Incidence, Virulence Markers and Antifungal Susceptibility Profile of *Candida* Species among Contraceptive Users in Benue and Niger States, Nigeria

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Abstract: *Candida* species are responsible for vulvovaginal candidiasis and resistance to antifungal drugs is a challenge. This study determined the incidence of vaginal candidiasis, antifungal susceptibility pattern and virulence markers of *Candida* species among contraceptive users. A total of 800 High Vaginal Swabs (HVS) were collected from women using contraceptive devices and inoculated onto Sabouraud Dextrose Agar (SDA) medium and CHROM agar. Candidal colonies were examined using lactophenol cotton blue and Germ tube test. The isolates were then subjected to a disc diffusion method using voriconazole, nystatin, and fluconazole on Mueller-Hinton agar to determine the susceptibility pattern of the isolates. Virulence markers which include heamolytic activity, coagulase production and biofilm formation were determined using standard microbiological methods. The incidence of vulvovaginal candidiasis in the study area is 44.88%. *Candida albicans* (33.98%) was the most frequent isolate while a preponderance of *C. glabrata* (16.99%) was observed among non-albicans *Candida* species. All the *Candida* species demonstrated at least one of the virulence markers, except *C. parapsilosis* which did not produce biofilm. *Candida albicans and Candida tropicalis* were 100% susceptible to nystatin, voriconazole and fluconazole. All the *Candida glabrata* isolates were susceptible (100%) to the three antifungal drugs. *Candida parapsilosis and Candida krusei* were 100% susceptible to nystatin. This current study revealed the incidence and the distribution of *Candida* species among contraceptive users. The isolates showed varying susceptibility patterns to the drugs except *Candida krusei* which was 100% resistant to voriconazole and fluconazole.

Key words: Antifungal drugs, Family planning, Resistance, Virulence.

INTRODUCTION

In recent times, the advocacy for family planning has become crucial as demand for reproductive and population reduction, economic and health care continue to grow. Family planning (FP) is one of the most cost-effective ways to prevent maternal, infant, and child mortality. It can reduce maternal mortality by reducing the number of unintended pregnancies, the number of abortions, and the proportion of births at high risk (Lule *et al.*, 2007). It has been estimated that the use of modern contraceptives would reduce maternal mortality rates by 25% thereby saving 140,000 to 150,000 lives per year (Singh *et al.,* 2003; Moronkola *et al.,* 2006).

A number of safe and effective contraceptive methods are available, and these include abstinence, barrier methods, oral contraceptives, Depo-Provera, Norplant implant, Intra-uterine devices, and sterilization methods (Greydanus *et al.,* 2001). However, the modern method mix predominantly comprises condoms, pills, and injectables. Despite the immense economic and health returns of family planning, maternal and infant mortality rates still remains on the increase; furthermore, family planning use has remained low, with only 10% of married women using a modern method of contraception (United Nations Development Programme, 2008).

In some studies conducted in Edo, Sokoto and Kano State, Nigeria, the prevalence of *Candida* infection among contraceptive users was 53.1%, 28.6% and 13% respectively (Egbe *et al*., 2011; Saidu *et al.,* 2017; Ali *et al.,* 2018).

In medical mycology, *Candida* species are one of the most important pathogens (Quindos *et al*., 2018) and a number of fungal species belonging to the genus *Candida* can cause acute Vulvovaginal Candidiasis infection (VVC) (Karami *et al.,* 2014). *Candida albicans*, an opportunistic polymorphic fungus and resident of the normal vaginal microbiota, is the leading causative agent of vulvovaginal candidiasis (VVC) and presents major issues for women worldwide (Achkar and Fries, 2010).

However, the emergence of non-*albicans Candida* species such as *Candida parapsilosis*, *Candida krusei*, *Candida glabrata*, *Candida dubliniensis, Candida tropicalis, Candida stellatoidea, Candida auris, Candida guilliermondii, Candida lusitaniae* and *Candida kyfer* has increased over the past decades (Da Matta *et al.,* 2017; Douglass *et al*., 2018).

