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Original Article **Open Access**

Antifungal resistance pattern of *Candida* species isolated from clinical specimens of patients in selected healthcare facilities in Yenagoa, Nigeria

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Abstract:

Background: *Candida* species are normal flora of humans that can be pathogenic and cause candidiasis. Candidiasis is a public health problem due to its high prevalence, mortality, and increased costs of care associated with serious systemic *Candida* infections. This study was designed to identify the antifungal resistance pattern of *Candida* species isolated from different clinical specimens in Yenagoa, Bayelsa State, Nigeria, and to detect resistant genes associated with the *Candida* species.
Methodology: A total of 500 patients from 5 different health facilities in Yenagoa, Bayelsa State, Nigeria, with clinical features of urinary tract infection (candiduria), cutaneous candidiasis, oral thrush, lower respiratory tract infection and HIV/TB were selected in the study. Urine, skin, sputum and mouth samples were obtained from the patients and cultured on Sabouraud Dextrose agar and Brilliance *Candida* agar. The isolates were phenotypically identified using standard mycological methods and genotypically confirmed by polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of the rRNA genes and sequencing. The semi-solid agar antifungal susceptibility testing method was performed on the isolates against commonly used antimycotic agents. The known antifungal resistance gene *Erg11* gene, was amplified by PCR.
Results: From clinical samples obtained from a total of 500 patients, 170 clinical isolates of *Candida* species were identified, giving the prevalence of *Candida* isolation of 34.0% in the study. *Candida albicans* was the predominant species (64.70%, n=110), followed by *Pichia kudriavzevii* (16.47%, n=28), *Nakaseomyces glabratus* (12.35%, n=21), *Candida akabanensis* (5.29%, n=9) and *Candida tropicalis* (1.17%, n=2). Female patients were more prone to *Candida* infection (36.6%, n=102/279) and age group 28-37 years (40.2%, n=41/102) had the highest prevalence of *Candida* species, but no statistically significant difference in *Candida* prevalence with respect to sex (p=0.2069) and age (p=0.1743). The three antimycotic agents to which *Candida* isolates exhibited the highest rates of resistance were griseofulvin (61.8%, n=105), ketoconazole (52.9%, n=90), and itraconazole (51.8%, n=88). Less than half of the isolates were resistant to fluconazole (n=70, 41.2%) and terbinafine (n=62, 36.5%). Nystatin had the lowest rates of resistance (19.4%, n=33). *Erg II* gene was detected in *P. kudriavzevii*, *C. albicans*, and *C. tropicalis* isolates.
Conclusion: Increased antifungal resistance by *Candida* species is a public health problem that can lead to failure in treatment, spread of infection and possibly death especially, in immune compromised individuals**.** *Erg* 11 genes contribute to *Candida* species resistance to antifungal drugs

Keywords: *Candida;* Normal flora; Antifungal agents; Anti-fungal resistance gene; PCR

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Profil de résistance aux antifongiques des espèces de *Candida* isolées à partir d'échantillons cliniques de patients dans certains établissements de santé à Yenagoa, au Nigéria

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Résumé:

Contexte: Les espèces de *Candida* constituent la flore normale de l'homme et peuvent être pathogènes et provoquer des candidoses. La candidose est un problème de santé publique en raison de sa prévalence élevée, de sa mortalité et des coûts accrus des soins associés aux infections systémiques graves à *Candida*. Cette étude a été conçue pour identifier le profil de résistance antifongique des espèces de Candida isolées à partir de différents échantillons cliniques à Yenagoa, dans l'État de Bayelsa, au Nigéria, et pour détecter les gènes de résistance associés aux espèces de *Candida*.
Méthodologie: Un total de 500 patients provenant de 5 établissements de santé différents à Yenagoa, dans l'État de Bayelsa, au Nigéria, présentant des signes cliniques d'infection des voies urinaires (candidurie), de candidose cutanée, de muguet buccal, d'infection des voies respiratoires inférieures et de VIH/TB ont été sélectionnés dans l'étude. Des échantillons d'urine, de peau, d'expectorations et de bouche ont été prélevés sur les patients et cultivés sur gélose Sabouraud Dextrose et sur gélose Brilliance *Candida*. Français Les isolats ont été identifiés phénotypiquement à l'aide de méthodes mycologiques standard et confirmés génotypiquement par amplification par réaction en chaîne par polymérase (PCR) de la région de l'espaceur interne transcrit (ITS) des gènes d'ARNr et séquençage. La méthode de test de sensibilité antifongique en gélose semi-solide a été réalisée sur les isolats contre des agents antimycosiques couramment utilisés. Le gène de résistance antifongique connu Erg11 a été amplifié par PCR.
Résultats: À partir d'échantillons cliniques obtenus auprès d'un total de 500 patients, 170 isolats cliniques d'espèces de *Candida* ont été identifiés, ce qui donne une prévalence d'isolement de *Candida* de 34,0 % dans l'étude. Français *Candida albicans* était l'espèce prédominante (64,7%, n=110), suivie de *Pichia kudriavzevii* (16,5%, n=28), *Nakaseomyces glabratus* (12,4%, n=21), *Candida akabanensis* (5,3%, n=9) et *Candida tropicalis* (1,2%, n=2). Les patientes étaient plus sujettes à l'infection à *Candida* (36,6%, n=102/279) et la tranche d'âge 28-37 ans (40,2%, n=41/102) présentait la prévalence la plus élevée d'espèces de *Candida*, mais aucune différence statistiquement significative dans la prévalence de *Candida* en fonction du sexe (p=0,2069) et de l'âge (p=0,1743). Les trois agents antimycosiques auxquels les isolats de *Candida* présentaient les taux de résistance les plus élevés étaient la griséofulvine (61,8%, n=105), le kétoconazole (52,9%, n=90) et l'itraconazole (51,8%, n=88). Moins de la moitié des isolats étaient résistants au fluconazole (41,2%, n=70) et à la terbinafine (36,5%, n=62). La nystatine présentait les taux de résistance les plus faibles (19,4%, n=33). Le gène *Erg* II a été détecté chez les isolats de *P. kudriavzevii, C. albicans* et *C. tropicalis*.
Conclusion: L'augmentation de la résistance antifongique des espèces de *Candida* est un problème de santé publique pouvant entraîner l'échec du traitement, la propagation de l'infection et potentiellement le décès, en particulier chez les personnes immunodéprimées. Les gènes *Erg* 11 contribuent à la résistance des espèces de *Candida* aux antifongiques.

