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Molecular Characterization and Biofilm Formation of *Candida Species* Isolated from Immunosuppressed HIV Seropositive Individuals in Calabar Metropolis, Nigeria

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Abstract **- Immunocompromised status of host creates an enabling environment for candida biofilm forming organisms to strive. The study aims to speciate clinical isolates from immunosuppressed subjects, detects their biofilm forming ability and antifungal resistance. This study was a cross sectional prospective study which ran for a period of 8months from January, 2023 to August, 2023. Isolates used in this study were isolated from sputum and urine samples. Biofilm production was tested by the Congo Red Agar method and Microtitre Plate Method. Antifungal susceptibility was studied using Kirby-Bauer disk diffusion technique. Polymerase Chain Reaction (PCR) technique was used to detect internal transcribed spacer (ITS) gene. A total of 200 consenting subjects were enrolled for the study. Candidiasis was significantly more prevalent among females than males (P=0.015*). Out of 40 candida isolates tested for biofilm formation 21(52.5%) formed biofilm. *Candida albicans* was the most encountered species 16(40%) among subjects and 11(52.4%) of *Candida albicans* produced biofilm. There was a statistically significant association between *Candida* infection and the rate of biofilm production (P=0.016*). The degree of biofilm formation by Candida isolates significantly correlates negatively with host immune status (r = -0.309, p = 0.001*). Twenty (20) *Candida* isolates were randomly selected and examined for Internal Transcribed Spacer (ITS) gene sequence, *Candida albicans* had the highest expression of the ITS genes of 11(55%) followed by *C. tropicalis* with 5(25%). The antifungal agents tested were amphotericin B, fluconazole, nystatin, itraconazole and voriconazole. The range of susceptibility of the isolates to antifungal agents was 10-90%. Isolates of *C. dubliniensis* were more susceptible to fluconazole (90%) followed by voricanozole (65%). *C. glabrata* was notable for multidrug resistance to antifungal agents tested. *C. rugosa* and *C. akabanensis* are emerging Candida pathogens in the area mis-identified by conventional methods.**

Keywords: Biofilms production, Candida species Antifungal resistance, Immunosuppression.

1. Introduction

Immunosuppressed status of host creates an enabling environment for candida biofilm forming organisms to strive especially in those with predisposing risk factors such as hormonal imbalance, ill-treated diabetes, immunosuppressing infections, glucocorticoids and genetic predisposition, [24].

Biofilms are heterogeneous and dynamic microbial communities undergoing continuous transformation. They are groups of microbial cells embedded in an extracellular matrix (ECM) forming a complex three dimensional architecture on biotic and abiotic surfaces. Biofilms formation is a complicated process which starts with adhesion on an abiotic surface, a tissue or the air-liquid interface. It is a continuous process which undergoes different stages of development such as conditioning, adhesion, quorum sensing-induced extracellular matrix synthesis, maturation and dispersion. These phases lead to the formation of a uniform structure surrounded by a matrix of polymers, [5]. Candida species forming biofilms have different properties than those of planktonic cells, which confer great resistance to antifungal treatment which is a global public health challenge, [21]. Each Candida species (C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. nivariensis, C. dubliensis and C. auris) shows significant peculiarities in terms of biofilm formation, which result in different morphologies, extracellular matrix composition and resistant to antifungal agents,[31].

1.1 Statement of the Problem

Biofilms are protected niche for microorganisms and are also important in clinical infections, especially due to the high antibiotic resistance associated with them, [5]. The emergence of drug resistance in *Candida* pathogens producing biofilms has a profound impact on human health given limited number of antifungal agent. Treatment with immunosuppressive drugs and infection with human immunodeficiency virus (HIV) has increased *Candida* biofilm formation in patients. Biofilms potentiate the establishment of unyielding infections in the human host as is the case of biofilms formed by *Candida* species, causing superficial and systemic fungal infections in immunosuppressed patients, [32].

Biofilms are an additional problem since they are usually found in medical devices, such as prostheses, cardioverter defibrillators, urinary and vascular catheters, and cardiac devices,[9]; [16], hindering the eradication of *Candida* infections.

