



**MOLECULAR CHARACTERIZATION AND PHYLOGENY OF CANDIDA SPECIES
ISOLATED FROM HIGH VAGINAL SWAB SAMPLES AMONG PATIENTS
PRESENTING WITH VULVOVAGINAL CANDIDIASIS IN PORT HARCOURT,
NIGERIA**

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ABSTRACT

Vulvovaginal candidiasis with the attendant morbidities remains a major reason for female visit to STI clinics. However, proper diagnosis has been a challenge. This study was conducted to carry out molecular characterization and phylogeny of *Candida species* from vulvovaginal patients. One hundred and eighty (180) high vaginal swab specimens from female patients presenting with vulvovaginal candidiasis, attending University of Port Harcourt Teaching Hospital were collected and cultured using standard procedures. The positive isolates were subcultured on the Sabouraud Dextrose Agar slant while DNA was extracted for molecular analysis. Internal transcribed spacer (ITS) gene extracted was amplified, product resolved on 1.5% agarose gel to measure the base pairs and then sequenced, edited and aligned with MEGA 6.0 to infer Phylogeny. A total of 72 positive isolates of *Candida* were assessed in this study. The result obtained from culture medium inoculation showed the *Candida species* were 72(40%), and non-*Candida species* were 108(60%). The positives isolates were subjected to germ tube test and the result showed that 24(33.3%) were *Candida albicans*, 24(33.3%) were non-albicans species and 24(33.3%) were unidentified. However, molecular analysis revealed 33(45.8%) *C.albicans*, 36(50%) non-albicans species which consists of *C.glabrata*, *C. parapsilosis*, *C.tropicalis*, *C.Krusei (Pichia kudriavzevii)*, *Candida akabenensis* and *Filobasidium uniguttulatum*; and 4.2% unidentified species. Most species phenotypically identified as *C.tropicalis* were genetically characterized as *C.albicans*. All the unidentified species by germ tube test were molecularly identified and the only isolate unidentified using molecular tool was characterized as *C.albicans* by conventional technique. Hence there was significant difference between results obtained from conventional method and that from molecular analysis. Molecular technique in this study was more discriminatory than routine conventional approach.

KEYWORDS: Vulvovaginal, Candida, Characterization, Phylogeny, Molecular.

INTRODUCTION

Candidiasis in humans has always been attributed to *Candida albicans*. However, reports have it that other species of *Candida* may also contribute to the burden of *Candida* infection in humans. There is therefore the need to scientifically prove this observation in order to ensure adequate and proper therapy for candidiasis. Identification of these species has largely depended on the phenotypical traits such as biochemical tests and on morphological characteristics observed microscopically. However, identification of emerging species by phenotypic techniques is mostly impossible or inconclusive. Hence, nucleic acid based sequence is necessary for taxonomic differentiation among *Candida species*.

Studies have indicated that *Candida albicans* is solely responsible for most invasive candidiasis from clinical specimens. However, in recent times, the occurrence of the infection as a result of non-albicans species has been reported to be on the increase (Horn *et al.*, 1999). Therefore is a shift towards non-albicans *Candida species* as emerging aetiologic agent of vaginal yeast infection which demonstrate decreased susceptibility to commonly used antifungal drugs (Pfaller,2010). Non-albicans species of *Candida* recently isolated from clinical specimens especially from genitourinary region have demonstrated the production of virulence attributes, and are highly resistant to azole group of antifungal agents and amphotericin B (Sachin *et al.*,2014).

Vulvovaginal yeast infection is a vaginal disease caused by the fungus belonging to the genus '*Candida*. It is

characterized by inflammation of vulvovaginal region in the presence of *Candida species*. The most prevalent cause of vaginitis is bacterial vaginosis and this is followed by vagina yeast infection which is responsible for about 33% of vaginitis infections (Workowski & Bolan, 2015).

Genetic differences in the ribosomal DNA internal transcribed spacer (ITS) region has been examined among *Candida species* but the number of ITS sequence polymorphisms in the various species and their relationship with sources of virulent fungal infections remain deficiently researched (Muriel *et al.*, 2011). Most cases of infectious candidiasis are ascribed to *Candida albicans* meanwhile the increase rates results from other species in various parts of the world (Vinitha & Mamatha, 2011).

Standard procedures used by reference centers for the description of medically important yeasts have been progressively repossessed by nucleic acid -based analysis and genomics (Arvanitis *et al.*, 2014). However, in under-resourced laboratories, commercial biochemical tests still represent the main tool for the identification of human yeast pathogens (Pincus *et al.*, 2007). These techniques take long time to produce results and have potential challenges with respect to proper identification to species level (Priya *et al.*, 2011).