Mots-clés: *Candida*; Flore normale; Agents antifongiques; Gène de résistance aux antifongiques; PCR

Introduction:

Twenty of the more than 150 *Candida* species are known to infect humans. Ignacio et al., (1) reported that adults and paediatric patients are mostly infected by *Candida albicans*, the fungus responsible for candidiasis. Among all types of sepsis, including those caused by fungus and bacteria, *C. albicans* has the highest death rate, at over 40% in the USA alone (2). Due to their rising prevalence as the cause of candidiasis, non-*albicans Candida* species like *Nakaseomyces glabratus* (formerly *Candida glabratus*), *Candida tropicalis, Candida parapsilosis, Candida kefyr, Candida famata, Candida auris*, and others have gained clinical significance (1).

Antimycotic drugs used for treatment of diseases caused by *Candida* species include polyenes, echinocandins, pyrimidine analogs and azoles (3). Azoles work by blocking the enzyme, lanosterol 14-demethylase (encoded by *Erg11*p gene), which is responsible for converting lanosterol to ergosterol (4). Echinocandins function by inhibiting β-1-3-glucan synthase, encoded by the *fks1* and *fks2* genes (5). Polyene binds to the sterol and causes permeability change in the membrane and disrupts membrane potential. They create transmembrane channels, which then act as an exit for the cell constituents such as Na^+ ions and K^+ until lyses of takes place. As a result of the low solubility and high toxicity to host, polyene use is limited (1,5). Flucytosine (5FC) interferes with pyrimidine metabolism thus inhibiting synthesis of DNA and RNA (5).

In recent years, the emergence of resistant strains to antimycotic agents, including strains resistant to multiple drugs, has been increasingly published (6,7). Also, it has been shown that phenotypes resistant to antibiotics can arise throughout an infection (8). Antifungal resistance is a major global public health issue that threatens the wellbeing of both human, crops, and the environment (9). As a result, antifungal resistance can lead to treatment failures, prolong illnesses and increased mortality (10). Antifungal resistance is also associated with increased healthcare costs for patients, healthcare system, and the country as a whole (11).

The objectives of this study are to determine the species composition and relative frequencies of *Candida* isolates recovered from

clinical specimens of patients with suspected fungi infections attending selected healthcare facilities in Yenagoa, Bayelsa State, Nigeria, and to evaluate their susceptibility profiles against commonly used antifungal agents.

Materials and method:

Study area:

The research was carried out in selected health facilities in Yenagoa, Bayelsa State, Nigeria from 2021 to 2023. The health facilities were; (i) Tuberculosis, Buruli Ulcer and Leprosy referral Center, (ii) Diete-Koki Memorial hospital, (iii) Niger Delta University Teaching Hospital, (iv) Federal Medical Center, and (v) Primary Health Center.

Ethical consideration:

Ethical approval with reference number BSHREC/Vol.1/21/12/005 was obtained from the Bayelsa State Ministry of Health, Nigeria. Informed consent was obtained from each study participant.

Study participants, sample size and method of sampling:

The study participants were symptomatic patients for suspected clinical infections including cough, chest pain, fever, frequent urination, skin rash, vaginal itching, weight loss and white patches on the tongue, with high suspicion for *Candida* infections. Sample size was determined using Yamane's formula as described by Adzani et al., (12); $n=N/(K+N(e)^2$, where 'n' is the calculated sample size, 'N' is the population of study, 'K' is a constant (1), and 'e' is the degree of error expected. This gave a sample size of 500.

Participants were selected by simple random sampling from the five health facilities; Tuberculosis, Buruli Ulcer and Leprosy referral Center (n=300), Diete-Koki Memorial hospital (n=100), Niger Delta University Teaching Hospital (n=30), Federal Medical Center (n=20), and Primary Health Center (n=50).

Specimen collection:

Specimens were collected from oral cavity, skin surface and urinary tract using appropriate techniques. Oral specimens were obtained using sterile swabs. Briefly, the inner cheek, the gum and the tongue surface were swabbed with a sterile cotton swab following the protocol described by Meagan et al., (13). For sputum collection, participants were asked to cough deeply into a wide-necked sterile container, early in the morning before the mouth was washed (14).

Skin specimens were collected in accordance with the technique outlined by the Center for Disease Control and Prevention (15). The soft end of the swab was firmly rubbed 3-5 times at the collection site. While swabbing the groin, the swab was rubbed over the surface of the left and right groin five times per hip crease, focusing on the inguinal crease, the skin fold that separates the leg and pelvis.

Clean catch early morning midstream urine sample was collected for microbiological analysis. In the case of females, participants were asked to to wash their hands and clean the urethral opening area, allowing it to dry before urine collection with the labia held apart. Samples were placed in leak-proof plastic bags were immediately transported to the laboratory for analysis. However, when there is a delay, samples were refrigerated at 4-6^{0}C.