Each *Candida* species exhibits differences in biofilm formation with relation to their morphology, characteristics of the extracellular matrix (ECM), and ability to confer antifungal resistance,[30]. This variability increases the challenge of finding an effective solution to tackle the threats of *Candida* biofilms as a unique problem. In fact, due to the emergence of these fungal infections, there is an urgent need to find adequate therapeutic approaches that might be able to treat patients more efficiently.

1.2 Objectives of the study

The study is backed by the following specific objectives;

1) To isolate and characterize *Candida species* from immunosuppressed individuals.
2) To determine degree of biofilms formation by isolates.
3) To determine the antifungal susceptibility patterns of isolates.

1.3 Research hypotheses

The hypotheses are stated in the null form as follows:

Ho: Biofilms produced by *Candida species* do not confer antibiotic resistance.
Ho: Biofilms formation by *Candida* has no correlation with host immunity.
Ho: Polymerase chain reaction techniques are not a reliable technique for identification of *Candida species*.

2. Literature Review

2.1 Overview of Candida Biofilms

Biofilms are structured communities of microorganisms attached to a surface. They are formed when unicellular microorganisms come together to form a community that is attached to a solid surface and enclosed in an exopolysaccharide matrix, [7]. This matrix contains polysaccharides, proteins and DNA originating from microbes and the microorganism family can be made up of one or more species living together, [23]. Biofilms can also be described as dynamic heterogeneous communities that are constantly changing, [11]. At the most basic level, a biofilm can be described as microorganism embedded in a thick, slimy barrier of sugars and proteins, [25].

The International Union of Pure and Applied Chemists, IUPAC, defines Biofilm as an “aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface”. The self-produced matrix of extracellular polymeric substance, also called slime, is a polymeric conglomeration generally composed of extracellular biopolymers in various structural forms, [35]. Biofilms may be composed of a single microbial species,[34], but more frequently, they are formed by a complex and diverse community of microorganisms.

Biofilms are greatly significant to public health, they exhibit decreased susceptibility to antimicrobial agents which may be intrinsic (natural outcome) or acquired due to transfer of extra chromosomal elements to susceptible microorganisms in the biofilm, [7]. The emergence of drug-resistant microorganism and the difficulty in killing some microorganism led to a re-evaluation of the microorganism lifestyle and it is now acknowledged that biofilms endow microorganism with mechanisms to resist antibiotics. These mechanisms may be delayed penetration of the antimicrobial agents through the biofilm matrix, altered growth rate of biofilm organism, and other physiological changes due to the biofilm mode of growth, [7].

Biofilms can form on many medical devices such as contact lenses, intrauterine device, catheters, prosthetic valves, due to their high resistance level to antimicrobials, [18]. Biofilm-producing microorganisms are far more resistant to antimicrobial agents than microorganisms which do not. In some extreme cases, the concentrations of antimicrobials required to achieve bactericidal effect against microorganisms can be three-to-four-fold higher than for planktonic forms depending on the species and drug combination, [8]. They also resist phagocytosis in the immune system.

2.2 *Candida* Biofilm formation

Biofilm formation by *Candida* species occurs in a series of events including initial cell-to-surface or cell-to-cell attachment, micro colony formation, biofilm maturation and dispersal. This process has been considered advantageous in biofilm protection, nutrient availability, metabolic cooperation and the acquisition of new metabolic traits (Davey and O’Toole, 2000). Within a biofilm, microorganisms communicate with each other by the production of chemical signals or inducer molecules, a phenomenon called ‘Quorum sensing’. Availability of key nutrients, chemotaxis towards the surface, motility of microorganism, presence of surface adhesions and surfactants are some of the key factors influencing biofilm formation, [12]. Biofilms are also sites where genetic materials are easily exchanged because of the proximity of the cells, thus, maintaining a large gene pool,[7].

