Prevalence and sensitivity of Candida albicans to fluconazole and nystatin

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Abstract
Vulvovaginal candidiasis (VVC) is a fungal infection of the female lower genital tract comprising the vulva and the vagina. The aim of this study is to assess the prevalence of vulvovaginal candidiasis (VVC) amongst female undergraduate students in a Nigerian university and also to evaluate the susceptibility profile of the organism. Validated questionnaires were used to collect demographic information of consenting study participants and to test the participants' level of knowledge and perception of VVC among female undergraduates. *Candida* isolates were identified using a germ tube test and Chromogenic *Candida* differential agar. *Candida* isolates were then subjected to a susceptibility test using antifungal discs. The result revealed that 120 participants were interviewed but 105 participants gave their consent. Yeasts were detected in 89 (84.76%) by culture. *Candida albicans* 79(88.76%) was the most common species, followed by *Candida glabrata* 9 (10.12%), and *Candida krusei* (1.12%). Susceptibility test showed that the isolates were majorly resistant to fluconazole and nystatin. It was seen that there was high prevalence of VVC despite good knowledge and average perception of the infection. *Candida albicans* was predominant in the study. Based on assessment, both fluconazole and nystatin were not exactly the most effective drug/antifungal drug of choice for therapy. The result of this study reveals that there is high prevalence of vulvovaginal candidiasis amongst female students in a Nigerian university and the isolates were largely resistant to fluconazole and nystatin.

Keywords: Vulvovaginal, Candidiasis, females, Institution, Susceptibility profile

Introduction

Vaginal infections are the most common women's health problem, and have been increasingly linked to a growing array of serious health risks (Yano *et al.,* 2019). Vulvovaginal candidiasis (VVC) is a widespread vaginal infection primarily caused by *candida albicans (C. albicans)* (Rosati *et al.,* 2020). Vulvovaginal candidiasis also known as vaginal thrush', 'monilia', 'vulvovaginal candidosis', 'candida vaginitis' refers to vaginal and vulval symptoms caused by a yeast, most often *Candida albicans*. It

affects 75% of women on at least one occasion over a lifetime (Waikhom *et al.,* 2020) and approximately 20% of women get an infection yearly. One additional episode of infection may occur among 40–50%, while no clinical manifestation is seen in 20–50% (Venugopal *et al.,* 2020).
Vulvovaginal candidiasis is due to an overgrowth of yeasts within the vagina. About 20% of non-pregnant women aged 15–55 harbour *C. albicans* in the vagina without any symptoms. Up to 9% of women in various populations experience more than three or four episodes within one year, which is regarded as RVVC (Recurrent Vulvovaginal Candidiasis) (Christopher *et al.,* 2021).

Candida spp thrives in the vagina because estrogen causes the maturation of the lining of the vagina. A mature vaginal lining contains glycogen which is a substrate on which *C. albicans* thrives on. Symptoms often occur in the second half of the menstrual cycle when there is also more progesterone. Progesterone has an inhibitory effect on the anti-*Candida* activity of neutrophils. On the other hand, oestrogen reduces the ability of vaginal epithelial cells to inhibit the growth of *Candida albicans* on them. Lack of oestrogen makes vulvovaginal candidiasis less common in younger and older postmenopausal women (Waikhom *et al.*, 2020)

Among all the fungal infections, vaginal candidiasis is the most common one that affects women of childbearing age (Sujit *et al.*, 2012). During the reproductive years of a healthy woman, the vagina maintains a moist environment that is in constant fluctuation. The secretion of an alkaline transudate from the vaginal epithelium and cervical glands maintains this moist environment to a pH ranging from 3.8 to 4.5 (Sujit *et al.*, 2012). The vaginal microbiota is characterized by relatively few microbial species compared with the intestinal microbiota. The stability of this microbial ecosystem is greatly affected in some women by the physiological variations of the menstrual cycle and hormones, and changes dramatically after menopause.

If untreated, vaginal candidiasis can lead to chorioamnionitis with subsequent abortion and prematurity in pregnant women, congenital infection of the neonate and pelvic inflammatory disease (PID) resulting in infertility in non-pregnant women (Oviasogie *et. al.,* 2019).