Isolation and phenotypic identification of *Candida* species:

The clinical samples were inoculated on Sabouraud Dextrose agar, incubated at 37^{0}C and examined for 24-72 hours. *Candida albicans* appeared white, creamy, soft, smooth, and wrinkled colonies. *Nakaseomyces glabratus* were small, shiny, round, smooth, and creamy-colored aromatic colonies. *Pichia kudriavzevii* on the other hand, were off-white, dull, and creamy colonies, with a rough surface and slightly raised with the production of wet, mucoid appearance. *Candida tropicalis* appeared white to a creamy, shiny, texture, and a smooth, domed appearance. *Candida akabanensis* appeared as white to cream, soft, smooth and glistening appearance.

Isolated *Candida* species were further sub-cultured on SDA to obtain pure colonies. Phenotypic identification was based on microscopy using KOH mount, Gram staining, Germ tube test and chromogenic media (brilliance *Candida* agar). KOH mount revealed budding yeast cells, pseudo-hyphae and hyphae. Microscopic examination revealed Gram positive cells that were purple colored while cells positive for Germ tube test showed filamentous outgrowth.

On brilliance *Candida* agar, *C. albicans* appeared green in colour, and *C. tropicalis, N. glabratus,* and *P. kudriavzevii* appeared dark blue, beige/brown/yellow and brown or pink colors respectively. *Candida akabanensis* was identified genotypically.

Genotypic identification of *Candida* species:

Genotypic identification to confirm the isolated *Candida* was done by PCR amplification of the internal transcribed spacer (ITS) region of the 16S rRNA gene, followed by sequencing.

DNA extraction:

Genomic DNA was extracted using the Inqaba, South Africa's ZR fungal/bacterial DNA mini-prep extraction kit. The pure culture of the *Candida* isolates was suspended during rapid growth in a ZR Bashing Bead Lysis tube with 200µl of isotonic buffer, and then 750µl of lysis solution was added to the combination.

For 5 minutes at full speed, the tubes were treated in a bead beater with a 2ml tube holder attachment. The tube with the ZR smashing bead lysis was spun at 10,000xg for 1 minute. Following its passage into a collecting tube containing 400µl of supernatant, the liquid in question was subjected to one-minute spinning at 10,000 rpm using an orange-topped Zymo-Spin IIIF spin filter. To make the filtrate volume in the collecting tubes 1600µl, 1200µl of fungal/bacterial DNA binding buffer was added. Later on, 800µl was moved to a Zymo-Spin IIC column in an independent collecting tube and spun at 10,000xg for one minute. Afterwards, the collecting tube had its flow through removed.

The DNA pre-wash buffer and Zymo-spin IIC were mixed in a new collection tube using 200µl of each. Then, for one minute, the mixture was spun at 10,000g. Prior to centrifugation at 10,000xg for 1 minute, 500µl of fungal/bacterial DNA Wash Buffer was added to the tube. Next, 100 µl of DNA elution buffer was added to the column matrix, and the Zymo-spin IIC column was centrifuged at 10,000g for 30 seconds. The column was then transferred to a fresh 1.50 µl centrifuge tube. Subsequent procedures requiring ultra-pure DNA were then executed and stored at -20^{o}C.

DNA quantification:

The amount of genomic DNA that was extracted was measured using a Nanodrop 1000 spectrophotometer. The software for the device was activated by double-clicking the Nanodrop icon. The device was initialized with two milliliters of sterile distilled water and blanked out with normal saline. After depositing two microliters of DNA extract onto the base pedestal, the top pedestal was lowered until it made contact with the DNA. The DNA concentration was quantified by pressing the "measure" button.

PCR amplification of ITS region of rRNA genes:

The ITS region of the rRNA genes of the isolates was amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5' TCCTCCGCTTATTGATATGC-3, primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50µl for 35 cycles. In the PCR mix, the template was extracted DNA, and the primers were X2 Dream Taq Master mix (Taq polymerase, dNTPs, $MgCl_2$) from Inqaba, South Africa, with a concentration of 0.4M.

The following were the PCR reaction cycle; denaturation at 95^{o}C for 5 mins, and continues for another 30 seconds, annealing at 53^{o}C for 30 seconds, and extension at 72^{o}C for 30 seconds, for 35 cycles, with final extension at 72^{o}C for 5 minutes. The 550 bp product was visualized using a blue light transilluminator after 15 minutes of resolution on 1% agarose gel at 200V.

Ribosomal RNA sequencing:

Sequencing of the ITS region of the rRNA gene amplicon was performed by Inqaba Biotechnological of Pretoria, South Africa in a 3510 ABI sequencer that was outfitted with the BigDye Terminator kit for the sequencing process. The following components were used in the sequencing process; 10 µl of BigDye® terminator v1.1/v3.1, 2.25 ul of 5 x BigDye sequencing buffer, 10 µM of primer PCR, and 2-10 ng of PCR template per 100 bp. Sequencing was performed under the following conditions; 32 cycles of once at 96°C for 10 sec, once at 55°C for 5 sec, and once at 60°C for 4 minutes.

The sequences were edited using the bioinformatics algorithm Trace Edit. This was used for base calling and sequence trimming after which the clean sequences were copied to the NCBI domain to check for sequences of organisms with the highest percentage similarity. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (16).

PCR amplification of *Erg 11* gene:

The *Erg* gene fragment was amplified using ErgF: 5'-GTAGAAATACTGAACGTCCGATAA-3' and ErgR: 5'-CCAATTCCACATTGTTTCGGTCTAA-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30µl for 35 cycles. The X2 Dream Taq Master mix (Taq polymerase, dNTPs, $MgCl_2$) supplied by Inqaba Biotec, South Africa, was used, with the extracted DNA as a template, and primers added at a concentration of 0.5µM to the PCR mix.

The following PCR conditions were applied; denaturation start at 95^{o}C for 5 sec followed by additional 30 sec at 95°C, annealing at 56°C for 30 seconds, and extension at 72^{o}C for 30 sec, for 35 cycles, with final extension at 72°C for 5 minutes. After 30 minutes of resolution on 1% agarose gel at 130 V, the gel was visualized in blue light transilluminator.