Formation of *Candida* biofilm is a developmental process that allows it to undergo a regulated lifestyle, changing from a unicellular form to a multicellular form, where subsequent growth results in structured communities and cellular differentiation, [28]. Surface-bound and free-floating microorganisms are also called sessile and planktonic forms respectively. Sessile *Candida* species can be attached to either abiotic materials such as those of implanted devices like catheters, prosthetic cardiac valves, intrauterine devices and biotic (living tissues or cells) surfaces, prevalent in natural, industrial and hospital settings, [17].

The ability of microorganisms to form biofilms is closely related to infectious diseases, environmental and biotechnological processes. The structural nature of the *Candida* biofilms and the characteristics of the sessile cells produce resistance towards antifungal agents, leading to a protected environment against adverse conditions and the host defenses.

The biofilm state is more predominant than the free-living planktonic state. This may be due to several reasons. First, biofilms can withstand harsh environmental conditions like shear forces or being washed off by water or blood stream by simply attaching to surfaces. Secondly, the EPS matrix protects the microorganism against antifungal agents this can be possible by delaying the antibiotics from reaching their targets. Thirdly, biofilms restrict fungal mobility thus increasing the chances of transfer of genetic materials,[27].

2.3 Stages of *Candida* biofilm formation

Biofilm formation takes place in a sequence of steps or distinct events. The stages of biofilm development are as follows: Initial attachment, irreversible attachment, maturation I, maturation II and dispersal,[4].

2.3.1 Virulence factors of *Candida* species

Polymorphism

Candida species are polymorphic fungi that can grow in several different forms, primarily yeast, pseudohyphae, and hyphae. For its pathogenicity, its ovoid-shaped budding yeast and parallel-walled true hyphae forms are the most important. The hyphae form is more prevalent for an infection, while the yeast form is believed to be important in the spread of *C.species*. The role of pseudohyphae is not very well understood, other than being an intermediate form between yeast and hyphae (Fig. 1). Several factors can cause a change in morphology, such as pH differences, temperature changes, carbon dioxide levels, starvation and quorum-sensing molecules such as farnesol, tyrosol, and dodecanol, [33].

Adhesins

Candidaspecieshave special sets of glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins that allow it to adhere to the surfaces of microorganisms. These glycoproteins are encoded by 8 sets of agglutinin-like sequence (ALS) genes, ranging from Als1-7 and Als9. For adhesion, the Als3 gene appears to the most important as it is up regulated during an infection of oral and vaginal epithelial cells. Also, it helps with biofilm formation by helping with adhesion to each other, [20].

Invasins

Along with adhesion, Als3 proteins can function as invasins that help with the invasion of *C. albicans and C. krusei* into host epithelial and endothelial cells. Another important invasin gene is Ssa1, which normally codes for heat-shock proteins. Basically, these specialized proteins on the pathogen’s surface mediate binding to host ligands, such as E-cadherin on epithelial cells and N-cadherin on endothelial cells, and it induces host cells to engulf the fungal pathogen. Another method of invasion is the active penetration of *C. glabrata* into host cells by an unknown mechanism involving hyphae, [20].

Biofilm formation

Candida specieshave the ability to form biofilms on living and non-living surfaces, such as mucosal membranes and catheters, respectively. After the adherence of yeast cells to the surface, there is development of hyphae cells in the upper part of the biofilm. Eventually, this leads to a more resistant, mature biofilm and the dispersion of yeast cells – both contributing to the pathogen’s virulence. In the process of biofilm formation, Bcr1, Tec1 and Efg1 function as important transcriptional factors. Recent studies show that biofilms protect *C. albicans* colonization from neutrophil attack and deter the formation of reactive oxygen species.

Secreted hydrolases

Candida albicans secrete 3 main classes of hydrolases: proteases, phospholipases and lipases. It is proposed that these hydrolases help facilitate the pathogen’s active penetration into host cells and the uptake of extracellular nutrients from the environment. There are about 10 known secreted aspartic proteases (Sap1-10), and their exact contribution to pathogenicity is controversial. For phospholipases, there are 4 major classes (A, B, C, and D), and all 5 members of the B class are involved with the disruption of a host cell surface. Thirdly, lipases are consisted of 10 members (LIP1-10), and studies show that there is decreased virulence in their absent, [20].

