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DETECTION OF VIRULENCE FACTORS AND ANTIFUNGAL RESISTANT PATTERNS OF *Candida* SPECIES ISOLATED FROM WOMEN WITH URINARY TRACT INFECTIONS

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ABSTRACT
The incidence of Candida species has increased among females of child bearing age over the years. There has also been an increase in the resistance of these Candida species to the antifungal drugs used to treat them. This study is aimed at detecting the prevalence of Candida species among patients with urinary tract infections (UTI) attending gynecological unit of Ekiti State University Teaching Hospital, Ado-Ekiti, Nigeria. The distribution of pathogenic factors and the antifungal resistance pattern of the isolates were also determined. A total of 61 subjects of different ages and socioeconomic status attending the health facility were enrolled in this study. Samples of high vaginal swab (HVS) were collected from each of the participant and screened. A total of 36 candidal isolates were recovered from the samples out of which 11 (30.5%) were predominantly Candida albican, 5 (13.9%) Candida krusei, 4 (11.1%) Candida glabrata, 2 (5.6%) Candida tropicalis and 14 (38.9%) were not identified beyond the genus level. Ten (27.8%) of the isolates were not able to produce biofilm. Out of those that produce biofilm 17 (47.2%) produced weak biofilm, 5 (8.2%) produced moderate biofilm, while 4 (11.1%) produced strong biofilm. Spectrophotometer was used to quantify those that produce biofilm 9 (25%) produced moderate biofilm while 16 (44.4%) produced strong biofilm. The isolates were subjected to various pathogenicity tests which include haemolysis, catalase, phospholipase and hydrolysis. This test shows that Candida species has the highest percentage to the entire test while none of C. glabrata produced haemolysin and phospholipase. Antifungal assay was then carried out on the entire organisms showed Candida albicans to have low resistance to the azoles drugs while the non-albican Candida shows higher resistance to it. Extremely high prevalence of Candida albicans and Candida species were documented in this study. These findings should be taken into account in further research concerning presence of Candida among patient with sexually transmitted disease in Nigeria.
Key words: Candida, urinary tract infections, antifungal, virulence factors, resistant, biofilm

INTRODUCTION

Members of the genus *Candida* has been earlier recognized as commensal and normal microbiota of human. Recent findings have shown that these organisms are now resistant to many antifungal and also possess different pathogenic factors (Deorukhkar *et al.,* 2014). They have been reported to be associated with different mycotic infections especially among the immunocompromised, severely ill patients and also immunocompetent individuals (Pfaller and Diekema, 2007). The extensive use of antibiotics, immunosuppressive agents and cancer chemotherapy has also been identified as risk factors to candidal infections. Members of the genus have been reported to cause mild to life threatening infections (Sardi *et al.,* 2013).
Candida genus forms blastospore, pseudohypha and septate hypha except (*Candida glabrata*). This dimorphic (morphological) nature enhances the ability of the pathogen to grow on and in the tissues or organs of the host.

In most cases *Candida* species exist normally as non-pathogenic fungi but under altered conditions they exhibit virulence. Conditions such warmth, moisture, nutritional deficiencies, broad spectrum antibiotics, sugar and a weakened defense system have accounted for the increase in candida infection (Molero *et al.,* 1998).

Drug resistance is strongly correlated with biofilm development. Resistance emerges relatively quickly after *Candida albicans* substrate adherence (Ramage *et al*., 2002). Extracellular matrix could slow antifungal drug penetration, limiting access to growing cells. There could be an increased expression of drug efflux pumps but the specific pumps involved have not been elucidated (Jabra-Rizk *et al*., 2000). A change in membrane sterol composition during biofilm development may create resistance to only specific anti-fungal medications. There is a possibility of phenotypic changes resulting from limited availability of nutrients and a slow growth rate (Jabra-Rizk *et al*, 2000). Since the drug resistance in *Candida albicans* biofilms cannot be attributed solely to matrix exclusion or slow growth rate, contact-induced gene expression for acquiring characteristic properties is probably an additional mechanism by which drug resistance is acquired (Baillie and Doublas, 2000, Moran *et al.,* 1998).