Antifungal susceptibility test:

Candida isolates were tested for their *in vitro* susceptibility to the azoles (fluconazole, itraconazole, and ketoconazole), allylamines (terbinafine), polyene (nystatin), and polyketide metabolite (griseofulvin). The isolates were suspended in 5ml sterile water, and the turbidity was adjusted to 0.5 McFarland standards. Antifungal susceptibility of *Candida* species isolates was tested using semi-solid agar antifungal susceptibility (SAAS) testing method. In this method, Brain Heart infusion broth (BHIB) with 0.5% Brain Heart infusion agar (BHIA) was used. The drugs were dissol-

ved either in water or dimethyl sulphoxide (DMSO). Five milliliters of semi-solid agar were transferred to different test tubes. Two-fold serial dilutions of the drugs were made in the different test tubes with semi-solid agar. Drug free media were prepared as positive control. The drug and drug free media were inoculated with a standard platinum loopful (0.001ml) of inoculum suspension by inserting the loop deep within the semisolid agar. The tubes were incubated at 35^0C for 24-48 hours (17).

The standard protocol described by the Clinical and Laboratory Standards Institute (18) was used to determine the minimum inhibitory concentration (MICs) of the anti-fungi agents against the *Candida* isolates. The interpretive breakpoints for sensitivity were as follows; fluconazole ≤8µg/ml; itraconazole ≤ 0.125µg/ml; ketoconazole ≤0.125µg/ml; nystatin ≤4µg/ml; terbinafine ≤4µg/ml; and griseofulvin ≤4µg/ml (18).

Statistical analysis:

Data were analyzed using SPSS Windows version 15.0. Descriptive statistics were used to express the data in percentages. Association of *Candida* prevalence and distribution with socio-demographic and other variables was determined by Chi square test, with statistical significance set at $p \leq 0.05$.

Results:

Prevalence distribution of *Candida* isolation from participants by gender and age:

Of the total 500 selected participants from the healthcare facilities, 221 (44.2%) were males and 279 (55.8%) females. *Candida* species were isolated from 170 (34.0%); 68 of 221 (30.8%) males, and 102 of 279 (36.6%) females. The age range of the participants was between 18-67 years. The age group 28–37 years had the highest *Candida* isolation rate (40.2%, 41/102), followed by age groups 48–57 (37.9%, 36/ 95), age group 58-67 (34.7%, 33/95), and age group 38–47 (33.0%, 33/100). The age group 18–27 years had the lowest prevalence, with isolation rate of 25.0% (27/108) (Table 1).

Prevalence distribution of *Candida* isolates by health facilities:

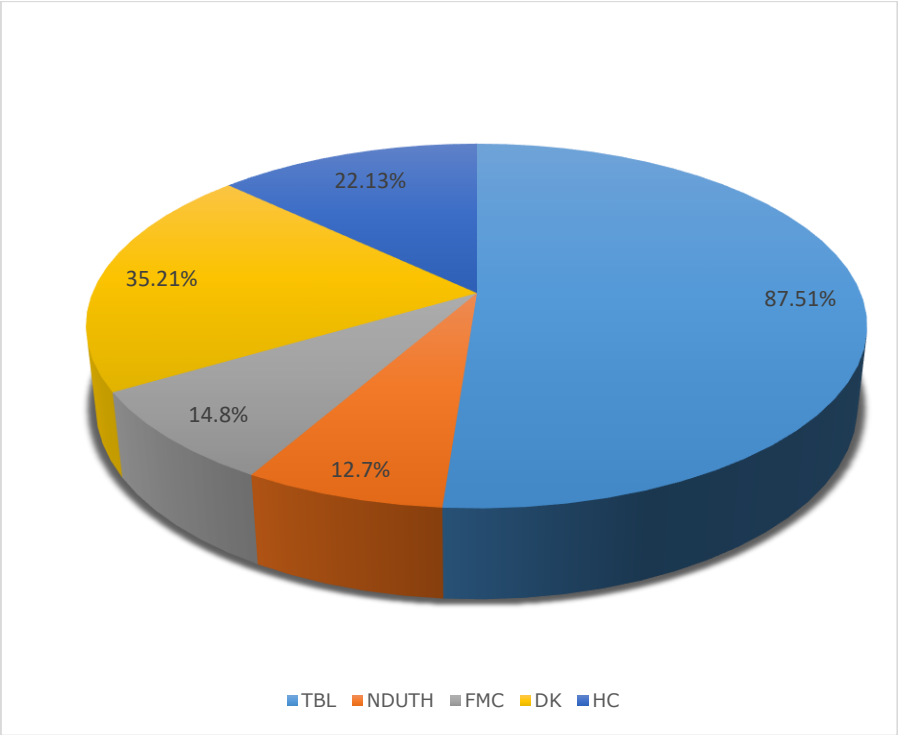
A total of 170 clinical isolates were recovered from the 500 selected patients giving a *Candida* prevalence of 34.0%. These include 110 *C. albicans,* 28 *P. kudriavzevii*, 21 *N. glabratus*, 9 *C. akabanensis* and 2 *C. tropicalis*. Of the 170 *Candida* isolates, 87 (29.0%) were from samples of 300 patients attending the Tuberculosis Buruli Ulcer and Leprosy Referral Hospital in Igbogene, 35 (35.0%) were from samples of 100 patients attending Diete-Koki Hospital in Opolo, 22 (44.0%) were from samples of 50 patients attending Primary Health Center, 14 (70.0%) were from samples of 20 patients attending Federal Medical Centre, and 12 (40.0%) were from samples of 30 patients attending Niger Delta University Teaching Hospital (Fig 1).