Recurrent vulvovaginal candidiasis does not correlate with mortality rates but the morbidity is dramatically increasing, and the costs associated with medical care rise accordingly. Hence, more effort needs to be made on the one hand to understand the immune- pathogenesis and on the other hand to treat VVC patients efficiently and prevent recurrences (Christopher *et al.,* 2021).

This study is aimed at assessment of the prevalence of vulvovaginal candidiasis (VVC) amongst female undergraduate students in a Nigerian university and the antifungal susceptibility profile of the isolates.

Materials and methods

Study Design and Area

This cross-sectional study was conducted in the Agbani campus of Enugu State University of Science and Technology, in Enugu State, Nigeria. Laboratory analysis was carried out in the Microbiology laboratory of the Faculty of Pharmaceutical Sciences, ESUT.

Study Population

A total of one hundred and five students were recruited in accordance with the populations in their different levels of study. Selection within levels was done purposively. Pre-enrolment briefing about the study was carried out for each participant, and thereafter, written informed consent was obtained in all participants.

Ethical Approval
Ethical approval for the study was obtained from the ethics committee of Enugu State University of Science and Technology Teaching Hospital with reference number ESUTHP/C-MAC/RA/034/VOL.2/173.The individual student's consent was sort for and those who consented were included in the study. The participants informed into their simple language about the infection, aim of the research and the benefits of the study.

Criteria
Inclusion criteria/ Exclusion criteria

Participants were included if they are between ages 16 years and 45 years, with or without signs and symptoms of vulvovaginal discomfort, and not pregnant.

Participants, under 15 years or over 45 years of age, pregnant, history of diabetics, and/or menstruating, those on antifungal antibiotics were excluded from the study. The procedure employed consisted of a questionnaire interview and the taking of patients' clinical history.

Data Collection
Pre-tested questionnaires were used to obtain data on age, marital status, antibiotic and contraceptive usage, students' sexual history, material used during menses, personal vaginal hygiene and on information about knowledge and perception. The overall percentage of level of knowledge and perception were obtained by calculating the percentage of positive answers to the questions asked on knowledge and perception of the infection respectively. The study subjects were then examined, and vaginal samples collected.

Sample Collection
High-vaginal swab specimens were collected from the study participants using sterile swab stick and transported in sterile containers to the Pharmaceutical Microbiology laboratory for microscopy and culture.

Methods

Microscopic Examination of Sample
Within an hour of collection, specimens were mixed with 10% w/v saline preparation and KOH
preparation in a 5 ml test tube, to expose the fungi. A drop of this mixture was viewed with low
power objectives (10× and 40×) after placing on a clean grease-free slide (Oli et al., 2017). The first swab was used to prepare wet mount and thin smear on microscopic slide for gram stain, Smears of the vaginal swab samples were prepared, and gram stained. Indirect Gram's stain was performed for yeast suspected colonies which revealed gram positive (G + ve) yeast cells. The slides were viewed under the microscope.

Culture of HVS specimen
Culture of the HVS was on SDA supplemented with chloramphenicol at 0.05 mg/ml

Identification of Isolated Fungi
Germ tube testing
From a freshly prepared Sabouraud dextrose agar plates, a loopful of a yeast colony was inoculated into 0.5 ml freshly prepared human serum and incubated at 35ºC for 3 hr. A wet smear of the yeast suspension was made and mounted on a microscope and viewed with the
40x objective lens. Sprouting non-septate germinating hyphae confirms the presence of *Candida albicans (*Baker *et al.,* 1967, Oli *et al.,* 2017)

The isolates were classified as either germ tube positive or germ tube negative. Filamentous extension from yeast cell with no constriction at the neck was considered as germ tube positive. Isolates with no

extensions or extensions with constrictions at the neck were considered germ tube negative.

Characterization of yeast isolates using CHROM agar candida

CHROMagar candida (Becton, Dickinson and Company, Maryland, USA) plates were prepared according to manufacturer's instructions (47.7 g/L). Positive isolates on Sabouraud dextrose agar plates were streaked on to the media, for species differentiation, and incubated at 35 ^{o}C for 48 hr. The different candida species were recognized by their different colours and shape according to manufacturer's instructions.