There is paucity of epidemiological data on vaginal candidiasis among contraceptive users therefore; this study was designed to determine the incidence of vaginal candidiasis, virulence factors and antifungal susceptibility pattern of *Candida* Species among contraceptive users.

MATERIALS AND METHODS

Study Area

This study was carried out in Niger and Benue States. Niger State is located on latitude 80^0 to $11^{0.}$ 30' North and longitude 3^0 30' to 7^0.40' East with Minna as its capital. Niger State covers a total land area of 83,266,779 square kilometers which represent 8% of the total land area of Nigeria. About 85% of the land is arable with a population of 4,082,558 (Niger State Health Statistics, 2011). Benue State is one of the North central states in Nigeria with a population density of about 145.1 p/km² in 2006 census. It is located between latitude$7^0$20N' and $8^0$38' E (NPC, 2006; NBS, 2006).

Study Design and Population

A cross sectional study design was used. Information on the type of contraceptive usage and bio-demographic data was obtained from the study participants using structured questionnaires after informed consent was obtained. Ethical clearance was sought for and obtained from the ethical committees of the hospitals. Reproductive - aged women between the age group of 18-45 years who use contraceptives were recruited for the study. All women who were currently menstruating or pregnant were excluded from the study.

Sample Collection and Processing

Vaginal swabs samples were collected from four hundred (400) contraceptive users in each state with the assistance of a gynecologist. Clinical samples were collected from vaginal walls with two sterile cotton-tipped swabs. The vaginal swabs were then inoculated into a tube containing approximately 2 ml of saline and transported to the Microbiology Laboratory of Federal University of Lafia for analysis. One of the swabs was used for direct smear examination while the second swab stick was pressed firmly against the inside wall of the tube above the fluid level to remove excess fluid from the swab, streaked on Sabouraud Dextrose Agar (SDA) and incubated aerobically at 37 ^{0}C for 48 hours (Cheesbrough, 2000).

Candida species were identified by conventional methods which include direct examination, lactophenol cotton blue examination with the microscope, colony morphology and germ tube test (Larone, 2002; Baradkar *et al*., 2010; Amar *et al.,* 2013). Purified single culture from Saboraud Dextrose Agar was inoculated on CHROM agar using an inoculating loop and incubated at 37^{o}C for 48 hours. *Candida* isolates were classified according to their colors on CHROM agar based on the manufacturer's protocol.

Antifungal susceptibility tests

Antifungal susceptibility tests were performed according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2004). The disc diffusion method was carried out using commercially available antifungal discs which include nystatin (10 mcg) voriconazole (10 mcg) and fluconazole (10 mcg), on Mueller-Hinton Agar plates supplemented with glucose and methylene blue dye and incubated at 37^{o}C for 24 hours. The diameters of zones of inhibition were measured in millimeters using a meter rule for each antifungal disc. Interpretation of all antifungal susceptibility were carried out and interpreted as Susceptible, Intermediate and Resistant according to NCCLS 2004 document.

Determination of Virulence Factors

Haemolysin activity was assessed by the method of Manns *et al*. (1994) on blood agar plate. Isolates were streaked onto freshly prepared blood agar plates supplemented with glucose at a final concentration of 3 % (w/v). The plates were incubated at 35^0C and observed for haemolysis after 24 and 48 hours respectively (Fidel *et al.,* 1999).

Coagulase production by *Candida* isolates was detected for each test isolate. A 0.5 mL of mycological broth in test tubes was inoculated with an overnight growth culture of *Candida* isolate and incubated at 35 ^{0}C for 24 hours. A 0.5 mL of rabbit plasma was added to the test tube and incubated at 35^0C (Cheesbrough, 2000).

Biofilm formation by *Candida* isolates was assessed by the tube method as described by Yigit *et al.* 2008. Colonies of *Candida* isolates from mycological agar plates were inoculated in saline and incubated overnight at 35^0C. A 0.5mL aliquot of the saline suspension was added into screw capped conical polystyrene tubes containing 5mL of mycological broth supplemented with glucose at a final concentration of 8%. The tubes were incubated at 35 ^{0}C for 48 hours without agitation. After incubation the broth from the tubes were aspirated gently with Pasteur pipette, washed twice with distilled water and stained with 2 % safranin. The stain was decanted after 10 minutes and rinsed with distilled water to remove excess stain.

