tract infection, *Candida* species, antifungal resistance, ketoconazole, fluconazole.

INTRODUCTION

Candida species are opportunistic yeast affecting the genitourinary tracts. It belongs to the subclass *Ascomycota* and measures 2 - 4 mm in diameter (Prescott et al., 2008). The genus *Candida* encompasses more than 160 species. The organism variously can be found among humans, other mammals, birds, insects, arthropods, fish, animal waste, plants, mushrooms, honey, necter, fresh water, sea water and in the air (Bennett et al., 1995). *Candida* is listed by the center for disease control (CDC) as a cause of sexually transmitted disease (Prescott et al., 2008). No other mycotic pathogen produces as diverse a spectrum of opportunistic disease in humans as does *Candida*. *Candida* species are important nosocomial pathogens and can be transmitted sexually (Tatfeng et al., 2004). *Candida* species are the normal microbiota within the gastrointestinal tracts, respiratory tracts, vaginal area and the mouth (Prescott et al., 2008). Candidiasis refers to a range of infection caused by species of fungal genus *Candida*. The infections can be acute or chronic, localized or systemic. Disseminated candidiasis is frequently life threatening.

The great majority of these infections are caused by *Candida albicans* (Greenwood et al., 1992). Pathogenic *Candida* infections mainly occur as opportunistic infec-

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tions due to altered conditions of the host, and at these altered conditions, the fungus proliferates faster (Elegbe et al., 1982). The incidence of genitourinary tract infection is much higher in females during adolescence and childbearing years (Sobel, 2004).

Candida is found in the vagina of 35 - 50% of healthy women. Under some conditions, such as reduced immunity, prolonged antibiotics therapy, use of contraceptives, malnutrition, pregnancy, diabetes, obesity, tissue transplant, use of immunosuppression drugs (Corticosteroids), neutropenia, *Candida* may become pathogenic and cause candidiasis (Okungbowa et al., 2003). Presence of indwelling central venous or pulmonary artery catheters and prior heamodialysis has also been identified as a risk factor (Barg, 1993). Sexual intercourse with an infected person is the most common mode of spread of genital candidiasis (Ogunbayo, 1988; Tatfeng et al., 2004). *Candida* species are the second most frequent isolates from blood cultures in hospitals with large populations of immunocompromised patients (Beck-Sague et al., 1993).

Vulva pruritis is the dorminant feature of vulvovaginal candidiasis. Women may complain of dysuria, soreness, irritation, dyspareunia, suprapubic pains, haematuria, white and clumpy vaginal discharge. The discharge is classically described as thick, adherent, and "cottage cheese- like" with a pH of 4.0 - 4.5 (Tatfeng et al., 2004). Fungus balls or bezoars may produce symptoms of urethral obstruction. Balanitis is a *Candida* infection of the male glans penis and occurs primarily in uncircumcised males (Prescott et al., 2008) . The diagnosis is confirmed by finding the organism on a wet mount of the discharge. Microscopy may be negative in up to 50% of patients with confirmed genitourinary candidiasis (Sobel et al., 2004). Genitourinary specimens are cultured on fungal media at room temperature or at 37°C. Yeast colonies are examined for the presence of pseudohyphae. *C. albicans* is identified by the production of germ tubes or chlamydospores. Other *Candida* isolates are speciated with a battery of biochemical reactions (Jawez et al., 2001). Clinical diagnosis is based on signs and symptoms as stated above.

The many drugs that are available at present to treat fungal infections can be divided into four broad groups on the basis of their mechanism of actions. These antifungal agents inhibit macromolecule synthesis (flucytosine), impair membrane barrier function (polyenes), inhibit ergosterol synthesis (allylamines, thiocarbamates, azole derivatives, and morpholines) or interact with microtubules (griseofulvin) (Vaden et al., 1997) . Currently, the azole drugs comprising of miconazole, ketoconazole, fluconazole and itraconazole are widely used for the treatment of fungal infections.

They have the advantage of being taken orally, increase potency, decreased toxicity and broader spectrum of activity (Ibrahim et al., 1998; Myers, 2006).