Antifungal susceptibility testing

Antifungal susceptibility testing was performed using Mueller Hinton Agar supplemented with 2% glucose and 0.5 mg/mL methylene blue. 0.5 Mcfarland standard was prepared by mixing 0.05ml of 1.175% barium chloride dehydrate (Bacl2.2H20) with 9.95ml of 1% sulphuric acid (H2SO4) together. Five distinct colonies were picked from a 48-hours old culture and inoculated into sterile water. The suspension was diluted to a 0.5 McFarland standard. With sterile cotton swab stick, the suspensions were streaked on to Mueller Hinton Agar (supplemented with 2% glucose and 0.5 mg/mL methylene blue) plates and allowed to dry and diffuse for about 5 minutes. The three antifungal discs (voriconazole, nystatin and fluconazole) were picked (separately) aseptically and dispensed on to the surface of the agar containing each isolate. The plates were then incubated for at 35^{o}C for 24 h. The assay was done in duplicates and Inhibition Zone Diameters (IZD) formed were measured and recorded. Susceptibility/resistance was interpreted according to Clinical and Laboratory Standard Institute (CLSI) (CLSI, 2012) guidelines.

Statistical analysis

Data generated from questionnaires and results of the laboratory analysis were analyzed using Microsoft Excel and SPSS software (version 23.0; IBM Corporation, Armonk, NY, USA). Results obtained were reduced to percentages, tables, and a figure. Categorical data variables were statistically described in the form of frequencies and percentages while continuous data variables were summarized as mean (Standard Deviation). P-value was considered significant if less than 0.05. The association between VVC and clinical presentations of study participants was determined using chi-square test.

Results

A total of 120 participants were interviewed, but only 105 of them were eligible and consented to be part of the study. The remaining 15 female students were either not eligible or verbally declined to be part of the study. Complete socio-demographic data, clinical presentations and vaginal swabs were taken from all 105 consenting participants**.** The age range of the participants was 18-42. (52.5 %) of the participants were within the age of 24-29 years, 34.2% 18-23 years, some (34.2%) were within the age range of 18-23 years while only 0.8% were aged ≥ 42 years. Majority (84.2%) of the participants were single while only few (15.8%) were married (Table 1).

Out of the 105 students examined, 89 (84.76 %) showed visible fungal growths after the incubation period while 16 (15.23 %) tested negative for vaginal candidiasis infection (Figure 1 & 2).The isolates constitute mixed candida species *C. albicans* in 79 (88.76 %), *C. krusei* in 1 (1.12 %), and *C. glabrata* in 9 (10.12 %). (Table 2)

Majority (90.8%) of the participants have heard of candidiasis before. A good number (65.8%) of the participants reported not

sexually active, 25.8% reported to be sexually active while only 1.7% were not sure if they were sexually active or not. More than half (58.3%) of the participants thought candidiasis cannot be gotten through sex, some (22.5%) thought that candidiasis can be gotten through sex while 13.3% had no idea if candidiasis can be spread through sexual intercourse. More than half (56.7%) of the participants thought candidiasis can be gotten through toilet. All (100.0%) of the participants use sanitary pad during menstruation. About 40.3% of the participants rarely take alcohol, some (40.0%) take alcohol sometimes while 16.7% never took alcohol. About one-third (36.7%) of the respondents sometimes wear tight underwear, 35.8% rarely put on tight underwear while 25.0% never wore tight underwear (Table 3).

Table 4 shows the relationship between participants' demographic data and candidiasis infection. Majority (93.3%) of participants who have had all the symptoms of candidiasis mentioned were aged 18-23 years while few others (6.7%) were aged ≥ 42 years. Nearly half (45.5%) of the participants who had some of the mentioned symptoms were aged 18-23 years, others (27.3% vs 27.3%) were aged 24-29 years and 30-35 years, respectively. A good number (64.2%) of those who had not experienced any symptom of candidiasis were aged 24-29 years. Most (93.3%) and all (100.0%) of the participants who have had respectively all and some of the symptoms of candidiasis mentioned were single. A significant ($P < 0.05$) relationship occurred between the demographic data (age and marital status) of the respondents and their history of candidiasis infection.