In recent years, the incidence of life-threatening mycoses caused by opportunistic fungal pathogens has increased dramatically. Many studies have showed that the prevalence of infection increased with age (Murray *et al.,* 2000). According to earlier reports, *Candida albicans* was the cause of 80-95% cases of symptomatic fungal vulvovaginitis, whereas other *Candida* species such as *C. glabrata, C. parapsilosis*, and *C. tropicalis* constitute the remaining cases.

Despite many scientific reports on association of *Candida* species with urinary tract infections, the reports on occurrence of the of Candida species with different forms of urinary tract infection among women within the child bearing age in the study location is still very scanty. Hence, the objective of this study was to assessing the prevalence of *Candida* spp among women seeking medical treatment for urinary tract infections (UTIs) in a tertiary health care institution in Ekiti State. Also, to determine the virulence factors and antifungal resistance of the fungal isolates revcovered from the subjects.

MATERIAL S AND METHODS

Subjects and Questionnaire

A structured questionnaire was used to collect data from the patients with Urinary Tract Infections (UTIs). The questionnaire used was evaluated, reviewed carefully, and then pre-tested on selected respondents. The questionnaire has two parts demographic and gynecological characteristics of respondents and the clinical presentation of the patients and the risk factors of UTIs among the respondents. At the onset of the study the intent of the study was disclosed to the subjects and those that gave a verbal consent were recruited. Subjects with signs and symptoms of UTIs and also between the child bearing and early menopausal age were screened in this study.

Study Area and Sample Collection

All the samples were collected from Ekiti State University Teaching Hospital (EKSUTH) Ado–Ekiti. One hundred and twenty nine non-repeat high vaginal swabs (HVS) of women diagnosed of UTI were collected with the aid of sterile swab sticks. The samples were immediately cultured on Saboroud dextrose agar (SDA) supplemented with chloramphenicol and incubated at 37^{o}C for 24 hours. Sugar fermentation tests, formation of germ tube and production of chlamydospores on cornmeal agar were carried out to confirm the identity of the isolates. Candida-Chrom Agar (Oxoid) was used to confirm the identification of the species as previously describe by Mohandas (2011).

Determination Phospholipase Production among the Isolates

The egg yolk medium consisted of 13.0 g Sabouraud Dextrose Agar (SDA), 11.7 g NaCl, 0.11 g $CaCl_2$ and 10% sterile egg yolk (all in 184 mL distilled water) was prepared as described previously by Erum *et al.* (2020) with minor modification, was prepared by sterilization the SDA and allowed to cool to around 50^{o}C then 10% sterile egg yolk was incorporated into it. The organism was then streak on the agar in the plates and incubated at 37^{o}C for 48 h. Visible precipitation zone around the colony on the plate was considered positive phospholipase activity.

Haemolytic Activity

As described by Manns *et al*. (1994) the haemolytic activity of the isolates was determined their ability to lyse the RBC using blood agar plates. Candidal isolates were streaked radially onto SDA supplemented with 7% of human blood at pH 5.6 ± 0.2 and incubated at 37^{o}C for 72 hours. Clear zone was seen around the colony after 48 hours.

Determination Caseinase Production among the Isolates

Caseinase production by the isolates was determined by incorporating 1% casein into SDA and sterilized at 121^{o}C for 15 mins as described by de Doroti *et al.* (2002). The agar was dispensed into the plates, inoculated and incubated for 48 h at 37^{o}C. After incubation 30% Trichloroacetic acid was flooded on the plates and any colony surrounded with the zone of clearance was recorded to be positive for caseinase production.

Determination of Hydrolase Production among the Isolates

0.7% skim milk was incorporated into PDA and sterilized at 121^{o}C for 15 mins. The organisms were streaked radially on the medium and incubated at 37^{o}C for

Determination of Catalase Production among the Isolates

Modified method of Larsen and White (1995) was used to determine catalase production in the isolates. A drop of 3% hydrogen peroxide was placed on a microscopic slide. The test organism was then introduced and smear was made using an inoculating wire loop. Catalase positive organisms produce bubbles while catalase negative organism does not produce bubble.