Elevated occurrence of infections attributed to non-albicans species and increase resistance to antifungal is of great concern. *C.tropicalis* and *C.parapsilosis* have exhibited susceptibility to fluconazole than is *C.albicans*. *Candida glabrata* is intrinsically more resistant to antifungal agents specifically fluconazole (Sardi *et al.*, 2013).

Transmission rates of infectious diseases including candidiasis has been traced using phylogenetics and its application to modern problems (Schmalreck *et al.*, 2014). Phylogeny is a history of organismal lineage as they change through time. Identification using molecular analysis and high number of patients who are severely ill and immune compromised have led to the detection and surfacing of uncommon, cryptic and new species of opportunistic fungi pathogenic to man and animal (Gittleman and Brooks, 2015).

Phylogenic allocation of causative organisms is critical in the provision of information necessary for proper selection of required antifungals (Schmalreck *et al.*, 2014).

In developing countries like Nigeria, there seems to be a paucity of information on the laboratory diagnosis of candidiasis due to non-albicans species, hence such infections are not readily reported, the species not properly identified and the infection is attributed to *Candida albicans*. Proper characterization of non-albicans candida species is critical in improving

diagnosis and therapy of candidiasis because different species possess varying virulent attributes as well as differences in their susceptibility to common antifungal drugs used against candidiasis. The aim of this study is to carry out molecular characterization and phylogeny of *Candida species* isolated from female patients presented with vulvovaginal candidiasis in the University Port Harcourt Teaching Hospital.

MATERIALS AND METHODS

Study Area

The research was done in Port Harcourt, Rivers State, Nigeria, a city that covers an area of about 369 square kilometers and located on the longitude 7°2'1''E and latitude 4°49'27''N. The analysis was carried out in three different locations which are Microbiology Laboratory unit of University of Port Harcourt Teaching Hospital (UPTH), Molecular Laboratory unit of Niger Delta University Amassoma, Bayelsa State and Inquaba Biotechnical Industries Limited Pretoria, South Africa.

Study Design

The study was carried out among 180 randomly selected female patients presenting with vulvovaginal Candidiasis in UPTH, Rivers State. The randomly selected subjects are supposed to represent a sub group of subjects in Port Harcourt and the choice of this hospital was made because it is one of the biggest hospitals accessible to the inhabitants of the State and people from nearby states.

Sample collection

One hundred and eighty (180) high vaginal swab specimens were collected from participants who met the inclusion criteria. The specimens were given laboratory identification number for analysis and retrieval of results. The specimens were cultured using Sabouraud Dextrose Agar (SDA) and Chocolate Agar. SDA is a special media for fungal growth. Chocolate Agar is an enriched medium for cultivation of fastidious organisms. Its use was to rule out organisms such as *Neisseria spp* and *Haemophilus spp*. The wet preparations of the specimens were also examined after culture for presence of yeast cells.

Sample Analysis

The samples collected were analysed using standard conventional procedures. Two sets of Slant of SDA were prepared and positive samples from cultural analysis were subculture on them. After 24 hours incubation, Germ tube test was carried out on the isolates of a set while the second set was sent to Molecular Laboratory, Niger Delta University Amassoma, Bayelsa state, for molecular analysis.

Germ Tube Test

0.5mls(12 drops) of pooled human serum was added in a clean test tube. Light suspension of suspected yeast colonies was made on the serum. The mixture in the tube was incubated at 37 degree centigrade for 3 hours. A drop of the suspension was placed on a clean glass slide

using Pasteur pipette and cover with cover slip. The wet mount was microscopically examined at 40* microscope objective for production of germ tubes.

DNA extraction

Extraction of DNA involves lysing of the cell, removal of the contaminant and recovery using elution buffer. A pure *Candida* isolates from HVS were cultured on Luria Bertani (LB) for 18hour to eliminate non-fungal isolates. Extraction was carried out with Zymo Research fungal DNA mini prep kit supplied by Inquaba Biotechnologies, Pretoria, South Africa.

In a bashing bead tubes, 200 microlitres of suspension (containing isolates and LB) was added. This was followed by the addition of 750 microlitres of lysing solution. The tubes were loaded on the high speed cell disrupter and processed for 5minutes to lyse the cells. The bashing bead lysis tubes were centrifuged for a minute in a microcentrifuge at 10000xg. 400 microlitre of supernatant from each tube was transferred to a Zymo-Spin IV filter in a collection tube and centrifuged for a minute at 7000xg. The Zymo-Spin IV filter has orange cover and a base which was snapped off before use. 1200 microlitres of fungal DNA binding buffer, already containing 0.5ml beta-mercaptoethanol in 100ml binding buffer was added for optimal performance. In another set of collection tubes with Zymo-Spin IIC column, 800 microlitres of the mixture from previous step was transferred and centrifuged at 10000Xg for one minute. This quantity was transferred because the Zymo-Spin IIC column has maximum capacity of 800 microlitres. Therefore, the flow through from the collection tube was discarded and the step was repeated.

