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Phylogenetic diversity and susceptibility of *Candida* species from women using contraceptive devices in northcentral Nigeria

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Abstract:

Background: The use of contraceptive devices predisposes women to vulvovaginal candidiasis (VVC) globally. Despite the high incidence of VVC and antifungal resistance to azoles*,* the genetic diversity and resistance pattern among contraceptive users in Nigeria is poorly investigated. This study therefore sought to characterize and determine the phylogenetic breadth of *Candida* species as well as their resistance to antifungal agents.
Methodology: This study recruited 1,600 women using contraceptive devices who visited selected gynaecology and obstetrics clinics in northcentral Nigeria. *Candida* species were isolated and characterized using conventional methods and sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). Bayesian phylogenetic analysis was used to characterize the diversity of *Candida* species and primer-specific PCR was used to detect the presence of resistant genes. Agar well diffusion technique was used for the determination of antifungal susceptibility profiles. Data analysis was done by Kruskal-Wallis Chi-square test on R Console software version 3.2.2, followed by post-hoc Wilcoxon rank sum test with Bonferroni correction for multiple pairwise comparisons of means where there was a significant difference between the antifungal agents. The level of significance was set at $p < 0.05$.
Results: A total of 710 (44.3%) out of the 1,600 women using contraceptive devices had VVC with five species of *Candida* identified in them. Although *Candida albicans* was the predominant (43.2%, n=307) species, other non-albicans *Candida* species include *Candida* (*Nakaseomyces*) *glabrata* (19.0%, n=135), *Candida tropicalis* (15.8%, n=112), *Candida parapsilosis* (8.9%, n=63), and *Candida akabanensis* (13.1%, n=93) which were phenotypically identified as *Candida* (*Nakaseomyces*) *glabrata*. All the *Candida* species showed varying degrees of susceptibilities to voriconazole, fluconazole and nystatin. However, resistance of *C. albicans* to fluconazole was 29.0%*, C. tropicalis* to nystatin (46.0%) and to voriconazole (14.0%), while *C. akabanensis* was 100.0% resistant to voriconazole and fluconazole. Kruskal-Wallis Chi-square test showed nystatin as the most effective antifungal agent against the *Candida* species (χ^2=786.03, df=2, p<0.001). Also, resistant gene *Erg11* was identified in all the *Candida* species that were phenotypically resistant to the antifungal agents tested.
Conclusion: Women using contraceptive devices in northcentral Nigeria harbor phylogenetically diverse *Candida* species including *C. akabanensis*, an uncommon cause of VVC. Of these *Candida* species, *C. albicans, C. tropicalis* and *C. akabanensis* were notable for multidrug drug resistance as well as harboring *Erg11* resistance gene.

Keywords: *Candida*, Mycobiome, Contraceptives, Resistance

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Diversité phylogénétique et sensibilité des espèces de *Candida* chez les femmes utilisant des dispositifs contraceptifs dans le centre-nord du Nigeria

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Résumé:

Contexte: L'utilisation de dispositifs contraceptifs prédispose les femmes à la candidose vulvo-vaginale (CVV) à l'échelle mondiale. Malgré l'incidence élevée de la CVV et de la résistance antifongique aux azoles, la diversité génétique et les modèles de résistance parmi les utilisatrices de contraceptifs au Nigéria sont peu étudiés. Cette étude a donc cherché à caractériser et déterminer l'étendue phylogénétique des espèces de *Candida* ainsi que leur résistance aux agents antifongiques.
Méthodologie: Cette étude a recruté 1600 femmes utilisant des dispositifs contraceptifs qui ont visité des cliniques de gynécologie et d'obstétrique sélectionnées dans le centre-nord du Nigeria. Les espèces de *Candida* ont été isolées et caractérisées à l'aide de méthodes conventionnelles et du séquençage de la région de l'espaceur transcrit interne (ITS) de l'ADN ribosomal (ADNr). L'analyse phylogénétique bayésienne a été utilisée pour caractériser la diversité des espèces de *Candida* et la PCR spécifique aux amorces a été utilisée pour détecter la présence de gènes résistants. La technique de diffusion dans des puits de gélose a été utilisée pour la détermination des profils de sensibilité aux antifongiques. L'analyse des données a été effectuée par le test du chi carré de Kruskal-Wallis sur la version 3.2.2 du logiciel R Console, suivi d'un test de somme de rangs de Wilcoxon post-hoc avec correction de Bonferroni pour de multiples comparaisons par paires de moyennes où il y avait une différence significative entre les agents antifongiques. Le niveau de signification a été fixé à $p<0,05$.
Résultats: Au total, 710 (44,3%) des 1600 femmes utilisant des dispositifs contraceptifs présentaient une VVC contenant cinq espèces de *Candida*. Bien que *Candida albicans* soit l'espèce prédominante (43,2%, n=307), d'autres espèces de *Candida* non albicans comprennent *Candida* (*Nakaseomyces*) *glabrata* (19,0%, n=135), *Candida tropicalis* (15,8%, n=112), *Candida parapsilosis* (8,9%, n=63) et *Candida akabanensis* (13,1%, n=93), phénotypiquement identifiés comme étant *Candida* (*Nakaseomyces*) *glabrata*. Toutes les espèces de *Candida* présentaient divers degrés de sensibilité au voriconazole, au fluconazole et à la nystatine. Cependant, la résistance de *C. albicans* au fluconazole était de 29,0%, celle de *C. tropicalis* à la nystatine (46,0%) et au voriconazole (14,0%), tandis que celle de *C. akabanensis* était de 100,0% résistante au voriconazole et au fluconazole. Le test du Chi carré de Kruskal-Wallis a montré que la nystatine était l'agent antifongique le plus efficace contre l'espèce *Candida* ($x^2=786,03$, df=2, $p<0,001$). En outre, le gène résistant *Erg11* a été identifié chez toutes les espèces de *Candida* qui étaient phénotypiquement résistantes aux agents antifongiques testés.
Conclusion: Les femmes utilisant des dispositifs contraceptifs dans le centre-nord du Nigéria abritent des espèces de *Candida* phylogénétiquement diverses, notamment *C. akabanensis*, une cause rare de CVV. Parmi ces espèces de *Candida*, *C. albicans*, *C. tropicalis* et *C. akabanensis* se distinguaient par leur multirésistance aux médicaments et par l'hébergement du gène de résistance *Erg11*.