Types of *Candida* species: There are over two hundred species of *Candida*, but only 10% are known to cause infections, [24]. The most common species that cause infections are *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei*. Another species is *C. duris* which is an emerging species that cause invasive candidiasis around the world. Other pathogenic candida species are *C. dubliniensis, C. guilliermondii, C. famata, C. kefyr* and *C. lusitanine*,[13].*Candida* species live as commensal on skin, the gastrointestinal and genitourinary tracts.

3. Materials and Methods

3.1 Study design

The study was a cross sectional prospective study which ran for a period of 8months from January, 2023 to August, 2023.

3.2 Study area

This study was carried out in Calabar Metropolis. Calabar Metropolis is made up of Calabar Municipal Council and Calabar South Local Government Area. It has an area of 406km^{2} and a population of 371,022 according to 2006 population census. The city is bounded in the North by Odukpani Local Government Area and in the North-East by the Great Kwa River. Southern shores are bounded by the Atlantic Ocean and in the West by the Calabar River. The pioneer dwellers speak Efik and Ejagam. Calabar is located in the Southern Senatorial District of Cross River State. The main occupations of the people are civil service and trading,[1].*Candidiasis* is an endemic disease in the area as they are many patients who are immunosuppressed due to high rate of HIV/AIDS, diabetic patients and Cancer patients undergoing chemotherapy.

3.3 Study Subjects

The study subjects were immunosuppressed patients enrolled from HIV/AIDS and TB units of University of Calabar Teaching Hospital (UCTH), Dr. Lawrence Henshaw Memorial Hospital and General Hospital, Calabar.

3.4 Study population

Adult seropositive subjects aged 18yrs and above attending antiretroviral therapy (ART) in University of Calabar Teaching Hospital (UCTH), Dr. Lawrence Henshaw Memorial Hospital and General Hospital, Calabar.

Phenotypic Identification of *Candida Species:* Sputum and urine samples were collected from HIV seropositive immunosuppressed individuals of both genders of different ages. The study samples were immediately transported to the laboratory in a transport medium. The samples were inoculated on sabouraud's medium with chloramphenicol. The inoculates were incubated at 37^{O}C for 48hours. The initial identification of yeast was based on macroscopic appearance of the colonies on sabouraud's medium and the growth of coloured colonies on CHROMagar *Candida*. The isolates that formed creamed coloured colonies, smooth or with slightly corrugated surface, convex, shiny, smelling of yeast were used for further study. The microscopic examination of Gram stained samples showed Gram-positive thin-walled, spherical, cylindrical or egg-shaped blastospores, 3-4μ*m* in diameter. The capacity of the *Candida species* to form biofilm *in vitro* were examined on Congo red agar, after incubation for 24-72hours at 37^{0C} and microtitre plate method.

Biofilm production by Microtitre plate method: Biofilm formation was performed on a sterile 96-well microtiter plate. A colony of each isolate was inoculated into tubes containing 2ml of Brain Heart Infusion Broth (BHIB) and incubated at 37^{0}C for 24 hours.All the broth cultures were diluted at a ratio of 1:20 using fresh BHIB and 200µl was placed into microtiter plates and incubated at 37^{0}C for 24 hours.

After the completion of incubation, microtiter plates was emptied, rinsed with distilled water three times and inverted to blot. Each well was filled with 200µl of 1% crystal violet and incubated for 15mins. After incubation, the microplates were again rinsed three times with distilled water. Then 200µl of ethanol acetone mixture (80:20w/v) was added to each well and read at 450nm using an ELISA reader and optical density (OD) recorded for each well. Sterile BHIB without isolates was used as the negative control. The cut-off value was determined by arithmetically averaging the optical density of the wells containing sterile BHIB and by adding a standard deviation of 0+2. Samples with any optical density (OD) higher than the cut-off value were considered positive whereas those with the lower optical density than the cut-off was deemed as negative (Munmun *et al.*, 2018).