C. albicans isolates obtained from sterile body sites tested against fluconazole, ketoconazole and miconazole using microdilution antifungal susceptibility testing method showed that all isolates were fluconazole susceptible. Emergence of drug resistance among yeast isolates and consequent increase in serious fungal infections have been reported (DeMuri et al., 1995). The mechanism of resistance to these antifungal agents by yeast isolates are purely chromosomal as *Candida* species lack plasmid or other natural mechanism capable of transferring genetic materials between strains (Odds et al., 2003). Candidiasis is not a communicable disease. The most important preventive measure is to avoid disturbing the normal balance of the microbial flora and intact host defences. Infected patients respond well to antifungal agents such as fluconazole, ketoconazole, amphotericin B, intraconazole and miconazole.

The aim of this study was to determine the resistance *Candida* species from the genitourinary tracts of female patients to some antifungal agents (Ketoconazole and fluconazole).

MATERIALS AND METHODS

Collection of sample

The sample comprised of 239 samples from the endocervix of the vagina (high vaginal swab, HVS) and 200 samples of urine collected from women clinically diagnosed of genitourinary tract infections from laboratories in Benin metropolis. Sterile speculum and swab sticks were used for the collection of HVS while urine samples were collected using sterile universal containers. Samples collected were examined microscopically by wet preparation direct mount. Both swab and urine were kept at room temperature for culture isolation. The women were grouped into four according to their ages as follows: 15 - 25, 26 - 35, 36 - 45, and 46 plus.

Culture procedure

Samples were cultured on Sabourand dextrose agar (SDA), (Lab.M) at 37°C. Inoculated plates were examined after 48 h incubation. Isolates from SDA were plated on CHROMagar (France) to ensure detection of mixed cultures. Cultures were incubated at 37°C for 72 h. Identification of *Candida* species were based on colony morphology and pigmentations on the CHROMagar.

Germ tube test (GTT)

This was done according to the method of Baker (1967) . Yeast isolates suspected to be *C. albicans* were inoculated into human serum, incubated for about 30 min at 37°C and examined microscopically for the production of germ tubes.

Sugar assimilatioin test

All isolates which could not be identified using CHROMagar and Germ tube test were subjected to sugar assimilation test as described by Baker (1967). Yeast was grown on a basal carbohydrate free medium supplemented with the test sugar. These were incubated at 30°C for 18 h. Opacity in the medium indicates the ability of the isolate to assimilate a sugar.

Antifungal susceptibility test

Susceptibility testing was carried on the banked isolates using broth

microdilution method of Hace et al. (1995) and based on the approved National Committee for Clinical Laboratory Standards (NCCLS) guidelines for a broth microdilution reference method, 2002.

Seven different concentrations of each drug were tested as follows; Fluconazole (0.10, 0.50, 1.0, 5.0, 10.0, 50.0, 100) ug/ml and ketoconazole (0.01, 0.05, 0.10, 0.50, 1.0, 5.0, 10) ug/ml. 0.1 ml yeast inoculum from Roswell Park Memorial Institute (RPMI) 1640 medium visually matched to 0.5 McFarland and incubated at 35°C for 48 h were added to each microdilution well. The trays were incubated at 35°C for 48 h.

A numerical score from 0 to 4 were assigned to each set of well using the following scale: 0 = optically clear, 1 = slightly hazy, 2 = Prominent reduction in turbidity, 3 = Slight reduction in turbidity, 4 = No reduction in turbidity. Scores 0 - 2 was regarded as sensitive while scores 3 and 4 were said to be resistant.

The MIC was regarded as the lowest antifungal concentration with substantially lower turbidity compared to growth in the antifungal free growth control well. A susceptible interpretation was given to any strain for which the MIC of fluconazole was 10 ug/ml, and ketoconazole 5 ug/ml (Galgiane et al., 1992).

The Chi- square Test was used to test the occurrence of *Candida* species as well as the significance of antifungal resistance among the yeast isolates.

RESULTS

Out of a total of 439 (HVS and urine) samples, 216 yielded growth of yeast isolates (Table 1 and Figure 1). These were identified as *C. albicans* 138 (63/9%), *Candida glabrata* 68 (31.5%), *Candida krusei* 6 (2.9%) and *Candida tropicalis* 4 (1.9%). A comparison of the occurrence of these species in HVS and urine (Table 1) showed that their rate of occurrence was significantly higher in HVS (83%) than in urine (17%) at $p > 0.05$.