Mots clés: *Candida*, Mycobiome, Contraceptifs, Résistance

Introduction:

The vaginal microecosystem consists of a complex and dynamic microbiome that coexists in a symbiotic relationship with the host (1) and the diversity of the vaginal microbiome plays a vital role in vaginal health. The vaginal normal flora of women within the reproductive age harbors 10^{10}–10^{11} bacteria predominantly *Lactobacillus* species (2,3). Despite the vast number of bacteria in the vaginal ecosystem, fungi contribute to the promotion of vaginal health. Using the sequences of the internal transcribed spacer 1 (ITS1) of vaginal samples from healthy women, Drell et al., (4) identified two fungal phyla as the major constituents of the vaginal mycobiome; Ascomycota (58.0%), predominated by the *Candida* genera, and Basidiomycota (3.0%). Similarly, *Candida* species colonization constitute 21.0-65.0% of the vaginal mycobiome of healthy women (4,5,6). However, these *Candida* species can transit from colonization and cause symptomatic infections including vulvovaginal candidiasis.

Vulvovaginal candidiasis (VVC) is caused by the overgrowth of *Candida* species in the vagina which is characterized by vulva irritation and may also present with white 'cheese-like' vaginal discharge (7). Globally, *Candida albicans* accounts for between 85-90.0% cases of VVC (8-11). However, recent studies have implicated non-*albicans Candida* (NAC) species including *Candida tropicalis, Candida* (*Nakaseomyces*) *glabrata, Candida krusei/ Pichia kudriavzevii* and *Candida parapsilosis* as emerging aetiological agents of VVC (12, 13).

VVC is a global health risk that contributes to significant morbidity and economic burden in women. It has been estimated that 70.0% of all women will have at least one episode of VVC, and 372 million women are affected by recurrent vulvovaginal candidiasis (RVVC) during their reproductive years (14, 15). Aging, pregnancy, use of contraceptives, diaphragms, vaginal douching, prolonged chemotherapy or antibiotic use, metabolic diseases especially diabetes mellitus and immunosuppression predispose women to VVC (16). The association between VVC and the use of injectables, intrauterine devices (IUD) and oral contraceptive pills has been documented (8,17,18).

In recent times, non-pathogenic species and emerging fungal agents are agents of

human disease. These emerging species express important virulence factors and possess antimicrobial resistant genes. The emerging fungal pathogen, *Candida auris*, known for invasive candidiasis was reported in a case of vulvovaginitis (19). There are various oral and topical treatments available for the treatment of VVC (20,21). However, local and global antifungal susceptibility surveillance has revealed decreased susceptibility of some *Candida* isolates to some antifungal in recent times (22,23,24).

In Nigeria, the increasing number of women using contraceptive devices can be linked to the increased advocacy on the use of devices to promote health and reduce infant and maternal mortality. However, the risk of vaginal microbiome modulation using these devices, pathogen's carriage, dissemination, biofilm formation, complex community interaction, changes in hormonal level are often neglected. We hypothesized that the use of contraceptive devices modifies the vaginal mycobiome leading to phylogenetic diverse species responsible for VVC among contraceptive users. In addition, the use of advance molecular technologies for the identification of *Candida* species is rarely carried out in most clinical settings in Nigeria because the procedure is relatively expensive, hence, patients are treated empirically on the basis of traditional diagnostic results thereby promoting drug resistance. Furthermore, there is paucity of epidemiological data on the phylogenetic diversity and susceptibility patterns of *Candida* species involved in VVC among contraceptive users in Nigeria, hence, this study was designed to bridge the gap by providing relevant information on VVC and contraceptive usage.

Materials and Method:

Study setting:

This research was carried out in three States of the northcentral Nigeria (Nasarawa, Niger, Benue) and the Federal Capital Territory (FCT) Abuja, which were selected by simple random sampling technique.

Ethical approval:

Ethical approval was obtained from the Health Research Ethics Committees of the hospitals with reference numbers (MOH/STA/204/Vol.1/96; STA/495/Vol/136; FHREC/2017/01/109/11-12-17; NHREC18/06/ 2017). The samples were obtained with the informed consent of the women.