Determination of CD4^{+} counts: The CD4^{+} T-cell counts were determined using the flow cytometer. Fifty (50µl) of coagulated venous blood was added to BDFACS COUNTER^{tm} tubes via reverse pipette. Reagents tubes were incubated for 30mins at 18-25^{0}C, after 30minutes fixation solution was added and the tubes were analysed with BDFACS count flow cytometry. The software identifies lymphocytes population and calculates CD4 count/µl of blood by comparing cellular events to beads events, at least 10,000 events were demanded for the procedure and results including

CD4^{+} counts and CD4 percentages were printed out after running the samples (Warrino *et al.*, 2005).

Antifungal susceptibility testing: The *Candida* isolates were tested by disk diffusion method using Muller-Hinton agar supplemented with 2% glucose and 0.5µg of methylene blue/ml. The agar surface was inoculated by using a swab dipped in a cell suspension adjusted to the turbidity of 0.5 McFarland standard. The following antifungal disks was used: Fluconazole (25µg), Vericonazole (lµg), Itraconazole, Nystatin (50µg) and Amphotericin B (100µg) (BioRad). Antifungal disks were placed on the inoculated plates and incubated at 37^{0}C for 24-48 hours. The diameter of the zones of inhibition were measured. Inhibition zones was interpreted using validated Clinical and Laboratory Standards Institute (CLSI, 2010) interpretative break points, [21].

Molecular Identification of *Candida species* by ITS gene Sequence

1. DNA extraction: Extraction was done using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. A heavy growth of the pure culture of the suspectedisolates was suspended in 200 microliters of isotonic buffer into a ZR Bashing Bead Lysis tubes, 750 microliter of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR bashingbead lysis tube was centrifuged at 10,000x*g* for 1 minute.

Four hundred (400) microliters of supernatant were transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000x*g* for 1 minute. One thousand two hundred (1200) microliters of fungal/bacterial DNA binding buffer were added to the filtrate in the collection tubes bringing the final volume to 1600 microliter, 800 microliter was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000x*g* for 1 minute, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microliter of the DNA Pre-Was buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000x*g* for 1 minute followed by the addition of 500 microliter of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000x*g* for 1 minute.

The Zymo-spin IIC column was transferred to a clean 1.5 microliter centrifuge tube, 100 microliter of DNA elution buffer was added to the column matrix and centrifuged at 10,000x*g* microliter for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degree for other downstream reaction.

2. DNA quantification: The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

3. Internal Transcribed Spacer (ITS) Amplification: The ITS region of the isolates was amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITSR: 5'-TCCTCCGCTTATTGATATGC-3,primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 53ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a blue light transilluminator.

4. Sequencing: Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows: 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

5. Phylogenetic Analysis: Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0, [29]. The bootstrap consensus tree inferred from 500 replicates,[10] is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method, [14].

3.5 Statistical analysis

Data were statistically analyzed in terms of frequencies and percentages. P-value was considered statistically significant if less than or equal to 0.05 (≤0.05). Comparison between the study groups was done using Chi-square (x^2) test and Pearson correlation using SPSS version 21.

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3.6 Results

There was a statistically significant association between *Candida* infection and the rate of biofilm production (**x^2=14.432, P=0.016**) (**Table.2**).

Table 1: Morphological characteristics of *candida species* on CHROMagar candia

Candida species	Colony characteristics on CHROM agar *Candida*	Total No. of isolates (%)
C. albicans	Light to metallic green colonies	16(40)
C. dublinensis	Blue to green colonies	6(15)
C. tropicalis	Deep blue that diffuses into surrounding agar	7(17.5)
C. glabrata	White large glossy to pale pink pigmentation	8(20)
C. krusei	Large, flat, spreading deep pink to purple colonies with rough surfaces	3(7.5)
Total		**40(100)**

Table 2: Biofilm formation results of 40 *Candida* isolates by Congo red agar

Candida species	No (%) of isolates	Biofilm Positive		(%)	x^2	p-value
		Strong	Weak	Total		
Candida albicans	16(40)	3(27.3)	8(72.7)	11(100)		
Candida tropicalis	7(17.5)	2(33.3)	4(66.7)	6(100)		
Candida dubliniensis	6(15)	0(0.0)	0(0.0)	0(0.0)		
Candida glabrata	8(20)	2(50)	2(50)	4(100)	14.332	0.016*
Candida krusei	3(7.5)	0(0.0)	0(0.0)	0(0.0)		
Total	**40(100)**	**7(33.3)**	**14(66.7)**	**21(100)**		