RESULTS

The distribution of birth control/family planning practices in the study area (Table 1) revealed that the major contraceptive method used is intrauterine device (29.1%) which was followed by the use of pills (25.0%), injectables (22.1%) and the use of implants (20.8%).

The incidence of vulvovaginal candidiasis in the study area is 44.88%. The highest incidence of 51.00% was recorded in Niger state while Benue State had an incidence of 39.25% as shown in Table 2.

A total of 359 women had vulvovaginal candidiasis out of which five species of *Candida* were isolated. The most frequent isolate was *Candida albicans* which accounted for 33.98% of the species isolated. Out of the non-*albicans Candida* species, 24.51% were *C. glabrata,* 16.99% were *C. tropicalis*, 10.58% were *C. parapsilosis* and *C. krusei* accounted for 14.76% of the total isolates (Table 3).

The results of the antifungal sensitivity tests are shown in Table 4. *Candida albicans* was 100% susceptible to nystatin and voriconazole, 81.0% susceptible to fluconazole and 12.3% resistant to fluconazole. *Candida glabrata* isolates were all susceptible (100%) to the three antifungal drugs.

Candidia tropicalis was susceptible to nystatin (83.6%), fluconazole (100%) and voriconazole (73.8%). Nevertheless, some isolates of *C. tropicalis* were 22.2% and 16.4% resistant against voriconazole and nystatin. *Candida parapsilosis* was 100% susceptible to nystatin and fluconazole while 23.7% were susceptible to voriconazole. *Candida krusei* was 100% susceptible to nystatin but 100% resistant to voriconazole and fluconazole.

Three hundred and fifty nine (359) *Candida* species isolated from this study was analyzed for virulence markers exhibited by the *Candida* isolates as shown on Table 5. Haemolytic activity was observed in 200 (55.7%) *Candida* isolates, coagulase activity was observed in 150 (41.7%) and biofilm formation was observed in 199 (55.4%) *Candida* isolates. All the *Candida* species demonstrated at least one of the virulence markers, except *C. parapsilosis* which did not produce biofilm.

The highest haemolytic activity was found in *C. glabrata* (70.5%) and the least haemolytic activity was found in *C. krusei* (16.9%). The highest coagulase activity (56.5%) was found in *C. glabrata* and the least coagulase activity (33%) was found in *C. parapsilosis*. All the *Candida* isolates produced biofilm except *C. parapsilosis* which did not produce biofilm. The highest biofilm production was demonstrated by *C. albicans* (81%) while *C. krusei* produced the least amount of biofilm.

Table 1: Distribution of Contraceptive Usage in the Study Area

Contraceptive Type	Niger State	Benue State	Total	Prevalence (%)
IUCD	75	158	233	29.1
Injectables	134	67	201	22.1
Implants	66	100	166	20.8
Pills	125	75	200	2.0
TOTAL	**400**	**400**	**800**	**100**

Key:
IUCD **-** Intrauterine device

Table 2: Incidence of Vulvovaginal Candidiasis (VVC) in the study area

States	Number Examined	Number Positive	Incidence (%)
Niger	400	202	25.25
Benue	400	157	19.63
Total	**800**	**359**	**44.88**

Table 3: Species Distribution of *Candida* Isolates among Patients with Vulvovaginal Candidiasis

Species	Number of isolates	Prevalence of the total isolates (%)
C. albicans	122	33.98
C. glabrata	85	24.51
C. tropicalis	61	16.99
C. parapsilosis	38	10.58
C. krusei	53	14.76
Total Isolates	359	100

Table 4: *In Vitro* Antifungal Susceptibility Pattern of the Isolates (n= 359)