Study design, participants & sampling method:

This was a descriptive cross-sectional study of 1600 randomly selected women on contraceptive devices conducted from January 2018 to May 2019. The sample size was determined by the Cochran formula (25) using a previous prevalence of 0.155% to determine the sample size per hospital in each State and the FCT. A total of 710 consenting women with contraceptive devices with vaginal *Candida* isolates but asymptomatic for VVC, across eight secondary health facilities in the study area were enrolled. Only women using contraceptives were included while those who were not contraceptive users and those pregnant were excluded.

Data and sample collection:

A structured questionnaire with both open and closed ended questions was used to collect risk factors and obtain biodemographic data from the study participants. With the assistance of a gynecologist, two cotton-tipped sterile swabs sticks were used to collect high vaginal swab (HVS) samples from each woman who met the inclusion criteria. The HVS was collected by inserting a sterile vaginal speculum into the vagina; a sterile cotton wool swab was inserted into the posterior vaginal fornix and rotated gently as previously described (26). The swab stick was withdrawn and replaced in its case and labeled appropriately with the participant's information.

Isolation and phenotypic identification of *Candida* species:

One swab was used subjected to 10% KOH direct smear examination while the other swab was cultured on Sabouraud Dextrose Agar (SDA, HiMedia, India) plates, supplemented with 50 mg/L chloramphenicol, and incubated for 48-72 hours at 35°C. Phenotypic identification of the isolated strains was carried out on the basis of microscopic and cultural features. All *Candida* species were differentiated on the CHROMagar *Candida* medium, (Difco^{TM}, CHROMagar^{TM}) after incubation for 48h at 37°C. In addition to Gram staining procedure, germ tube test was performed by inoculating the *Candida* isolate into 0.5 mL human serum, incubating at 37°C for 3 hours, and observing for sprouting yeast cells under the microscope.

Antifungal susceptibility test:

Antifungal susceptibility was performed by the modified disc diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI) guideline (27), which required the addition of 2% glucose and 0.5 µg/mL methylene blue dye into Mueller-Hinton agar (Oxoid, UK). A suspension was prepared by adding five distinct *Candida* colonies into a 5ml test tube containing 0.85% sterile saline solution and incubated overnight. The suspension was then compared to 0.5 McFarland standards. Cotton swab moistened with the fungal suspension was streaked on modified Mueller-Hinton media. Commercially avai-

lable antifungal disks for nystatin (100 units), voriconazole (10µg) and fluconazole (10µg) (Oxoid, UK) were aseptically dispensed onto the surface of the inoculated agar plates and placed in an incubator at 37^0 C for 24 hours.

The diameters of zone of inhibition were measured in millimetres and reported as susceptible (S), susceptible dose dependent (SSD) or resistant (R) in accordance with CLSI M44A document guideline (27). Quality control tests were performed daily to check for the precision and accuracy of the results of disk diffusion testing.

Genomic DNA extraction:

The genomic DNA was extracted from the *Candida* isolates using a ZymoResearch (ZR) fungal/bacterial DNA mini prep extraction kit (Cat.D6005; South Africa) according to the manufacturer's instructions. Briefly, into each ZR BashingBead Lysis tubes, colonies from the pure culture of the isolates were added into 200 µL of isotonic buffer and 750µL of lysis buffer was added to the tube secured in the 2mL tube holder assembly of the Disruptor Genie™ and centrifuged at 10,000x*g* for 1 min.

Four hundred microlitres (400µL) of supernatant was transferred to a Zymo-Spin IV spin filter in a collection tube and centrifuged at 7000x*g* for 60sec. One thousand two hundred microlitre (1200µL) of fungal DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600µL, 800µL was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000x*g* for 60sec, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred microlitres (200µL) of the DNA pre-wash buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000x*g* for 60sec followed by the addition of 500 µL of fungal DNA and wash buffer. This was centrifuged at 10,000x*g* for 60sec. The Zymo-spin IIC column was transferred to a clean 1.5µL centrifuge tube, 100µL DNA elution buffer was added to the column matrix and centrifuged at 10,000x*g* for 30sec to elute the DNA. The pure DNA was then stored at -20°C for further analysis.

The quantity and quality of extracted DNA was estimated using a NanoDrop™1000 spectrophotometer (Thermo Fisher Scientific, USA) at 260 nm. The purified DNA was maintained at -20 °C until used in the PCR assay.

PCR amplification of ITS region:

The ITS region of the isolates was amplified using the specific primer pair of ITS1 forward (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 reverse (5'-TCCTCCGCTTAT TGATA TGC-3') primers (28). The PCR conditions were as follows; initial denaturation at 95ºC for 5 min; final denaturation at 95ºC for 30 sec; annealing at 53ºC for 30 sec; initial extension at 72ºC for 30sec for 35 cycles and final extension at 72ºC for 5min in a Thermocycler (Applied Biosystems, UK). The integrity of the amplified product was evaluated by electrophoresis in a 1% (w/v) agarose gel at 120V for 15 min in 1 X Tris-borate-EDTA (TBE) buffer, stained with 2µL ethidium bromide and visualized on a blue light transilluminator.