200 microlitre of the DNA Pre-Wash buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000xg for 1 minute. This was followed by the addition of 500 microlitres of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000xg for 1 minute.

The Zymo-spin IIC column was transferred to a clean 1.5 microlitre centrifuge tube, 50 microlitres of DNA elution buffer was added to the column matrix and centrifuged at 10,000Xg for 30 seconds to elute the DNA. The ultra pure DNA was then stored at -20 degree for other downstream reaction.

DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer connected to a system. It measures the DNA in nanogram per microlitre. This machine also reveals the purity of the DNA extracted at 1.7-2.0 purity level. At this state, extracted DNA is suitable for PCR amplification and other downstream applications.

Internal Transcribed Space (ITS) Amplification

The ITS region of the rRNA genes of the isolates were amplified using the

ITS1(TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 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, 95 degree for 5 minutes; denaturation, 95 degree for 30 seconds; annealing, 53 degree for 30 seconds; extension, 72 degree for 30 seconds and final extension, 72 degree for 5 minutes. The product was resolved on a 1.5% agarose gel at 120V for 15 minutes. In the electrophoresis machine, 100base pair molecular ladder was used to measure the amplified gene to confirm that the actual gene of interest was amplified. The ladder was placed at the centre of the amplicons dropped on the electrophoretic gel inside the tank. The result was visualized on a UV transilluminator.

Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa.

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 (Saitou and Neil, 1987). The evolutionary history of the taxa analyzed was represented using the bootstrap consensus tree inferred from 500 replicates. The evolutionary distances were computed using the Jukes-Cantor method (Jukes *et al.*, 1969).

Statistical Analysis

The statistical tools used for statistical analysis of this study include;

- Microsoft excel (Windows 10)
- Percentage occurrence
- Clustal X

Microsoft excel was used to compute the tables and bar chart to illustrate the percentage occurrence of different species isolated.

Clustal X is a bioinformatics tool applied to infer Phylogeny of the *Candida species* isolated in this study.

RESULTS

The 72 positive isolates from inoculation onto culture medium were tested using Germ tube test (Figure 4.1) and the result obtained showed that 24 (33.3%) were negative indicating unidentified, 24 (33.3%) were positive indicating *Candida albicans* and 24(33.3%) revealed pseudohyphae, indicating *Candida tropicalis*.

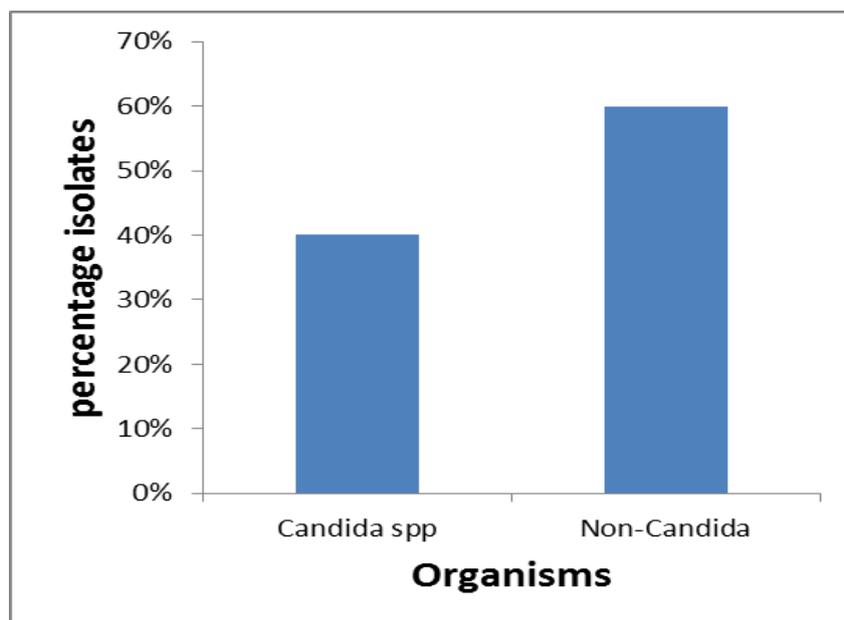


Figure 1: Graph of Organisms Identified Using Inoculation on Sabouraud Dextrose Agar.