Table 1: Participants' demographic data

Variable	Frequency	Percentage (%)
Age		
18-23 years	41	34.2
24-29 years	63	52.5
30-35 years	10	8.3
36-41 years	5	4.2
≥ 42 years	1	0.8
Marital status		
Single	101	84.2
Married	19	15.8
Sexually active		
Yes	31	25.8
No	79	65.8
I think so	8	6.7
I am not sure	2	1.7
Taken any antibiotics in the past three months		
Yes	42	35.0
No	66	55.0
I think so	4	3.3
I am not sure	8	6.7
Used any contraceptive in the		

past three months		
Yes	109	90.8
No	8	6.7
I am not sure	3	2.5
Material used during menstruation		
Sanitary pad	120	100.0
Total	**120**	**100.0**

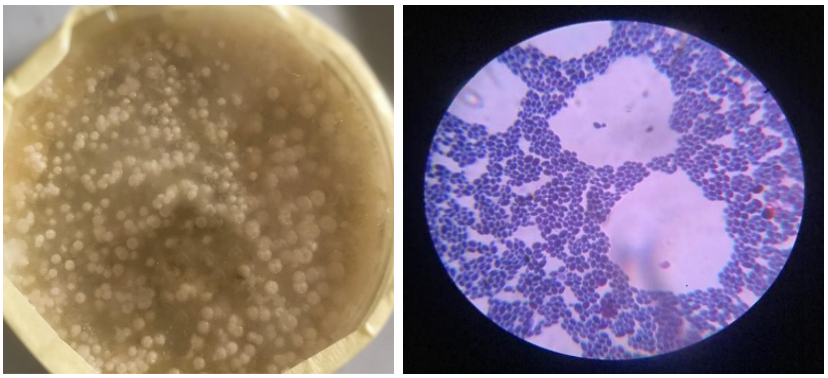

Fig.1a Macroscopic morphology of *Candida albicans* (pale cream, smooth, glistening, convex colonies) on Sabouraud Dextrose Agar after 48 h of aerobic incubation at 37 °C
Fig.1b. Micrograph of *Candida albicans* in direct Gram smear of HVS(X100)

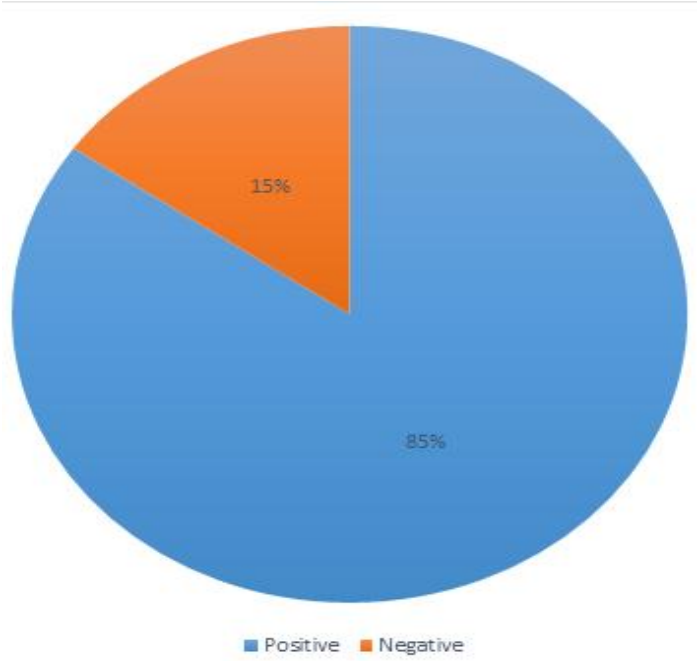

Figure 2. Prevalence of vaginal candidiasis in female students attending ESUT .