Species	Antifungal agent	% susceptible of the isolates	% intermediate of the isolates	% resistant of the isolates
C. albicans (122)	Nystatin	122(100)	0 (0)	0 (0)
	Voriconazole	122(100)	0 (0)	0 (0)
	Fluconazole	99(81.0)	8(6.6)	15 (12.3)
C. glabrata (85)	Nystatin	85(100)	0 (0)	0 (0)
	Voriconazole	85(100)	0 (0)	0 (0)
	Fluconazole	85(100)	0 (0)	0 (0)
C. tropicalis (61)	Nystatin	51(83.6)	0 (0)	10(16.4)
	Voriconazole	45(73.8)	0 (0)	16(22.2)
	Fluconazole	61(100)	0 (0)	0 (0)
C. parapsilosis (38)	Nystatin	38(100)	0 (0)	0 (0)
	Voriconazole	29(76.3)	9(23.7)	0 (0)
	Fluconazole	63(100)	0 (0)	0 (0)
C. krusei (53)	Nystatin	53(100)	0 (0)	0 (0)
	Voriconazole	0 (0)	0 (0)	53(100)
	Fluconazole	0 (0)	0 (0)	53(100)

Table 5: Production of Virulence Factors by *Candida* Species

Candida Isolates	Haemolytic activity	Coagulase activity	Biofilm Formation
C. albicans (122)	79 (65%)	50 (40%)	99 (81%)
C. glabrata (85)	60(71%)	48 (57%)	45 (53%)
C. tropicalis (61)	35 (57%)	23 (38%)	37 (61%)
C. parapsilosis (38)	17 (45%)	9 (24%)	0 (0%)
C. krusei (53)	9 (17%)	20 (38%)	18 (34%)
Total 359	**200 (56%)**	**150(42%)**	**199 (55%)**

DISCUSSION

The distribution of birth control/family planning practices in the study area revealed that the major contraceptive method used is intrauterine device (29.1%). This occurrence may be due to the convenient use of intrauterine device which requires a onetime insertion and removal when ready for conception unlike the use of oral contraceptive pills and injectables which require regular intake.

The incidence of vulvovaginal candidiasis in the study area is 44.88%. Niger state recorded a prevalence of 25.25% while Benue State had a prevalence of 19.63%. This variation may be due geographical difference, nutritional factors or the personal hygiene of the women. The incidence of 44.88% obtained in this study is higher than the incidence rates of 39.23% and 38.08% reported by Muthusamy *et al*. (2016) and Tapati *et al.* (2018) in separate studies. However, Nnadi and Singh, (2017) recorded a prevalence of 60.8% among pregnant women in North-west Nigeria.

Candida albicans is the most common cause of candidiasis which still remains the most frequent specie isolated as recorded in this study. *Candida albicans* accounted for 33.98% of the species isolated in this study. This could be attributed to the fact that *Candida* species are members of the vaginal mycobiota and *Candida albicans* posses the distinctive characteristic feature of dimorphic transition whereby morphological transition from yeast form which is usually found in healthy asymptomatic women can transist to hyphal form, which has consistently been isolated from cases of severe VVC. The distribution of *Candida albicans* in this study agrees with the findings of ElFeky et *al.* (2016) Sida et *al.* (2017) Bitew and Abebaw (2018), Alem and Feleke (2019), Seyoum *et al.* (2020).

Candida glabrata is reported to be the most common cause of non-albicans *Candida* vulvovaginal candidiasis. In this study, out of the non-*albicans Candida* species, *C. glabrata* was the most recovered species with an occurrence of 24.5%. This can be attributed to tolerance against phagocytosis and antifungal resistance (Santos *et al.*, 2017). Out of the non-albicans *Candida* species *C. glabrata* was observed to have the highest occurrence of 24.5%. This agrees with the report of Tchana *et al.* (2017), Kalaiarasan *et al.* (2018) and Ziba *et al.* (2018) who observed that *C. glabrata* was the most recovered non-albicans *Candida* specie with a distribution of 8.6%, 46% and 77.1% respectively.