Sequencing of amplified products and bioinformatic analysis:

Sequencing of the purified and amplified products was performed as previously described (29), using standard methods. Sequencing was done in Pretoria, South Africa, with BigDye Terminator kit on 3510 ABI sequencer (Applied Biosystems, UK) using standard protocols and previously designed primers. The sequencing was performed at a final volume of 10µL, the elements included 0.25 µL BigDye® terminator v1.1/v3.1, 2.25µL of 5xBigDye sequencing buffer, 10µL primers, PCR primers and 2-10ng PCR template per 100bp.

The bioinformatics algorithm Trace edit was used to edit the obtained sequences and similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLASTn) for species identification. The sequences obtained were aligned using Multiple Alignment Fast Fourier Transform (MAFFT).

Identification of ergosterol resistant (*Erg11*) gene:

Detection of *Erg11* resistant gene was done on representative isolates from each *Candida* species. Amplification of the gene was performed using the Erg F: 5'–GTTGA AACTGTCATTGAT-3' and Erg R: 5'-TCAGAACA CTGAACTGAAA-3' primers on ABI 9700 Thermal Cycler (Applied Biosystems, UK). The PCR conditions were strictly followed, and the resulting products were resolved on 1% agarose gel at 120V for 25min and visually observed on UV transilluminator with expected *Erg11* amplicon size of 500bp (30).

Bioinformatic and statistical analyses:

Bayesian phylogenetic analysis was used, and the evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (31,32). Confidence limits for phylogenetic trees were estimated by bootstrap consensus tree inferred from 500 replicates (33). This was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the method of Jukes and Cantor (34).

Other data obtained were analyzed

using R Console software (version 3.2.2). Shapiro-wilk normality test was carried out and the data was observed not to be normally distributed. Hence, Kruskal-Wallis Chi-square test was used to compare the mean response of the fungi species in relation to antifungal agents. Kruskal-Wallis Chi-square test was followed by a post-hoc Wilcoxon rank sum test with Bonferroni correction was used for multiple pairwise comparisons of means where there was a significant difference between the treatments. The level of significance was set at $p<0.05$.

Results:

A total of 710 (44.3%) women using contraceptive devices had VVC and five species of *Candida* were phenotypically identified; *C. albicans, C. glabrata (Nakaseomyces glabrata*), *C. tropicalis, C. parapsilosis* and *C. krusei* (*Pichia kudriavzevii*). *Candida albicans* (43.2%, n=307) was the most predominant species isolated, other non-albicans *Candida* species included *C. glabrata* (*N. glabrata*) (19.0%, n=135), *C. tropicalis* (15.8%, n=112), *C. parapsilosis* (8.9%, n=63) and *C. krusei (P. kudriavzevii*) (13.1%, n=93).

Candida albicans was the most predominant species (49.4%) identified among women within the age group 20-24 years. The highest distribution of *C. tropicalis* was seen in older women aged 45-50 years. A high frequency of *C. parapsilosis* (16.0%) was observed within the age group 30-35 years, no *C. parapsilosis* was isolated amongst women within age group 15-19 and 45-50 years. *Candida* (*Nakaseomyces) glabrata* was observed to have a high frequency distribution (32.0%) among women within the age group 30-34 years. *Candida krusei* (*P. kudriavzevii*) had a high frequency (22.0%) among women within the age group 15-19 years (Table 1).

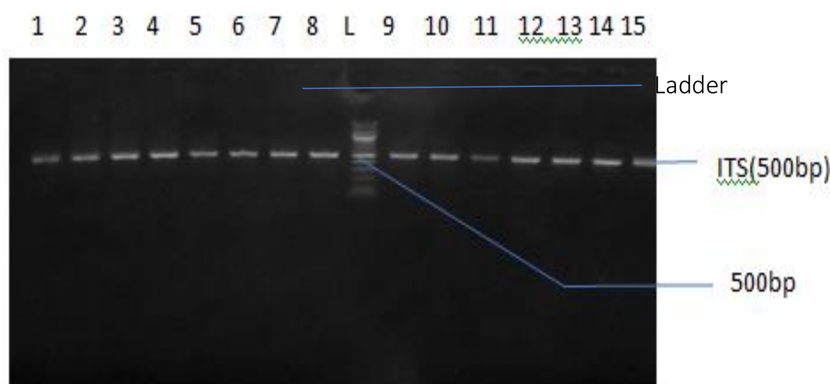
The most predominant *Candida* species solated from women who use contraceptive pills was *C. albicans. Candida tropicalis* and *C. glabrata* (*N. glabrata*) (25.0%) were the most frequent isolates recovered from the study participants that used injectable contraceptives. *Candida parapsilosis* (18.0%) and *C. glabrata* (*N. glabrata)* (25.0%) were mostly isolated from IUCD and implant users. The diversity of *Candida* species may be attributed to the use of contraceptive devices which modified the vaginal mycobiome. *Candida albicans* was the most recovered isolate from participants with informal secondary and tertiary education. The highest frequency of *C. tropicalis* was seen amongst women with primary education. The distribution of *Candida* species among the married women was higher than the single ladies (Table 1).