Table 2: Differentiation of species of *candida isolate on C*HROM agar

Species	Culture positive (no of isolates)	Culture negative	Percentage isolate (%)
C. glabarta	9	96	10.12
C. krusei	1	104	1.12
C. albicans	79	26	88.76

Table 3: Knowledge, perception of vaginal candidiasis and lifestyles among the students (n = 120)

Variable/ Question asked	**1.Have you Heard of candidiasis before**			**2. Have you Had any symptom(s) of candidiasis**				**3. Gone for candidiasis test**			**4 Think candida can be gotten through toilet**				**5. Number of person(s) sharing toilet**				**6. How often toilet is washed**			
Response	Yes	ITS	INS	ATM	STM	OS	NTM	Yes	No	ITS	Yes	No	ITS	NI	0	1-2	3-4	5-6	ED	TW	OW	IAU
	109	5	6	15	11	13	81	63	49	8	68	23	15	14	27	53	33	7	6	45	62	7
% Response	90.8	4.2	5.0	12.5	9.2	10.8	67.5	52.6	40.8	6.7	56.7	19.2	12.5	11.7	22.5	44.2	27.5	5.8	5.0	37.5	51.7	5.8

Variable/ Question asked	**7.Sexually active**				**8.Number of sexual partner**				**9.Take any broad spectrum antibiotics**			**10.Ever been pregnant**				**11.Wear tight underwear**				**12.Take alcohol**		
Response	Yes	No	ITS	INS	0	1	2	3	Yes	No	INS	O	T	MTT	N	OT	ST	R	N	R	ST	N
	31	79	8	2	85	27	7	1	79	38	3	1	11	3	105	3	44	43	30	48	52	20
% Response	25.8	65.8	6.7	1.7	70.8	22.5	5.8	0.8	65.8	31.7	2.5	0.8	9.2	2.5	87.5	2.5	36.7	35.8	25.0	40.0	43.3	16.7

I think so =ITS, I am not sure = INS, All of the mentioned = ATM, Some of the mentioned = STM, One symptom = OS, None of the mentioned = NTM, No idea = NI, Every day=ED, Twice a week = TW, Once a week = OW, Immediately after use = IAU, Once = O, T=Twice, OT=Oftentimes
ST = Sometimes, Rarely = R, N = Never

Table 4: Relationship between participants' demographic data and candidiasis infection

	Had any symptom (s) of candidiasis				
Demographic variables	**All of the mentioned F (%)**	**Some of the mentioned F (%)**	**One symptom F (%)**	**None of the symptoms F (%)**	**Total F (%)**
Age					
18-23 years	14(93.3)	5(45.5)	5(38.5)	17(21.0)	41(34.2)
24-29 years	0(0.0)	3(27.3)	8(61.5)	52(64.2)	63(52.5)
30-35 years	0(0.0)	3(27.3)	0(0.0)	7(8.6)	10(8.3)
36-41 years	0(0.0)	0(0.0)	0(0.0)	5(6.2)	5(4.2)
≥ 42 years	1(6.7)	0(0.0)	0(0.0)	0(0.0)	1(0.8)
Total	**15(100.0)**	**11(100.0)**	**13(100.0)**	**81(100.0)**	**120(100.0)**
	***X*²=47.974**		**df=12**		**p=0.000***
Marital status					
Single	14 (93.3)	11(100.0)	13 (100.0)	63(77.8)	101(84.2)
Married	1(6.7)	0(0.0)	0(0.0)	18(22.2)	19 (15.8)
Total	**15(100.0)**	**11(100.0)**	**13(100.0)**	**81(100.0)**	**120(100.0)**
	***X*²=7.942**		**df=3**		**p=0.047***

F=frequency, %=percentage, df = degree of freedom, x^2=chi-square, p=probability, * = statistically significant ($p < 0.05$).

Antifungal susceptibility outcome

The highest overall resistance rate of *Candida species* was seen against fluconazole (100%), and nystatin (100%).