Detection of Biofilm formation among the isolates

Biofilm formation among the candida isolates was detected by the method of Chandra *et al*. (2007). The isolates were radically streaked on nutrient agar supplemented with 4.0 % Congo red dye. The plates were incubated for 24 h at 37^{o}C. Isolates with black colonies on Congo red agar were taken for biofilm producers.

Antifungal Susceptibility Test

Each of the isolates was grown at 37°C in Malt extract broth (Oxoid) for 18 h and adjusted to an optical density of 0.5 McFarland Standard. The microdilution method was used for susceptibility testing as described by Clinical and Laboratory Standard Institute, CLSI (2012). The growth of the isolates in each of the microtitre plate was observed visually. The first well in the series with no visible growth was taken as the MIC.

RESULTS

A total of 129 subjects were recruited during this study. Subjects within the age bracket of 31-40 years recoded the highest frequency of 61 (47.55%) followed by those within the ages 41-50 years. A total of 70 (54.10%) of the subjects were civil servants, 34 (26.23 %) were traders while 13 (9.84%) were artisans). Only 4 (3.10%) of the subjects have not given birth to children, 32(24.81%) reported that they have had either one or two children while 74(57.36%) of the subject has three of four children. Sixty three (49.21%) out of the subjects had had abortion while 66 reported that they had never had an abortion. All the subjects reported that they had at least one of the signs of UTIs ranging from abdominal vaginal discharge, painful sexual intercourse to vagina/vulva itching as shown in Table 2. The risk factors among the subjects are shown in Table 3.

A total of 79 *Candida* species were isolated from the subjects as shown in Table 4. The isolates include different *Candida* species *Candida albicans* [n=46 (58.23%)], *Candida krusei* [n=15 (18.99%)], *Candida glabrata* [n=12 (15.19%)] and *Candida tropicalis* had [n=4 (5.06%)]. Two of the candidal isolates could not be identified beyond the genus. Fifty three of the isolates produce biofilm using Congo red qualitative method. The highest number of biofilm former was noticed among *Candida albicans*, while *Candida tropicalis* has the least occurrence. The quantitative determination of biofilm formed by the isolates showed 2, 20 and 10 of the isolates produces strong, moderate and weak biofilm respectively as shown in Table 4.

Five pathogenic factors which include catalase, gelatinase, haemolysin, hydrolysin and phospholipase were determined in the isolates. The distribution of the pathogenic factors varied among the isolates. *Candida albicans* among the isolates had the highest prevalence of the pathogenic factors with the occurrence of hydrolase (72.73%), haemolysin (63.64%) and catalase (54.55 %). The observed incidence of the pathogenic factors in the candidal isolates were presented as catalase > haemolysin > hydrolase > gelatinase > phospholipase. Three different antifungals are tested on the isolates. Fluconazole followed by itraconazole had the highest minimum inhibitory activity on the isolates. Comparatively, the antifungal tested had the highest MICs on *albicans* species of the *Candida.*

Table 1: Demographic and gynecological profiles of the patients attending Ekiti State University Teaching Hospital

Characteristics	**No Tested (%)**
Marital status: Married	95 (74.64)
Single	31 (24.03)
Widow	3 (2.33)
Age group (yr)	
≤30	27(21.31)
31-40	61(47.55)
41-50	38(29.51)
≥51	2(1.64)
Occupation	
Civil- servant	70(54.10)
Traders	34(26.23)
Pastors	8(6.56)
Artisan	13(9.84)
Undisclosed	4(3.28)
Number of pregnancy	
0	4(3.14)
1-2	23(17.80)
3-4	28(21.47)
5-6	23(17.80)
7-8	10(7.85)
10-11	41(31.94)
Number of Children (Parity)	
0	4(3.10)
1-2	32(24.81)
3-4	74(57.36)
5-6	15(11.63)
7-8	4(3.10)
Cases of previous abortion	
No	63(49.21)
Yes	66(51.16)
Frequency: One	40(31.10)
Two	13(9.80)
Three	9 (6.60)
Four	4 (3.30)