The amplification of the ITS DNA region revealed that all the isolates have a fragment of 500bp which indicated they were all *Candida* species (Fig 1). The obtained ITS sequence from the isolate produced an exact match during the MegaBlast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. *Candida* isolates designated 1,3,5,6,8,9,11,12,13,14, 15,17,23,25 and 27 were identified as *C. albicans;* isolates 24,2,22,4,19 and 20 were *C. glabrata* (*N. glabrata)*; isolates 16,21 and 26 were *C. tropicalis*; and isolate 18 was identified as *C. parapsilosis.* The ITS sequence identified isolates 7 and 10 as *Candida akabanensis* but were phenotypically identified as *C. krusei* (*P. kudriavzevii*).

The susceptibility pattern of *Candida* species against the 3 antifungal drugs tested is shown in Table 2. *Candida albicans* was 100.0% susceptible to nystatin, 92.1% susceptible to voriconazole and 71.1% susceptible to fluconazole (28.9% resistant to fluconazole). All isolates of *C. glabrata* (*N. glabrata)* were 100.0% susceptible to nystatin, voriconazole and fluconazole. *Candida tropicalis* was susceptible to nystatin (53.5%), voriconazole (85.7%) and fluconazole (100.0%) although some isolates of *C. tropicalis* were 46.4% and 14.3% resistant to nystatin and voriconazole. *Candida parapsilosis* was 100.0% susceptible to nystatin and fluconazole while 82.5% were susceptible to voriconazole. *Candida akabanensis* was 100.0% susceptible to nystatin but 100.0% resistant to voriconazole and fluconazole.

Table 1: Frequency distribution of *Candida* species with respect to demographic characteristics of the women with contraceptive devices in northcentral Nigeria

Parameters	**Frequency of *Candida* isolation (%)**					
	Total	***Candida albicans***	***Candida tropicalis***	***Candida parapsilosis***	***Candida krusei* (*Pichia kudriavzevii*)**	***Candida* (*Nakaseomyces*) *glabrata***
Age group in years						
15-19	63	29 (46.0)	11 (17.5)	0	14 (22.2)	9 (14.3)
20-24	81	40 (49.4)	20 (24.7)	6 (7.4)	10 (12.3)	5 (6.2)
25-29	208	93 (44.7)	38 (18.3)	18 (8.7)	21 (10.1)	38 (18.3)
30-34	187	72 (38.5)	21 (11.2)	14 (7.5)	19 (10.2)	61 (32.6)
35-39	123	51 (41.5)	10 (8.1)	20 (16.3)	21 (17.1)	21 (17.1)
40-44	42	20 (47.6)	9 (21.4)	5 (11.9)	8 (19.0)	0
45-50	6	2 (33.3)	3 (50.0)	0	0	1 (16.7)
Contraceptive types						
IUCD	157	70 (44.6)	17 (10.8)	29 (18.5)	21 (13.4)	10 (6.4)
Injectables	252	76 (30.1)	64 (25.4)	33 (13.1)	15 (6.0)	64 (25.4)
Implants	96	45 (46.9)	11 (11.5)	0	24 (25.0)	16 (16.7)
Pills	205	116 (56.6)	10 (4.9)	1 (0.5)	33 (16.1)	45 (21.9)
Educational status						
Informal	69	30 (43.5)	21 (30.4)	0	0	18 (26.1)
Primary	195	89 (45.6)	38 (19.5)	11 (5.6)	15 (7.7)	42 (21.5)
Secondary	178	82 (46.1)	23 (12.9)	13 (7.3)	39 (21.9)	21 (11.8)
Tertiary	268	106 (39.6)	30 (11.2)	39 (14.5)	39 (14.5)	54 (20.2)
Marital status						
Single	64	14 (21.9)	11 (17.2)	11 (17.2)	12 (18.8)	16 (25.0)
Married	646	293 (45.4)	101 (15.6)	52 (8.0)	81 (12.5)	119 (18.4)
Total number of participants with VVC	**710**	**307 (43.2)**	**112 (15.8)**	**63 (8.9)**	**93 (13.1)**	**135 (19.0)**



Lanes 1-15: ITS bands at 500bp; lane L: 500bp molecular ladder

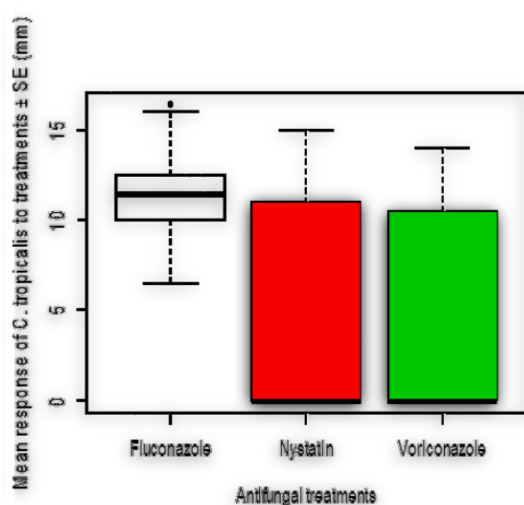
Fig 1. Agarose gel electrophoresis picture of amplified ITS bands of representative *Candida* isolates

Table 2: *In vitro* antifungal susceptibility and mean inhibitory response of the isolates