Table 3: Distribution of CD_4^+ count among subjects infected with *candida species*

CD_4^+ count of subjects (cells/µl)	No (%) of subjects	No. of Infected subjects (%)	x^2	p-value
250-300	43(21.5)	12(30)		
301-350	52(26)	10(25)		
351-400	25(12.5)	7(17.5)	6.451	0.033*
401-450	30(15)	5(12.5)		
451-500	50(25)	6(15)		
Total	**200**	**40**		

Table 3: Shows the distribution of $CD4^+$counts among subjects. Most of the subjects 52(26%) enrolled into the study had $CD4^+$ counts 301-350cells/µl, followed by 50(25%) subjects with 451-500cells/µl. The $CD4^+$ group 250-300cells/µl had the highest infection rate of 44.2% among infected subjects. There was statistically significant association between subjects $CD4^+$ counts and the rate of *Candida* infection (x^2=6.251, P=0.035*).

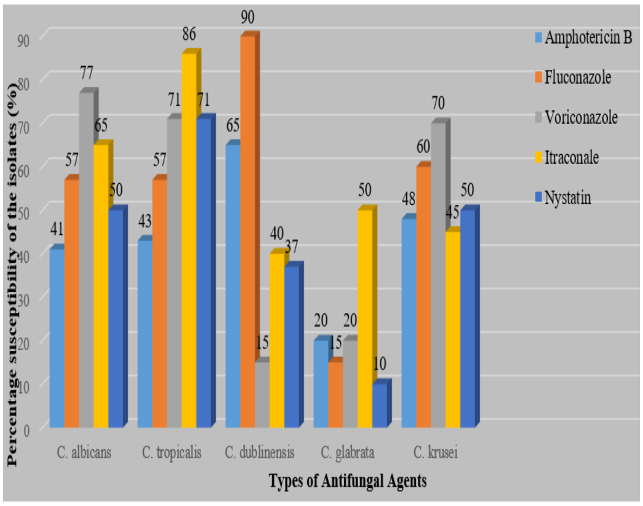

Figure 1: Antifungal Susceptibility Pattern

Figure 1 shows the susceptibility pattern of *Candida* species to antifungal agents. The antifungal agents tested were Amphotericin B, Fluconazole, Nystatin, Itraconazole and Voriconazole. The range of susceptibility of the isolates to antifungal agents was 10 - 90%. Isolates of *C. dublinensis* were more susceptible to fluconazole (90%) followed by Amphotericin B (65.0%). Isolates of *C. albicans* were more susceptible to voriconazole (77%) followed by itraconazole (65%). Other non albicans species varying susceptibility rates ranging from 10-87%. *C. glabrata* was 90%,85% and 80% resistant to nystatin, fluconazoleand amphotericin B respectively. The rank of potency of the antifungal agents against *Candida* isolates was Fluconazole>Amphotericin B > Nystatin > Voriconazole >Itraconazole.

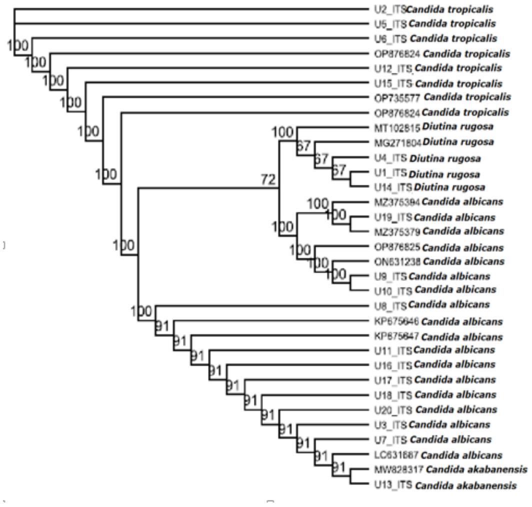

Figure 2: Phylogenetic tree showing the evolutionary distance between the fungal isolates

The obtained ITS sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The ITS of the isolates showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the ITS of the isolates within the *Candida* and *Diutina* sp and revealed a closely relatedness to *Candida albicans, Candida tropicalis, Candida akabanensis* and *Diutina rugosa* (FIG.2).