Table 2: Clinical presentation of the patients attending gynecological Unit of Ekiti State University Teaching Hospital

Clinical Presentation	**Yes (%)**	**No (%)**
Abdominal vaginal discharge	36 (27.91)	93 (72.09)
Painful urination	6 (4.65)	123 (95.35)
Vagina/vulva itching	17 (13.18)	112 (86.82)
Burning and irritation of the virginal	37 (28.68)	92 (71.32)
Low abdominal pain	32 (24.81)	97 (75.19)
Irregular Menstruation	30 (23.26)	99 (76.74)
Painful sexual intercourse	11 (8.53)	118 (91.47)
Suprapubic tenderness	21 (16.28)	108 (83.72)
Menstrual pain	6 (4.65)	123 (95.35)
Vaginal bleeding after sexual intercourse	2 (1.55)	127 (98.45)
Dysuria	24 (18.60)	105 (81.40)
Loin pain	32 (24.81)	97 (75.19)

Table 3: Risk factors among the patients attending gynecological unit of Ekiti State University Teaching Hospital

Risk factor	**Yes** (%)	**No (%)**
Multiple sexual partners	17(13.18)	112(86.82)
Spouse with multiple sexual partners	40(42.11)	55(57.89)
Previous termination of pregnancy	66(51.16)	63(49.21)
Family history of diabetes	59(45.74)	70(54.26)
Frequent vaginal washing	95(73.64)	34(26.36)
Intrauterine contraceptive device (coil) usage as family planning method	17(13.18)	112(86.82)

Table 4: Distribution of candidal isolates and formation of biofilm among them

		Biofilm formation			
		Formers			Non-formers
Isolates	N (%)	Strong	Moderate	Weak	
C. albicans	46 (58.23)	13(28.26)	10 (21.74)	6 (13.04)	17 (36.96)
C. krusei	15 (18.99)	9 (60.00)	0 (0)	2 (13.33)	4 (26.67)
C. glabrata	12 (15.19)	0	10 (83.33)	2 (16.67)	0
C. tropicalis	4 (5.06)	1(25)	0	0	3 (75)
Candida species	2 (2.53)	0	0	0	2
	Total	23 (29.11)	20 (25.32)	10 (12.66)	26(32.91)

Table 5: Distribution of virulence factors among candidal isolates

Virulence Factors	***C. albicans* (n=46)**	***C. krusei* (n=15)**	***C. glabrata* (n=12)**	***C. tropicalis* (n=4)**	***Candida* species (n=2)**
Haemolysin	29(63.04%)	5(33.33%)	4(33.33%)	1(25.00%)	1(50.00%)
Catalase	25(54.35%)	7(46.67%)	5(41.67%)	2(50.00%)	0
Phospholipase	13(28.26%)	3(20.00%)	2(16.67%)	2(50.00%)	0
Hydrolase	33(71.74%)	2(13.33%)	3(25.00%)	0	0
Gelatinase	24 (52.17%)	3(20.00%)	4(33.33%)	1(25.00%)	0

Table 6: Minimum inhibitory concentrations (µg/ml) of the *Candida* species isolated women to some antifungals

Antifungal	**Candida Isolates**				
	C. albicans	***C. tropicalis***	***C. glabrata***	***C. krusei***	***Candida* species**
Fluconazole	18.75-50.00	12.5-37.50	18.75-25.00	≥100.00	6.25-50.00
Ketoconazole	0.125 – 4.00	0.125-0.50	0.5-2.00	1.00-4.00	0.125-2.00
Itraconazole	0.125 - 4.00	0.25-2.00	0.5 ≥ 8.00	0.125≥8.00	0.063-4.00

DISCUSSION

During this study the demographic profiles and clinical presentation of the patient at the teaching hospital was obtained. The age group with the highest presentation was 31-40 years with a total of 29 (47.9%) of the subjects. At reproductive stage women tend to have the highest colorization of *Candida* species (Alli *et al*. (2011). In this work the highest colorization was noticed among married women with 60 (98.4%) of the samples. Feyi-Waboso and Ahmadi (2001) reported that 42.9% of vaginal candidiasis was observed among pregnant and they also observed that primigravida and younger age group suffered more from vaginal candidiasis. Candidiasis is the most common opportunistic fungal infection (Marcano and Feo, 1983). Vaginities is one of the principal reasons that make women to visit an obstetrician or gynecologist. Despite the introduction of antifungals the incidence of urinary tract infection remains high. This may be partly due to increase in population of immunocompromise patients. It could also be due to overuse of broad-spectrum antibiotics. *Candida* is a normal commensal organism colonizing in the vagina, particularly the *albicans* species.