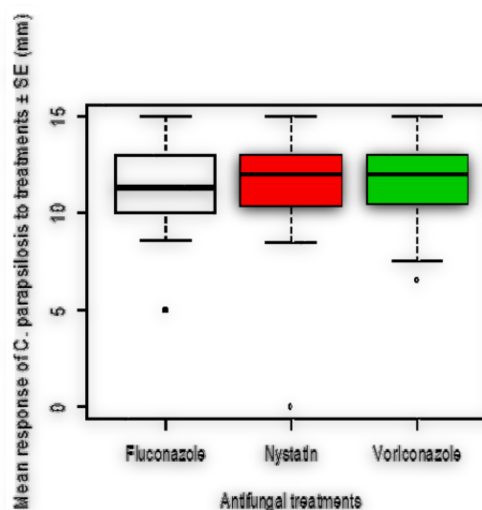
***Candida* species**	**Antifungal agents**	**No (%) of susceptible isolates**	**No (%) of intermediate isolates**	**No (%) of resistant isolates**	**Kruskal-Wallis (x^2)**
Candida albicans (n=307)	Nystatin	307 (100.0)	0	0	181.71
	Voriconazole	283 (92.1)	24 (7.8)	0	
	Fluconazole	218 (71.1)	0	89 (28.9)	
Candida (*Nakaseomyces*) *glabrata* (n=135)	Nystatin	135	0	0	9.091
	Voriconazole	135	0	0	
	Fluconazole	135	0	0	
Candida tropicalis (n=112)	Nystatin	60 (53.5)	0	52 (46.4)	229.52
	Voriconazole	96 (85.7)	0	16 (14.3)	
	Fluconazole	112 (100.0)	0	0	
Candida parapsilosis (n=63)	Nystatin	63 (100.0)	0	0	9.091
	Voriconazole	52 (82.5)	11 (17.5)	0	
	Fluconazole	63 (100.0)	0	0	
Candida akabanensis (n=93)	Nystatin	93 (100.0)	0	0	786.03
	Voriconazole	0	0	93 (100.0)	
	Fluconazole	0	0	93 (100.0)	

Fig 3 shows the mean diameters of zones of inhibition. Nystatin was the most effective antifungal agent against *C. albicans* while fluconazole was the least effective. Consequently, the mean inhibitory response of *C. albicans* to antifungal agents showed a very high significant difference (Kruskal-Wallis χ^2= 181.71, df=2, *p*<0.0001). The mean zones of inhibition revealed that nystatin was the most effective antifungal agent against *N. glabrata* (*C. glabrata)* followed by fluconazole and voriconazole respectively (Kruskal-Wallis χ^2=9.09 df=2, *p*=0.01061). The mean zone of inhibition showed that fluconazole was the most effective antifungal agent against *C. tropicalis* while voriconazole was the least effective (Kruskal-Wallis χ^2=229.52, df=2, *p*<0.0001).

The mean zones of inhibition revealed that nystatin was the most effective antifungal agent against *C. parapsilosis,* thus, the mean inhibitory response of *C. parapsilosis* to antifungal agents showed a significant difference (Kruskal-Wallis χ^2=6.9564, df=2, *p*=0.0307). The mean zones of inhibition revealed that nystatin was the most effective antifungal agent against *C. akabanensis* whereas voriconazole and fluconazole were not effective. Therefore, there was a very high significant difference (Kruskal-Wallis χ^2=792.14, df=2, *p*<0.001) on the mean inhibitory response of *C. akabanensis* to antifungal agents.



(a)



(b)