3.7 Discussion

The findings indicated that the most common *Candida species* causing *Candida* infection in HIV seropositive patients in Calabar were *Candida albicans* 16(40%) with light to metallic green (smooth), *Candida glabrata* 8(20%) with white large glossy to pale pink pigmentation, *Candida tropicalis* 7(17.5%) with deep blue colonies that diffuses into the surrounding agar, *Candida dubliniensis* 6(15%) with blue to dark green colonies (rough) and *Candida krusei* with large, flat, spreading deep pink to purple colonies having rough surfaces (Table.1). this study corroborated with the findings of Seyedeh *et al.,* (2018) who reported that *Candida albicans* (62.4%) was the most common isolate followed by *Candida glabrata* (17.5%) respectively causing Candidemia in Iran. However, 20 *Candida* isolates were randomly selected out of the 40 C. isolates identified phenotypically for molecular identification via PCR “internal transcribed spacer (ITS)” sequence which revealed that 11(55%) were *Candida albicans*, 5(25%) were *Candida tropicalis,* 3(15%) were *C. rugosa* (reclassified as *Diutina rugosa*) and 1(5%) was *Candida akabanensis* respectively (fig.2).

Consequently, phenotypic and molecular methods had resulted in the identification of 80% and 100% of the isolates respectively. This is similar with the study of [31], who reported 65.2% phenotypic and 96.6% molecular identification of candida species. This has shown that the molecular method is more reliable for identification of candida species which will aid better diagnosis of candidiasis. The PCR - Internal Transcribed Spacer (ITS) sequence was able to identified emerging *Candida* pathogen such as *Candidarugosa* and *C. akabanensis* which were phenotypically misidentified as *C. albicians* and *Candidadublinensis* respectively (Table 1). The phylogenetic tree for *Candidaspecies* was constructed based on the similarity value, the strains were determined as identical (100%), genetically related (80-99%) or unrelated (<80%). The phylogenetic diversity and relatedness of candida species can be seen among *Candidaalbicans, Candidatropicalis, C. akabanensis* and *C. rugosa* (reclassified as *Diutinarugosa*) (Fig.2). This finding correlated with the study of [15], who reported that there is genetic diversity among medically important and emerging *Candida* species causing invasive infection.

Candidiasis is an opportunistic disease that has emerged at alarming rate because there is an increase in number of HIV patients who are immunosuppressed. The ability of *Candidaspecies* to form biofilms is associated with the pathogenicity and as such should be considered as a vital virulence determinant. Biofilm may help fungi to maintain their role as pathogen by evading host immune mechanism and resisting antifungal therapy. Biofilm formation by *Candidaspecies* is also associated with high level of antifungal resistance. Out of 40 candida isolates tested in this study, 16(40%) were *C. albicans* and 24(60%) were non-albicans species. Among the non-albicans species, *C. glabrata* (8(20%) was the most common isolate. These isolates were tested by *invitro* screening test for biofilm production by congo red agar (CRA) method and microtitre plate method. The CRA method is a simple, reliable and qualitative test to determine whether an isolate has the potential for biofilm production or not. Whereas microliter plate method is a quantitative test to determine the degree of biofilm production by the isolates. In this study biofilm positivity occurred most frequently in isolates of *C.tropicalis* followed by *C.albicans* and *C.glabrata* 21 of 40 (52. 5%) of the isolates formed biofilm. This is correlated with Merit *etal.,* (2014) in which a total of 61(55.5%) out of 110 *Candidaspecies* obtained from the clinical isolates produced biofilm. About 69% (11 of 16) of *C. albicans* isolates produced biofilm which was significantly higher than the non-albicans candida isolates producing biofilm (41.7%), 10 of 24; ($p = 0.016$). Strong biofilm formation was observed more in *Candidaglabrata* (50%) followed by *Candidatropicalis* with (33.3%) and weak biofilm production was observed more in *C. albicans*. In contrast, [20], reported that isolates of *C. krusei,C. glabrata* and *C. tropicalis* gave significantly weak biofilm growth ($p < 0.001$) than the more pathogenic *C. albicans.* Quantitatively, *C. glabrata* formed higher degree of biofilm production which ranged from $0.300 \leq 570\text{nm} \geq 0.500\text{nm}$. There was significant negative correlation between the degree of candida biofilm production and host immune status ($r = -301$, $p = 0.001$).