Table 5: Result of Sensitivity test on *candida albicans* on Mueller Hinton Agar

Antifungal agents	N(susceptibility)	N(resistant)	N(Dose dependent susceptible)
Fluconazole	0(0.00%)	89(100%)	0(0.00%)
Nystatin	0(0.00%)	89(100%)	0(0.00%)

NOTE: There was no growth or zone of inhibition on Mueller Hinton Agar

Table 6: Result of Sensitivity test on *candida albicans* on SDA Agar

Antifungal agents	N(susceptibility)	N(resistant)	N(Dose dependent susceptible)
Fluconazole	0(0%)	69(64.42%)	20(22.27%)
Nystatin	0(0%)	47(52.81%)	42(47.19%)

Discussion

This study revealed that students who are at the beginning and peak of their reproductive years are more vulnerable to infections. This observation is consistent with the reports of (Muller *et al.,* 2015, Sobel *et al,.*1998), who revealed that women in their reproductive years were more prone to vaginal candidiasis compared to other age groups. From this study, it can be seen that up to 89(84.76%) of women examined were infected with vaginal candidiasis. Similarly, (Brande *et al*., 1996) estimated that 75% of all women will experience at least one symptomatic yeast infection during their lifetimes.

High oestrogen activity within this age bracket which primes the vaginal lining for Candida colonisation, high incidence of antibiotics abuse (Table 3) and contraceptive usage associated with this age group may be responsible for the high percentage recorded. This is also a pointer that sexual activity is a risk factor for VVC (vulvovaginal candidiasis) even though it is not a sexually transmitted disease. This supports the report of (Emeribe *et al.,* 2015)., and (Ezeigbo *et al.,* 2010), but in contrast to (Alo *et al*.,2012) who reported 36-40 years old women as the highest VVC (vulvovaginal candidiasis) prevalent age group.

The highest prevalence rate 79(65.8%) (table 3) was found in students who admitted that they had prolonged use of broad spectrum antibiotics followed by 9(7.5%) (table 3) among students under regular use of tight fitting net pants. According to Richard (2009), prolonged use of broad spectrum antibiotics can destroy the normal flora in the vagina which ordinarily would have suppressed the growth of *Candida albicans*. This microbial flora has been known to secrete acidic materials which help to keep the pH of the vagina under check. Alteration in the normal pH influence the over growth of this fungus leading to candidiasis (Spinillo *et al.,* 2001*).* Similarly, the use of non-ventilating tight fitting nylon pant has been reported to foster fungal growth (Ferrer, 2000). This is because these tight fittings nylon pants discourage vagina aeration, decreasing the moisture of the region and making the vagina warmth. These conditions have been observed to support and promote the growth of *Candida albicans* in the vagina, resulting in infections (Akingbade *et al.,* 2013).

Also, oral and vaginal anti-fungal agents that are frequently used in our locality have been found to be less effective against the non-*Candida albicans* infections (Jumbo *et al* 2010; Ugochukwu *et al.,* 2012) possibly due to observed high miss-use of fluconazole and nystatin (personal communication). Both drugs are prescription drugs but are easily obtained over-the-counter. They are often taken in sub-therapeutic dose by the users. Several other researchers also reported the development of resistance to nystatin, imidazole and triazole antifungal agents (Loeffler & Stevens, 2003; Pfaller & Diekema, 2004).

The knowledge of the respondents concerning vulvovaginal candidiasis was impressive as 109 (90.8%) had heard about the infection and also 63(52.6%) has gone for a candidiasis test. This can be attributed to the current free access to all sorts of information on the internet.

Many of our study population 68 (56.7%)(table 3) still believed or thought that VVC is a kind of toilet disease. It is therefore expected that the general (uneducated) population may even have worse perception. This is confirmed by a similar study in the study environment (Ugochukwu *et al.,* 2012) and in other regions of Nigeria. This actually calls for the need for increased awareness on VVC via all available social media, schools and churches. This will help to reduce possible complications of untreated cases (Christine *et al.,* 2011).

Conclusion

From the findings of this study, the studied population had good knowledge and perception of the infection. However, there was a high prevalence of vulvovaginal candidiasis. Different types of *candida species* were isolated and identified. *Candida albicans* dominated the etiology in the studied population. Isolated *Candida* spp were observed to be highly resistant to fluconazole and nystatin.

Conflict of interest statement: The authors declare no conflict of interest.

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