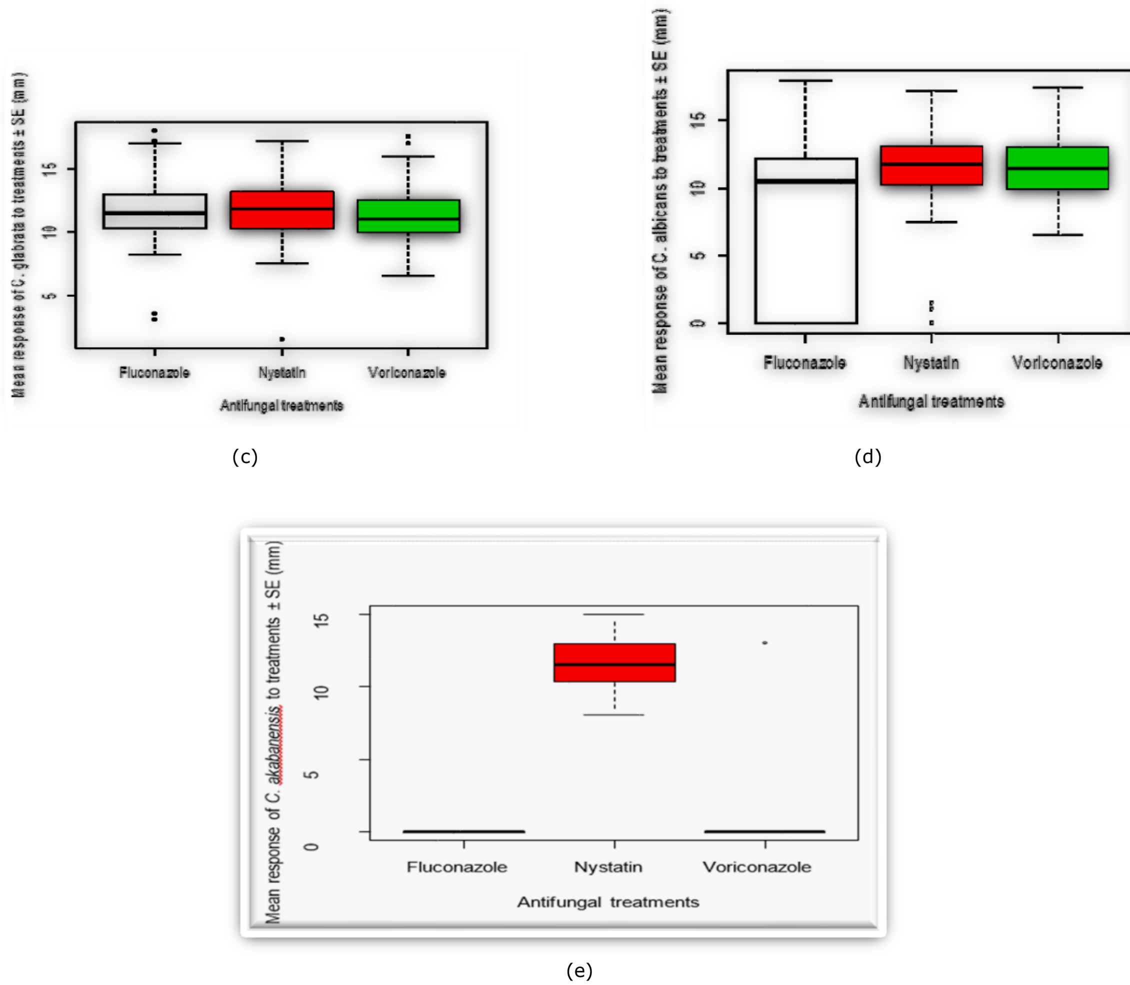


Fig 3: Mean inhibitory responses to antifungal agents of; (a) *Candida tropicalis*; (b) *Candida parapsilosis*; (c) *Nakaseomyces* (*Candida*) *glabrata*; (d) *Candida albicans*; (e) *Candida akabanensis*

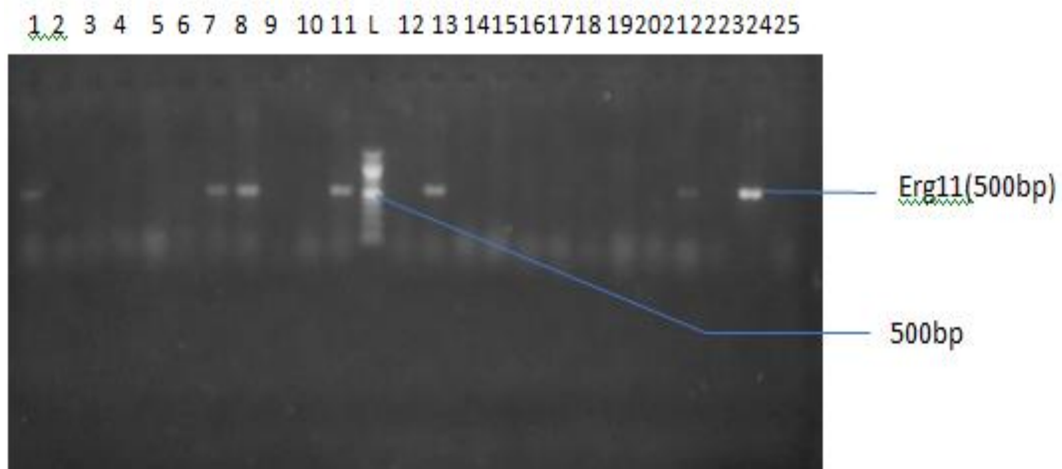


Fig 4: Agarose gel electrophoresis picture showing the bands of the amplified *Erg11* genes

Fig 5: Phylogenetic analysis showing the evolutionary distance between the *Candida* isolates

The resistant gene, *Erg11,* was identified in all the *Candida* species resistant to the antifungal agents tested (Fig 4). *Candida albicans, C. glabrata (N. glabrata) and C. akabanensis* depicted on lanes isolates 1,7,8,11,13, 22 and 24 carried the *Erg11* gene. Lane L represents the 500bp molecular ladder. Fig 5 shows the evolutionary distances of the *Candida* isolates expressed on a phylogenetic tree. The *Candida* isolates from this study are coded as H1 to H25 while the others are reference strains obtained from the GenBank.

Discussion:

Our study observed the diversity of *Candida* species among the study participants regardless of the contraceptive type used, age marital or educational status (demographic information). Contraceptive users are more prone to VVC. Younger women had a high distribution of *Candida* species which suggest the role of estrogen in contraceptives while menopausal (45-50 years) women had the least distribution of *Candida* species. Similarly, our results revealed a high frequency of diverse *Candida* species among users of oral contraceptive pills which has been reported to increase the glycogen content of the vagina thereby increasing the accessibility of sugars that enhance *Candida* replication (35,36). We hypothesized that the use of contraceptive devices modifies the vaginal mycobiome by decreasing bacterial diversity leading to phylogenetic diverse *Candida* species responsible for VVC.