In this study, people having 3-4 pregnancy has the highest presentation having 20 (32.8%) of the sample obtained. From previous work the occurrence of *Candida* is common among those at their early stage of child bearing. Also in this work people having 3-4 children has the highest incidence of 35 (57.4%) among the subjects. In this study, 17 (27.9%) of the subjects presented with the abdominal vaginal discharge. 8(13.1%) presented with vaginal vulva itching. Vulvovaginal candidiasis is a common condition and an estimated 75% of all women experience an infection with Candida yeast during their lifetime (Akah *et al*., 2010). Rizvi *et al.* (2003) reported that most women attending attended routine antenatal clinic are asymptomatic with no noticeable sign. High prevalence of vaginal candidiasis has been observed in developing countries (Parveen *et al.* 2008).

From the risk factors subject using intrauterine contraceptive device (coil) or involved in family planning has the highest risk factor having 45 (73.8%) of the subject followed by patient with frequent vaginal wash having 28 (45.9%) of the subject followed by patient which their spouse has multiple sexual partners 7 (14.8%) and people with family history of diabetes 6 (9.8%) of the subjects. This is followed by people with multiple sexual partners. During this study a total of 36 candidal isolates were obtained in this study which include *Candida albicans* 5 (13.9%), *Candida krusei* 4 (11.1%), *Candida glabrata* 2 (5.6%), *Candida tropicalis* and 14(38.9%) were *Candida* species. These correlate with the work of Pfaller, *et al*. (2007) which states that 90% of invasive infections are caused by these species. Out of the isolates identify to the species level *Candida albicans* and *Candida* species has the highest incidence having the occurrence of 11 (30.6%) and 14 (38.9%) of the isolates. Grigoriou *et al.* (2006) isolated *Candida albicans* in 60% of pregnant women who had complaints of vaginal discharge and with diabetes mellitus, a possible risk factor to colonization of *Candida* spp.

Ability to produce biofilm was carried out on all the isolate. It was observed that greater percentage 25 (69%) of isolate form biofilm indicating the roles of biofilm in their pathogenicity. This correlates to the report of Mah and O'Toole (2001) who described the roles of biofilm formation in the pathogenesis and antimicrobial resistance. Also biofilm quantification of biofilm formers showed that majority 9 (25%) of those that form biofilm produces moderate biofilm.

Pathogenecity test was then carried out on this isolates. 19 (52.8%) of the isolates produce haemolyses human RBC, while 23 (77.8%) of the isolates produce catalase 16 (44.4%) produce phospholipase and 20 (55.5%) produced hydrolysis. This report supports the work of Tsang *et al.,* (2007). Antifungal sensitivity was then carried out on this isolates *Candida* albicans showed low resistance to azole drugs which include fluconazole while higher rate of resistance was observed in other species like *Candida tropicalis*, *Candida glabrata* , *Candida krusei* has a moderate resistance to this drug which ranges from 50%-60%. Secondary exposure was highlighted as the main cause of the emergence of resistance to azoles seen in few years (Jabra-Rizk, *et al*., 2004). We were unable to support this work by using the molecular method to detect the identity of the isolates and to also determine the virulence factors in isolates.

CONCLUSION

In this study *Candida albicans* is the most common *Candida* associated with urinary tract infections (UTIs) in the recent time. The risk factors in this study helps to know the category of women of child bearing age that are predisposed to candidal infections. Extracellular enzymes help to provide information to establish a causal relationship between the species.

Conflict of interest

The authors declares no conflicts of interest.

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