There was a statistically significant difference (x^2=17.647, p=0.0014) in *Candida* isolation rate between the health facilities, with the highest isolation rate of 70.0% in Primary Health Center and lowest isolation rate of 29.0% in Tuberculosis Buruli Ulcer and Leprosy Referral Hospital.

Table 1: Prevalence distribution of *Candida* isolation among participants with respect to sex and age

Variable	Total number examined	No with *Candida* isolated (%)	No without *Candida* isolated (%)	x^2	*p* value
Sex					
Male	221	68 (30.8)	153 (69.2)	1.593	0.2069^{ns}
Female	279	102 (36.6)	177 (63.4)		
Age group (years)					
18-27	108	27 (25.0)	81 (75.0)	6.353	0.1743^{ns}
28-37	102	41 (40.2)	61 (59.8)		
38-47	100	33 (33.0)	67 (67.0)		
48-57	95	36 (37.9)	59 (62.1)		
58-67	95	33 (34.7)	62 (65.3)		
Total	**500**	**170 (34.0)**	**330 (66.0)**		

ns = not statistically significant



TBL: Tuberculosis Buruli Ulcer and Leprosy Referral Hospital, NDUTH: Niger Delta University Teaching Hospital, FMC: Federal Medical Centre, DK: Diete-Koki Memorial Hospital, HC: Primary Health Centre

Fig. 1: Distribution of *Candida* isolates by health facilities

Frequency of *Candida* species isolated from patients:

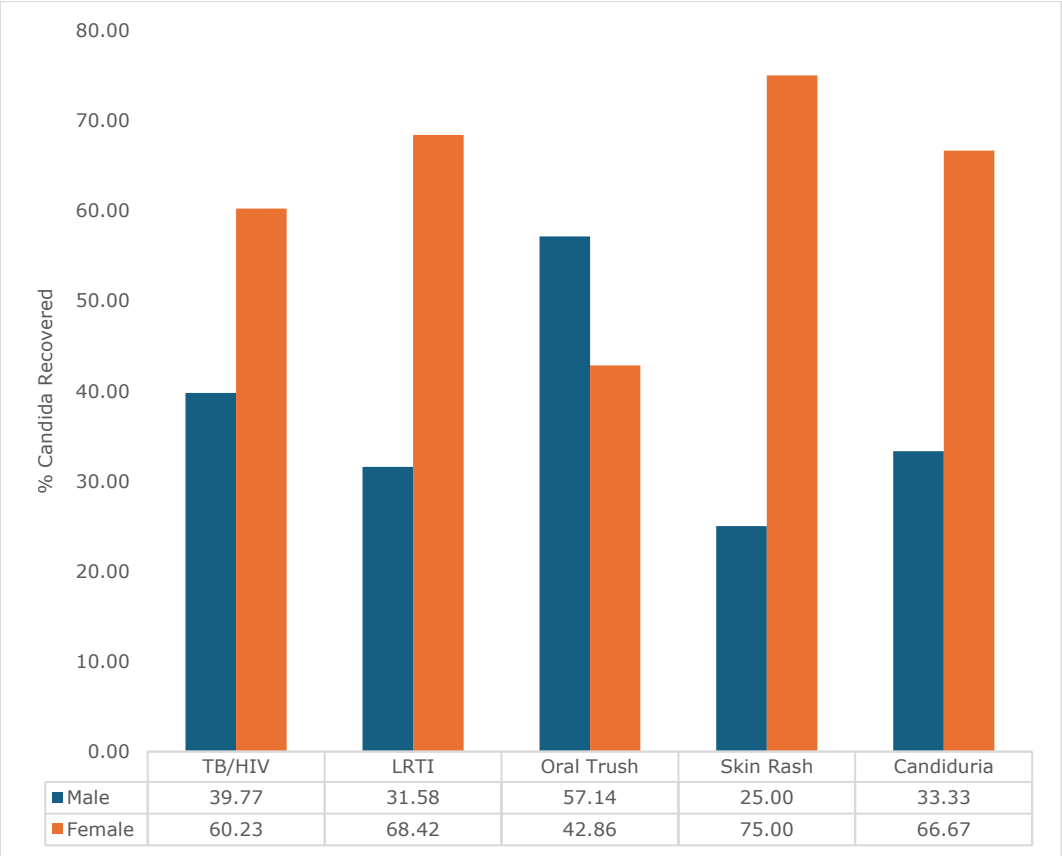
Out of 170 *Candida* species isolated clinical samples, *Candida albicans* was the most frequent, with 110 isolates (64.7%). Of these, 95 (86.4%) were isolated from sputum, 12 (10.9%) from urine, and 3 (2.7%) from skin. From the mouth, 28 species of *Pichia kudriavzevii* were identified, accounting for 16.5% of the total isolates, 21 (13.4%) and 9 (5.3%) were *Nakaseomyces glabratus* and *Candida akabanensis* from sputum respectively. *Candida tropicalis* was the least recovered, with only 2 isolates (1.17%); 1 (50.0%) isolated from the skin and 1 (50.0%) from sputum (Table 2).

Distribution of *Candida* species based on clinical conditions of patients:

A total of 88 *Candida* isolates were isolated from patients with HIV and TB diagnosis, with 35 (39.8%) in males and 53 (60.2%) in females. A total of 38 *Candida* isolates (26 from females and 12 from males) were recovered from individuals with lower respiratory tract infections. Patients with oral infections had 28 *Candida* isolates recovered from them, male and 12 from females. Twelve *Candida* isolates were recovered from individuals with candiduria; 4 from in males and 8 from females. Four species of *Candida* were identified from patients with skin rash, three of whom were females and one a male (Fig 2).