Distribution of *Candida* species among different age groups is shown in Figure 2 and Table 2. The age bracket 26 - 35 years had the highest frequency of *Candida* species with a total of 124 (57.4%), followed by the age group 16 - 25 years with a total of 64 (29.6%) yeast isolates. Ages 46 and above recorded the lowest, 2(1.0%). Prevalence of *Candida* species was significantly higher within age group 26 - 35 years than other age groups at $p > 0.05$.

Table 3 shows the summary of MIC of isolates against different concentrations (0.1 - 100 g/ml) of fluconazole. With reference to the NCCLS standards, isolates giving clarity of growth (optical clarity) at concentrations 10 g/ml were regarded as susceptible while those giving such clarity at concentrations > 10 g/ml were regarded as resistant. 132 (95.7%) *Candida albicans* isolates gave optical clarity at lower concentrations 10 g/ml and were regarded as susceptible. The remaining 6(4.3%) *C. albicans* isolates which gave optical clarity at higher concentrations >10 g/ml were regarded as resistant. *Candida tropicalis*, was 100% susceptible to fluconazole since all the isolates showed optical clarity at concentrations 10 g/ml. High resistant rate (100%) was recorded for *Candida krusei* since all the 6 isolates had their optical clarity at concentrations >10 g/ml. These observations were further summarized in Table 5.

Table 1. Occurrence of *Candida* species in the genitourinary tract.

Species	Urine No (%)	HVS No (%)	Total No (%)
Candida albicans	26(18.8)	112(81.2)	138(63.9)
Candida glabrata	11(16.20)	57(83.8)	68(31.5)
Candida krusei	0(0)	6(100)	6(2.9)
Candida tropicalis	0(0)	4(100)	4(1.9)
Total	37(17.1)	179(82.9)	216(100)

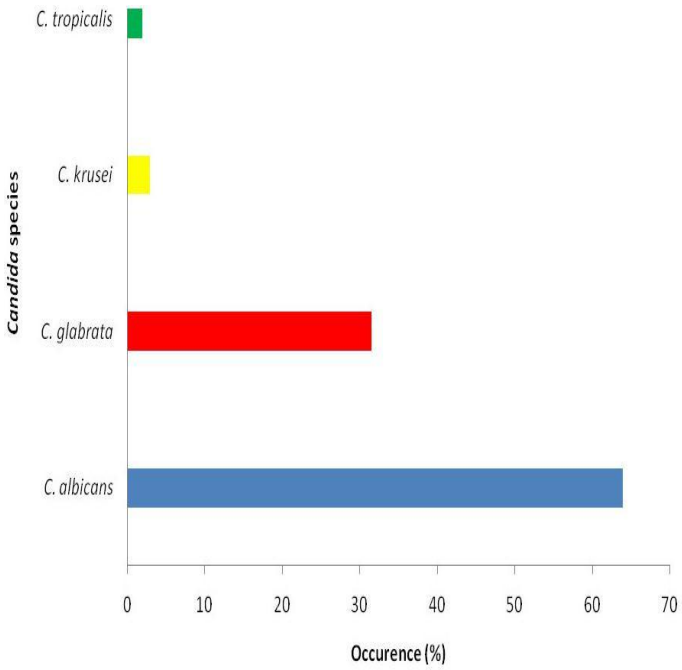

Figure 1. Occurrence of *Candida* species in the genitourinary tract.