The antifungal susceptibility results showed that amphotericin B and fluconazole had good efficacy for isolates of *Candida dubliniensis*, *C. Krusei*, *C. albicans* and *C. tropicalis*. In this study, C. *glabrata*was notable for multidrug resistance to antifungal agents tested which is in contrast with the report by [3], in India who reported *Candidaspecies* to be remarkably susceptible to polygenes, azoles and echinocardins.Although they recorded 92% susceptibility to Amphotericin B while this study had 43.4% susceptibility to Amphotericin B. Resistance rate was 42.8% to itraconazole, 44.2% to fluconazole, 56.4% to nystatin and 56.6% to amphotericin B which may be as a result of prolonged use by patients. This is in contrast with [2], who reported that the resistance rate to fluconazole was 23% and 4% to itraconazole respectively in Khartoum, Sudan. There was higher degree of resistance to all the antifungals in biofilm producers as compared to non-producers of biofilm as reported by several authors. The possible resistance mechanism may involve restricted penetration of drugs through biofilm matrix, phenotypic changes resulting from decreased growth, expression of resistance genes (Erg11) induced by prolonged used of azoles and polygenes by immunosuppressed patients. Non-albicans *Candidaspp*. Predominated (60%) in this study which is well correlated with [3], that had reported 85.3% of non-albicans species from immunosuppressed patients at a tertiary care Hospital in North India. The predominant non albicans *Candidaspecies* in this study were *C. glabrata* 8(18.6%) followed by *C. tropicalis* 7(16.3%) which is in contrast with [26], reported *C. tropicalis 13*(39%) followed by *C. parapsilosis* 7(20%). Immunosuppressed patients in Calabar, Metropolis-Nigeria habour phylogenetically diverse *Candidaspecies*. *Candida akabanensis* and *Candidarugosa* are emerging cause of Candidiasis in the area, which was detected using molecular techniques via internal transcribed spacer (ITS) genes. Out of the *Candida* species isolated; *C. glabrata*, *C. akabanensis* and *C. rugosa* are notable for multidrug resistance because they formed stronger biofilm which corroborated with the findings of [19], who reported multidrug resistance *Candida* in India.

4. Conclusion

The accurate identification of candida species is important for selecting the most effective treatment strategies to control invasive fungal infections. In this research, the phenotypic methods used effectively identify clinically important candida species, including *C. albicans, C. tropicalis, C, glabrata, C. dubliniensis,* and *C. krusei.* However, the chrome agar candida method failed to identify *C.rugosa* and *C. akabanensis* isolates. Conventional identification of the candida rugosa species is still limited, because it is not included in the database of current commercial biochemical systems such as chrom agar candida ID. Nevertheless, the reliable identification of rare and emerging candida species that cause C. infection in immunosuppressed patients such as candida rugosa and candida akabanensis was made possible using DNA sequencing. For epidemiological purposes, DNA-based methods have been used to differentiate *Candidaspecies.* Out of the 16 isolates phenotypically identified as candida albicans 3 were identify as *C. rugosa* well as one isolate out of those identify phenotypically as candida dubliniensis was found to be *C. akabanensis* by internal transcribed spacer (ITS) sequencing. Biofilm production by *Candida species* is a virulence determinant and it also contributes significantly to antifungal resistance as observed in *Candida glabrata.*

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