Table 2: Frequency of *Candida* species isolated from samples of patients

Sample type	**Clinical isolates (%)**				
	C. albicans	***N. glabratus***	***C. tropicalis***	***C. akabanensis***	***P. kudriavzevii***
Mouth	-	-	-	-	28 (100.0)
Sputum	95 (86.4)	21 (100.0)	1 (50.0)	9 (100.0)	-
Urine	12 (8.7)	-	-	-	-
Skin	3 (2.2)	-	1 (50.0)	-	-
Total	**110 (64.7)**	**21 (12.4)**	**2 (1.2)**	**9 (5.3)**	**28 (16.5)**



	TB/HIV	LRTI	Oral Trush	Skin Rash	Candiduria
Male	39.77	31.58	57.14	25.00	33.33
Female	60.23	68.42	42.86	75.00	66.67

TB/HIV: Tuberculosis/Human Immunodeficiency Virus; LRTI: Lower Respiratory Tract infection; Candiduria: Urinary Tract infection.

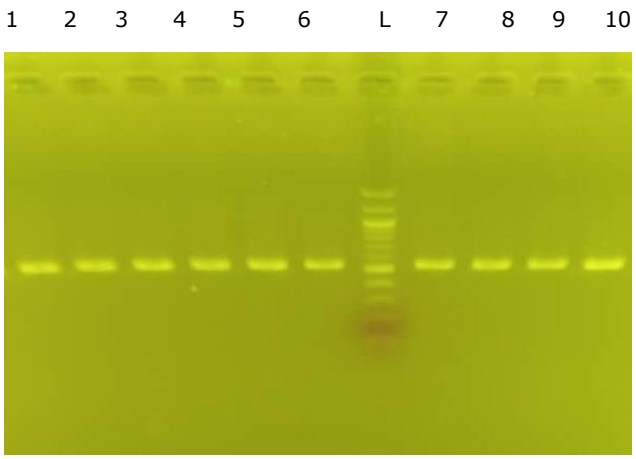
Fig 2: Recovery of *Candida* species from clinical conditions

ITS 16S rRNA amplification and sequencing of *Candida* isolates:

The 550 bp product of the PCR amplified ITS rRNA region of representative *Candida* isolates is shown in Fig 3. A Megablast search for remarkably similar ITS sequences from the National Centre for Biotechnology Information' (NCBI) database showed perfect match with the ITS sequences obtained from the *Candida* isolates as shown in the phylogenetic tree in Fig 4.

Detection of *Erg II* gene amplicon:

The 400 bp PCR amplicon of *Erg11* gene for *P. kudriavzevii*, *C. albicans*, and *C. tropicalis* isolates is shown in Fig 5.



Lanes 1-10 represent the ITS bands at 500bp while lane L represents the 100bp molecular ladder.

Fig. 3: Agarose gel electrophoresis showing the amplified ITS fragment of 16S rRNA gene of representative *Candida* isolates

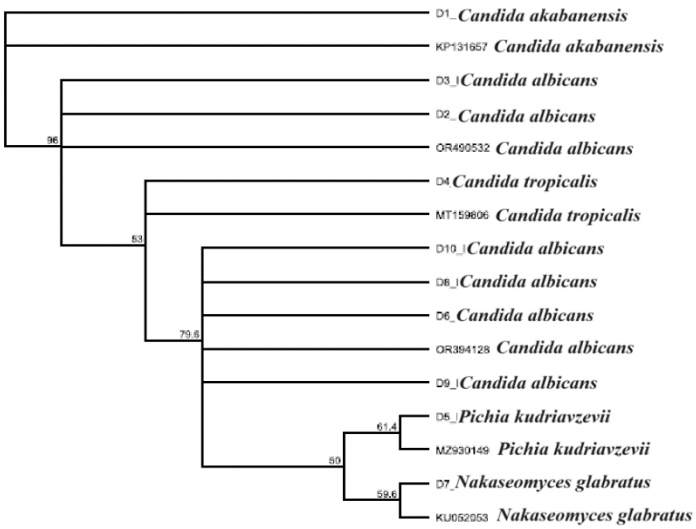
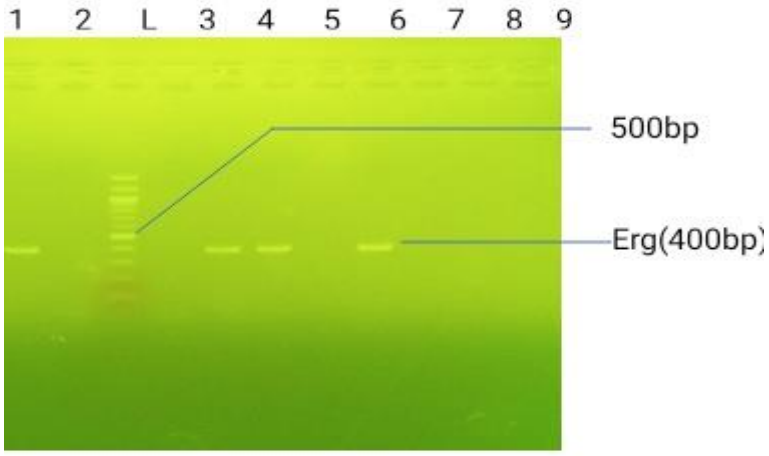


Fig.4: Phylogenetic tree showing the evolutionary distance between the fungal isolates



Lanes 1, 4, 5 and 7 represent the *Erg11* genes bands at 400bp while lane L represents the 100bp molecular ladder.

Fig. 5: Agarose gel electrophoresis showing the amplified *Erg11* gene of some *Candida* isolates

Antifungal susceptibility profiles of isolated *Candida species:*

Table 3 shows the results of *in vitro* antifungal susceptibility testing of the *Candida* isolates to six antifungal agents. Of the 110 *C. albicans* tested, 69 (62.7%) were susceptible to fluconazole, 51 (46.4%) to itraconazole, 50 (45.5%) to ketoconazole, 86 (78.18%) to nystatin, and 72 (65.5%) to terbinafine. Isolates were more resistant to griseofulvin (71.8%, n=79), with only 31 (28.8%) isolates susceptible.