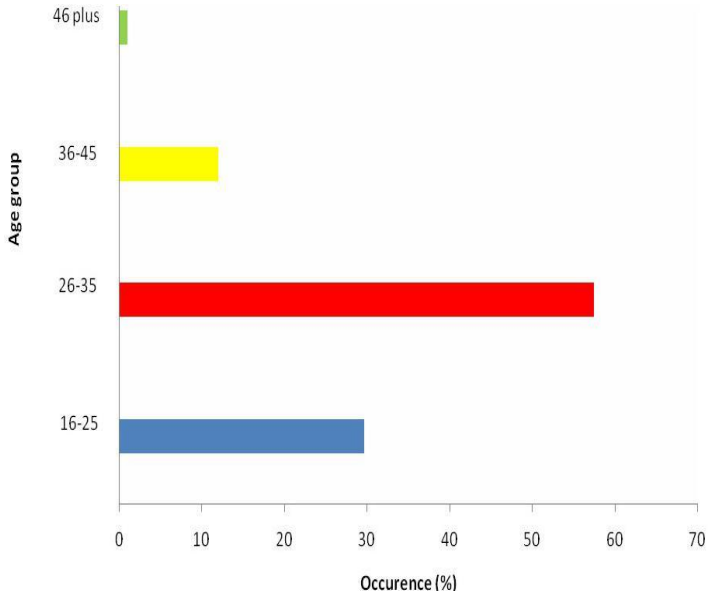

Figure 2. Distribution of candidiasis within different age groups.

Table 2. Distribution of *Candida* species within different age groups.

Age Group	*C. albicans*	*C. glabrata*	*C. krusei*	*C. tropicalis*	Total (%)
16 - 25	40	20	2	2	64(29.6)
26 - 35	78	40	4	2	124(57.4)
36 - 45	18	8	0	0	26(12.0)
46 plus	2	0	0	0	2(1.0)
Total	138	68	6	4	216(100)

Table 3. Susceptibility of *Candida* species to fluconazole (%).

Fluconazole g/ml	*C. albicans* No (%)	*C. glabrata* No (%)	*C. krusei* No (%)	*C. tropicalis* No (%)
0.1	35(25.4)	33(48.5)	Nil	Nil
0.5	60(43.5)	11(16.2)	Nil	Nil
1.0	20(14.5)	15(22.1)	Nil	4(100)
5.0	11(8.0)	9(13.2)	Nil	Nil
10.0	6(4.3)	Nil	Nil	Nil
50.0	4(2.9)	Nil	1(16.7)	Nil
100	2(1.4)	Nil	5(83.3)	Nil
Total	138(100)	68(100)	6(100)	4(100)

Nil = No organism was tested at that concentration.

Table 4 shows the summary of the MIC of isolates against different concentrations of ketonazole. According to NCCLS standards, optical clarity at concentrations 5 g/ml was regarded as susceptible while optical clarity at concentrations >5 g/ml was regarded as resistant. High rate of susceptibility was recorded for *C. albicans* (92.8%), *C. tropicalis* (100%) and *C. glabrata* (97.6%); since all their isolates gave optical clarity at lower concentrations (5 g/ml). *C. krusei* showed moderate resistance since only 50% of its isolates gave optical clarity at higher concentrations (>5 g/ml). These observations were again summarized in Table 5.

Both antifungals (fuconazole and ketonazole) showed high activity (95.7 and 98.2% respectively) against *C. albicans* isolates (Table 5). Their resistance rates (4.3 and 7.2% respectively) were however quite low. Antifungal resistance in non-albicans species was observed in case of *C. krusei* where high (100%) and moderate (50%) resistance rates were recorded against both antifungals. There was no significant difference between the activities of both drugs at >0.05.

DISCUSSION

The observation in this study that *C. albicans* had the highest incidence rate (63.9%) among the yeast isolates studied is in agreement with the reports of other workers (Richter et al., 2005; Sobel, 1995; Tafteng et al., 2003). Richter et al. (2004) reported a 76% incidence rate among his yeast isolates. Sobel (1995) reported an 80 - 90% incidence rate. Tatfeng et al. (2003) reported *C. albicans* to be the most incriminated yeast isolate in urinary tract infections. This finding however contradicted the earlier report of Okungbowa et al. (2003) who reported *C. glabrata* as the most common *Candida* species among symptomatic individuals in Nigerian cities. Also in this study, an incidence rate of 36% was observed for non-albicans species. Reports from other workers showed similar observations (Hollandia et al., 2003; Spinillo et al., 1997; Nyirjesy et al., 1995). An overall non-albicans percentage of 24, 17, 11 and 32 respectively were reported by each of these researchers. This variation in reports may be attributed to the period of specimen collection and differences in population types (Enweani et al., 1987).