Candida albicans was the most prominent species isolated from the women diagnosed with VVC followed by non-albicans isolates, *C. glabrata* (*N. glabrata*) (19.0%) and *C. tropicalis* (16.0%). Our study results are consistent with those of Amouri et al., (37) and Yassin et al., (38) who reported that *C. albicans* represented the predominant strain responsible for VVC. This frequency of *C. albicans* may be attributed to the role of estrogen which has been reported to decrease the ability of vaginal epithelial cells to inhibit the growth of *C. albicans* or its ability to exhibit unique virulence factors which elicits robust immunopathogenicity (39-43).

Of the non-albicans *Candida* (NAC) species, *C. glabrata* (*N. glabrata*) had the highest distribution which may be due to the genomic plasticity reported in *C. glabrata* (44,45). The loss and gain of relevant genes may be crucial for its adaptation in the vaginal microbiota. The high occurrence of *C. tropicalis* may

be due to its proficient biofilm production which promotes antifungal resistance and promotes the acquisition of genetic modification in the vaginal ecosystem (46,47). Furthermore, the transition from commensalism to pathogenicity of *Candida* species may result to dysbiosis of the microbiome. Similarly, fungal-bacterial interaction may result in the modulation of the vaginal ecosystem (48).

Phenotypically, 5 *Candida* species (*C. albicans*, *C. glabrata (N. glabrata)*, *C. tropicalis*, *C. parapsilosis* and *P. kudriavzevii*/*C. krusei*) were identified from contraceptive users. DNA sequencing confirmed the phenotypic classification of 4 *Candida* isolates using CHROMagar used in our study. However, the results of the DNA sequencing revealed that the previous classification of *P. kudriavzevii* (*C. krusei*) was misrepresented as the linear order of nucleotide bases in the DNA of the isolates and MegaBlast search revealed 100% similar sequence with *C. akabanensis* which buttress the relevance of molecular diagnosis. Historically, *C. albicans*, *C. glabrata* (*N. glabrata*), *C. tropicalis* and *C. parapsilosis* have been frequently identified in women with VVC (15,49). Interestingly, *C. akabanensis*, a non-pathogenic species with no history of VVC was identified. In our opinion, the use of contraceptive devices modified the vaginal mycobiome leading to this phylogenetic diverse species responsible for VVC among the study participants.

All the *Candida* species showed varied susceptibility patterns to the three antifungal drugs and this variation may be an indication that these drugs are still potent for the treatment of VVC. However, *C. albicans* recorded 28.9% resistance to fluconazole. This may be due to the role of hormonal contraceptives which have been reported to modulate *Candida* vaginal isolates biofilm formation and decrease their susceptibility to azoles. Our observation is similar to Ruchi et al., (50) who reported a higher resistance of 40.6% to fluconazole by *C. albicans*. The robust formation of biofilm by *C. tropicalis* may be responsible for increased resistance to nystatin and voriconazole.

Ergosterol (Erg) is a vital constituent of fungal cell membranes, consequently inhibition of Erg11 protein (cytochrome P450 lanosterol 14-α-demethylase) reduces cellular ergosterol and results in the accumulation of toxic methylated sterol intermediates in the cell membrane, thereby halting cell growth (51). *Candida albicans* expressed the resistant *Erg11* gene which is consistent with the findings of Caban et al., (52). The *Erg11* resistant gene was not expressed in *C. tropicalis* yet 46.4% and 14.3% resistance to nystatin and voriconazole were phenotypically observed. This may be due to the expression of other resistant genes which were not evaluated in this study. Similarly, *C. glabrata* (*N. glabrata*) demonstrated 100.0% susceptibility to the azoles phenotypically, nevertheless, *C. glabrata* (*N. glabrata*) isolates carried the *Erg11* gene and this phenomenon may be due to mutations or increased expression of *Erg11*. The study of Yang et al., (53) reported the carriage of *Erg11* resistant gene in *C. glabrata* (*N. glabrata*).

Candida akabanensis was 100% resistant to fluconazole and voriconazole. Although *C. akabanensis* is reported to be non-pathogenic, this *Candida* specie may employ the mechanism of horizontal gene transfer through natural genetic transformation with transfer of vital genes such as antifungal resistance or virulence genes, thereby constituting a public health concern. The phylogenetic analysis confirmed the evolutionary relationships among the *Candida* isolates examined.

Conclusion:

Women using contraceptive devices in Central Nigeria harbors phylogenetically diverse *Candida* species including *C. akabanensis* an uncommon cause of VVC. Of these *Candida* species, *C. albicans*, *C. tropicalis* and *C. akabanensis* were noted for multidrug drug resistance as well as harboring *Erg11* resistant gene. The susceptibility and resistance patterns of the *Candida* species to azole antifungal drugs observed in this study can guide the appropriate treatment protocol to be initiated.

Contributions of authors:

ALY prepared the manuscript, AC and NF reviewed the manuscript, ALY and AB carried out data collection and analysis. OA and RCR performed data analysis. All authors reviewed the results and approved the final version of the manuscript.

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