Fifteen (71.4%) of 21 *N. glabratus* were sensitive to fluconazole, and 18 (85.7%) to nystatin. Resistance was high for the other drugs, for example, 12 (57.1%) isolates were resistant to itraconazole, 14 (66.7%) to terbinafine, 15 (71.4%) to griseofulvin, and 11 (52.3%) to ketoconazole. Five of 9 (55.6%) *C. akabanensis* isolates were sensitive to fluconazole and all 9 (100.0%) isolates were susceptible to nystatin, terbinafine and itraconazole. Griseofulvin and ketoconazole showed less activity, with percentage susceptibility of 44.4% and 33.3% respectively.

All 2 *C. tropicalis* isolates were susceptible (100.0%) to terbinafine and nystatin, but resistant to itraconazole. On the other hand, 50.0% of the isolates were resistant to fluconazole, ketoconazole and griseofulvin. *Pichia kudriavzevii* isolates were more susceptible to nystatin (n=22, 78.6%), followed by terbinafine (n=18, 64.3%), and itraconazole (n=15, 53.5%). However, resistance to other antifungal agents was high, for example, 23 (82.1%) of the 28 isolates were resistant to griseofulvin, 16 (57.14%) to ketoconazole, and 18 (64.3%) to fluconazole.

Table 3: *In vitro* susceptibility of clinical *Candida* isolates to selected antifungal agents

Antifungal agent		*C. albicans* (n=110)	*N. glabratus* (n=21)	*C. tropicalis* (n=2)	*C. akabanensis* (n=9)	*P. kudriavzevii* (n=28)	Total isolates (n=170)
FLU	S	69 (62.7)	15 (71.4)	1 (50.0)	5 (55.6)	10 (35.7)	100 (58.8)
	R	41 (37.3)	6 (28.5)	1 (50.0)	4 (44.4)	18 (64.3)	70 (41.2)
ITR	S	51 (46.4)	9 (42.9)	0	9 (100.0)	13 (46.4)	82 (48.2)
	R	59 (53.6)	12 (57.1)	2 (100.0)	0	15 (53.6)	88 (51.8)
KET	S	50 (45.5)	10 (47.6)	1 (50.0)	3 (33.3)	16 (57.1)	80 (47.1)
	R	60 (54.5)	11 (52.4)	1 (50.0)	6 (66.7)	12 (42.9)	90 (52.9)
TER	S	72 (65.5)	7 (33.3)	2 (100.0)	9 (100.0)	18 (64.3)	108 (63.5)
	R	38 (34.5)	14 (66.7)	0	0	10 (35.7)	62 (36.5)
NYS	S	86 (78.2)	18 (85.7)	2 (100.0)	9 (100.0)	22 (78.6)	137 (80.6)
	R	24 (21.8)	3 (14.3)	0	0	6 (21.4)	33 (19.4)
GRI	S	31 (28.2)	6 (28.6)	1 (50.0)	4 (44.4)	23 (82.1)	65 (38.2)
	R	79 (71.8)	15 (71.4)	1 (50.0)	5 (55.6)	5 (17.9)	105 (61.8)

FLU= Fluconazole, ITR=Itraconazole, KET=Ketoconazole, TER=Terbinafine, NYS=Nystatin, GRI=Griseofulvin, S=Sensitive R=Resistance, N=Number of isolates tested.

Discussion:

The purpose of this study was to evaluate how isolated *Candida* species respond to common antimycotic drugs *in vitro*. Overall, the isolated *Candida* species were more sensitive to nystatin (80.6%), followed by terbinafine (63.5%), and fluconazole (58.8%) in the current study. This agrees with the earlier findings of Alkhars et al., (19) who reported 94.4% susceptibility of *Candida* species among mother-child dyads, and Kessler et al., (20). Terbinafine demonstrated remarkable *in vitro* efficacy against *C. albicans* and *C. tropicalis* in their studies. However, our study finding does not agree with findings of several studies. For example, Lawal et al., (21) reported that *Candida* species were more susceptible to ketoconazole, itraconazole, and nystatin. Mahnaz et al., (22) reported terbinafine resistance was highest in *Candida* species. This finding implies that when *Candida* species are resistant to azoles, nystatin and terbinafine may be used as an alternative to treat candidiasis in this setting.

The resistance patterns of *C. albicans* to the azoles showed that, except for fluconazole, which has a comparatively lower resistance rate of 37.3%, the isolates were highly resistant to ketoconazole (54.5%) and itraconazole (53.6%). The susceptibility patterns documented in earlier research by Berkow and Lockhart (23), which reported that fluconazole resistance in *C, albicans* was minimal, ranging from 0.5 to 2% among isolates from the United States of America, are corroborated by our findings but the resistance rate in our study surpasses theirs. Our study contrasts with a 10-year analysis of isolates from vulvovaginal infections by Sobel (24), which reported lower fluconazole resistance in *C. albicans* of 22.8%, despite variations in the annual trends of resistance. Fluconazole resistance, for instance, was 33.3% in 2017 and increased to 35.7% at the end of 2021, four years later, which is consistent with the results of the current study.

Fluconazole resistance was seen in 64.3% of *P. kudriavzevii* isolates tested in our study, ketoconazole resistance in 42.85%, and itraconazole resistance in 46.42%. These patterns of resistance exhibited by *P. kudriavzevii* isolates from Yenagoa, Nigeria to azoles are similar to those reported from other regions of the world. As reported by Seyoum et al., (25) and Chen et al., (26), *P. kudriavzevii* is thought to be naturally resistant to fluconazole, and this result is in line with those findings.