A higher frequency of *Candida* species (57%) within age bracket 26 - 35 years as observed in this study is in agreement with report of other workers (Sehgal, 1990; Okungbowa et al., 2003). Sehgal (1990) reported a 54% incidence rate within age bracket 20 - 30 years in Northern Nigeria. 35% incidence rate was reported within age group 26 - 36 years in Benin City by Okungbowa et al., 2003. These reports points to this age group as a vulnerable group probably due to sexual promiscuity, drug abuse and use of contraceptives. The high fluconazole susceptibility rate (95.7%) in *C. albicans* found in this study is consistent with other reports. Ogubanjo (1988) reported that fluconazole resistance was observed infrequently (3.7%) among his yeast isolates. No fluconazole resistance was reported among yeast isolates in earlier works on vulvovaginitis conducted in the U.S.,

Table 4. Susceptibly of *Candida* species to ketoconazole (%).

Ketoconazole g/ml	*C. albicans* No (%)	*C. glabrata* No (%)	*C. krusei* No (%)	*C. tropicalis* No (%)
0.01	72(52.2)	9(27.9)	Nil	4(100)
0.05	12(8.7)	2(2.9)	Nil	Nil
0.1	32(23.2)	12(8.7)	1(16.7)	Nil
0.5	3(2.2)	33(23.9)	2(33.3)	Nil
1.0	9(6.5)	Nil	Nil	Nil
5.0	5(3.6)	Nil	2(33.3)	Nil
10.0	5(3.6)	2(2.9)	1(16.7)	Nil
Total	138(100)	68(100)	6(100)	4(100)

Nil = No organism was tested at that concentration.

Table 5. Susceptibility of isolates to fluconazole and ketoconazole (%).

Isolates	Fluconazole		Ketoconazole	
	No. R (%)	No. S (%)	No. R (%)	No. S (%)
Candida albicans	6 (4.3)	132 (95.7)	10 (7.2)	128 (92.8)
Candida glabrata	0 (0)	68 (100)	2 (2.9)	66 (97.6)
Candida krusei	6 (100)	0 (0)	3 (50)	3 (50)
Candida tropicalis	0 (0)	4 (100)	0 (0)	4 (100)
Total	12 (2.8)	204 (97.2)	15 (5.6)	201 (94.4)

R = resistance.
S = susceptibility.

England and Brazil (Sobel et al., 2004; Lynch, 1994; El-Din et al., 2001; Ribeiro et al., 2000).

The low fluconazole-resistance rate (4.3%) in *C. albicans* found in this study is consistent with other research findings. A U.S. Study reported fluconazole resistance in 3.6% *C. albicans* isolates (Sobel et al., 2004). A 2.1% *C. albicans* resistance rate was reported in New York by Mathema et al., 2001. Azole resistant candidiasis appears to be on the increase, and the reasons for resistance may include incomplete therapy, overgrowth of resistant strains, and induction of drug resistance in the particular species, colonization and subsequent infection with a resistant organism (Powderly, 1994; Rex et al., 1995). *C. krusei* is naturally resistant to fluconazole even at high doses (Goa and Barradell, 1995; Klastersky, 1995). In this study, a 100% resistance rate was observed for *C. krusei,* which is consistent with research reports. The second azole antifungal studied in this work was ketonazole. Similar susceptibility pattern was observed in this drug as in fluconazole. The 94.4% susceptibility and 5.6% resistance observed for keto-nazole were also consistent with previous research works. The similarity in the activity of these two anti-fungals shows that they both belong to same azole antifungal, the imidazoles

The research findings of this study, support previous observations that clinical *Candida* species and related yeast infections are increasing and that the widespread use of imidazoles (such as fluconazole and ketonazole) appears to be associated with emerging resistance tothese important antifungal agents in yeasts. As a conesquence, *in vitro* testing of the susceptibility of yeasts to antifungal agents will likely play an ever increasing role in the appropriate selection of antifungal agents for the treatment of fungal infections. Nonetheless, the high susceptibility rate of *Candida* species to azole drugs as observed in this work supports the continued use of azole antifungals for the treatment of genitourinary tract infections among women.

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