The antifungal sensitivity profile reveals that *Candida albicans* was 100% susceptible to nystatin and voriconazole, 81.0% susceptible to fluconazole. *Candida glabrata* were all susceptible (100%) to the three antifungal drugs. *Candidia tropicalis* was susceptible to nystatin (83.6%), fluconazole (100%) and voriconazole (73.8%). *Candida parapsilosis* was 100% susceptible to nystatin and fluconazole while *Candida krusei* was 100% susceptible to nystatin.

The variation in susceptibilities to the azole drugs may be due to the inhibition of the cytochrome P450 enzyme lanosterol demethylase which hinders ergosterol biosynthesis thereby inhibiting cell growth (Heimark *et al.,* 2002; Liu *et al.,* 2016).

The efficacies of azoles of these isolates is relatively similar to the reports of Hasanvand *et al*., 2017; Seyoum *et al.,* 2020; Waikhom *et al*., 2020).

Candida albicans was 12.3% resistant to fluconazole. Some isolates of *C. tropicalis* were 22.2% and 16.4% resistant against voriconazole and nystatin. *Candida krusei* was 100% resistant to voriconazole and fluconazole. The antifungal resistance to these azoles may be due to the formation of biofilm which consists of an extracellular matrix which participates in the sequestration of antifungal drugs especially fluconazole (Desai *et al.,* 2014; Mitchell *et al.,* 2015). The resistance of *Candida albicans* to fluconazole is lower than the 13.3% resistance reported by Zaidi *et al*. (2018) and higher than the reports of Ghaddar *et al.,* 2020 who observed 2.5% resistance to fluconazole in Lebanon. In a separate study, Ikenyi *et al.* (2020) reported 11.1% resistance to fluconazole in Nigeria.

The resistance of *Candida krusei* to fluconazole in this work agrees with the findings of several authors who reported that *Candida krusei* is intrinsically resistant to fluconazole (Mukasa *et al*., 2015; Alexander *et al*., 2017; Khan *et al*., 2018; Seyoum *et al.*, 2020).

All the *Candida* species identified in this study expressed virulence markers except *C. parapsilosis* which did not produce biofilm. The highest haemolytic activity was shown by *C. glabrata* species (70.5%) and this may be due to its ability to degrade hemoglobin using hemolysins in order to obtain iron. Deorukhkar *et al.* (2014) also observed that *C. glabrata* demonstrated the highest hemolysin production. Similarly the highest coagulase activity (56.5%) was expressed by *C. glabrata* species which may be attributed to the host physiology conditions, particularly, the state of its immune system. The coagulase activity of *C. glabrata* species in this study is in concordance with the reports of Ebhodaghe *et al.* (2016) and Jafari *et al.* (2017) who recorded the highest coagulase activity of 32% in *C. glabrata.*

All the *Candida* isolates produced biofilm except *C. parapsilosis* which did not produce biofilm. In this study all *Candida* isolates produced biofilm *except C. parapsilosis* which did not produce biofilm. This may be attributed to the fact that *C. parapsilosis* exhibits a biofilm formed by clusters of yeast cells and pseudohyphae morphologies which is mainly composed by carbohydrates with mimimum levels of proteins which adhered to surfaces, with minimal extracellular matrix whereas other *Candida* isolates produce biofilm which is composed of yeast, pseudohyphae, and hyphae, with intense hyphal budding composed by carbohydrates with high levels of proteins with maximum extracellular matrix. The result of biofilm formation in this study contradicts the reports of Soldini *et al.* (2018) and Borges *et al.* (2018) who observed biofilm formation in *C. parapsilosis* in separate studies. The highest biofilm production was demonstrated by *C. albicans* (81%). Emira *et al.* (2015) reported that vaginal *C. albicans* (70.58%) had the highest biofilm producing strains.

CONCLUSION

The incidence rate of 44.88% of vulvovaginal candidiasis among contraceptive users was established in this study. This work also revealed that the expression of virulence markers by the *Candida* isolates is an indication of pathogenicity and not mere colonization. The resistance to azole was observed amongst the isolates especially *Candida krusei,* Hence, the search for an alternative drug or herbal remedy is highly recommended.

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