Low *in vitro* activity of the azoles; fluconazole (n=5, 55.6%) and ketoconazole (n=3, 33.3%) was seen in the majority of the *C. akabanensis* isolates in this study. This result agrees with the study by Adogo et al., (27), who reported that every isolated *C. akabanensis* from northcentral Nigeria was resistant to both fluconazole and voriconazole. Nevertheless, itraconazole was found to be effective against the *C. akabanensis* isolates as all (100.00%) the isolates were susceptible to it. As a result, itraconazole should be one of the preferred antimycotic for treating candidiasis that are identified to be *C. akabanensis.*

Our study found that one each of the two *C. tropicalis* (50.0%) isolates were resistant to fluconazole and ketoconazole. In comparison, all 2 (100%) were resistant to itraconazole. The high level of resistance to azoles among *C. tropicalis* is comparable to that reported by a study in Taiwan (28) but contrasted the low levels of fluconazole and voriconazole resistance reported by studies in Middle East (29), Italy and Spain (30). Emerging antifungal resistant isolates are primarily caused by prior and extended exposure to antifungal agents (31,32). Together with the potential connection between antibiotic prophylaxis and fluconazole-resistant strains (33), there is also the inference that either the host conditions could be caused by alternative pathways leading to resistance (34) or the azoles-resistant

strains were obtained from the hands, especially the contaminated hands of healthcare workers (35). On the other hand, a study conducted in Taiwan found that fruit-related azole-resistant *C. tropicalis* isolates grouped with the fluconazole non-susceptible blood isolates. This was observed at the same time that the country's use of fungicides in agricultural applications increased fourfold (28). Therefore, as previously reported by (28,36), we opined that the isolates in our study might have acquired azoles resistance from the environment.

None of the *C. akabanensis* and *C. tropicalis* isolates were resistant to terbinafine, however, *P. kudriavzevii* (35.7%, 10/28) and *C. albicans* (34.5%, 38/110) showed low resistance. Cross-resistance between terbinafine and azoles has already been reported by other researchers (37,38). Our study suggests that cross-resistance to the azoles may also be the cause of resistance observed in *P. kudriavzevii* and the high degree of resistance reported in the isolates of *N. glabratus*. Similar to the earlier study by Yassin et al., (39), terbinafine proved to be the most active *in vitro* against *C. akabanensis*, *C*. *tropicalis*, and *C. albicans* in our study, despite the high prevalence of multidrug-resistant *Candida* strains. As a result, before starting medical therapy, it is recommended that hospitals perform antifungal sensitivity testing.

Majority of the *Candida* species (61.8%) were resistant to griseofulvin, with *C. albicans* (71.8%, 79/110), *N. glabratus* (71.4%, 15/21), *C. akabanensis* (66.7%, 6/9), and *C. tropicalis* (50.0%, 1/2) being resistant, and 23 of 28 (82.1%) *P. kudriavzevii* were also resistant to this agent. This could be explained by the fact that griseofulvin is easily obtained over the counter and is commonly prescribed to treat dermatophytes (40), which makes it easy for people to misuse the prescription as a self-medication. However, a study indicates that a combination of azoles and griseofulvin might be effective in infection due to *Candida* species (41).

Our study show high susceptibility of *Candida* isolates to nystatin (80.6%), with *P. kudriavzevii* (78.6%, 22/28), *N. glabratus* (85.7%, 18/21), and *C. albicans* (78.2%, 86/110), but this high nystatin susceptibility is lower when compared with previous studies, such as De-la-Torre et al., (42) with 95.0%, Nguyen et al., (43) with 93.7% and Zida et al., (44) with 93.7%. These results also show that some strains of *Candida* can express resistance to nystatin (although at a relatively low percentage). Numerous researchers have reported the benefits of using nystatin to treat candidiasis (45,46,47). Although most of the isolates in our study exhibited low resistance to nystatin, it is noteworthy that all the isolated *C. akabanensis* (100.0%, 9/9) and *C. tropicalis* (100.0%, 2/2) were totally sensitive to nystatin. As a result, nystatin is considered an effective medication and is advised to be used either as a first-line treatment or as a "backup" treatment for candidiasis in Yenagoa.

Azoles inhibit ergosterol production pathway by blocking cytochrome P450-dependent enzyme, lanosterol 14-alpha-demethylase, encoded by *Erg11* in yeasts. Inhibiting this pathway impedes ergosterol synthesis, resulting in the accumulation of toxic sterol intermediates that undermine membrane integrity and block fungal growth. The modification of the target 14-alpha-demethylase constitutes a mechanism of azole resistance. These modifications result in diminished affinity of 14-alpha-demethylase for azoles or provoke conformational changes that hinder the antifungal agent access to the active site, hence azoles cannot perform their inhibitory role. The modifications arise from point mutations in the *Erg11* gene, which encodes 14-alpha-demethylase (48). Over expression of *Erg11* genes can occur as a result of gain-of-function mutation (49). Our study found that azoles resistance may have occurred via this mechanism as *Erg11* were detected by PCR in *P. kudriavzevii, C. albicans,* and *C. tropicalis* isolates.

Conclusion:

Though, considered normal flora of humans, *Candida* species can cause candidiasis in humans with compromised immunity. Increased antifungal resistance by *Candida* species is a public health challenge that can lead to failure of treatment, spread of resistant infections and potential mortality from such infections.

Erg11 genes detected in some *Candida* species in our study encode resistance to anti-fungal agents and can complicate treatment of candidiasis in the study setting. Nystatin is recommended as a first-line or as a backup treatment for candidiasis.

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Contribution of authors:

BD was involved in sample collection from medical centers and in laboratory bench work, CDO was involved in writing the manuscript and laboratory bench work, and ABA was the project supervisor and also analyzed data. All authors approved the manuscript submitted for publication.

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