Candida species Isolated using Molecular Technique

The same isolates tested were subjected to molecular analysis. The result (Figure 2) showed that 33(45.8%) were identified as *Candida albicans*, 36(50%) as non-*albicans* species and 3(4.2%) was not identified. Non-*albicans* species identified are *Candida glabrata* (25%), *Candida akabensis* (8.4%), *Candida tropicalis* (4.2%), *Candida parapsilosis* (4.2%), *Filobasidium uniguttulatum* (4.2%), and 4.2% *Candida krusei* (*Pichia kudriavzevii*).

Phylogenetic Analysis

Figure 3 shows the evolutionary relationship between the *Candida species* using Neighbour-joining phylogenetic tree. F1, F8, F3, F23, F5, F13, and F17 were more

closely related (*Candida albicans*) though different strains. F21 is related closely to these isolates above than others. This is followed by F12 and 15; F9 and 14. F4, F20, F19, F24 and F22 were related while F2, F7 and F11 did not relate directly to any species. The following isolates were added to the genebank.

Agarose Gel Electrophoresis

The ITS of the isolate showed a percentage similarity to other species at 99%. 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 species* and revealed a closely relatedness to *Candida tropicalis* strain CBS 6632(gb: KY102481.1) than other *Candida species*.

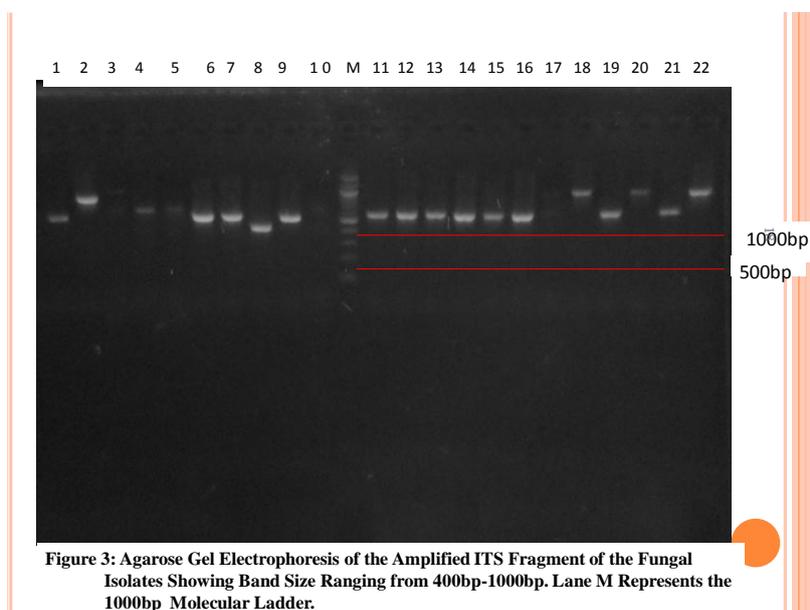


Figure 3: Agarose Gel Electrophoresis of the Amplified ITS Fragment of the Fungal Isolates Showing Band Size Ranging from 400bp-1000bp. Lane M Represents the 1000bp Molecular Ladder.

Plate 1: Agarose Gel Electrophoresis of the Amplified ITS Fragment of the Fungal Isolates Showing Band Size Ranging from 400p-Lane M Requirements the 100bp Molecular Ladder.

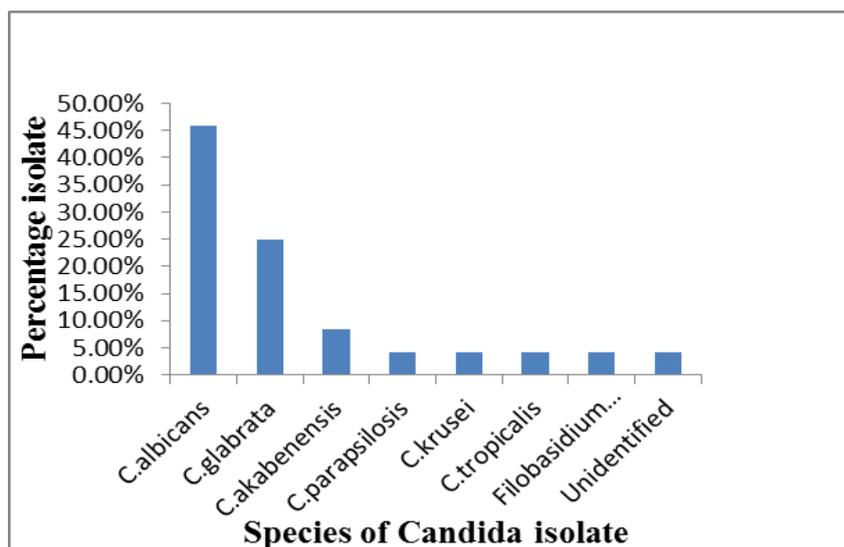


Figure 2: Graph of Molecular Characterization of species Isolated.

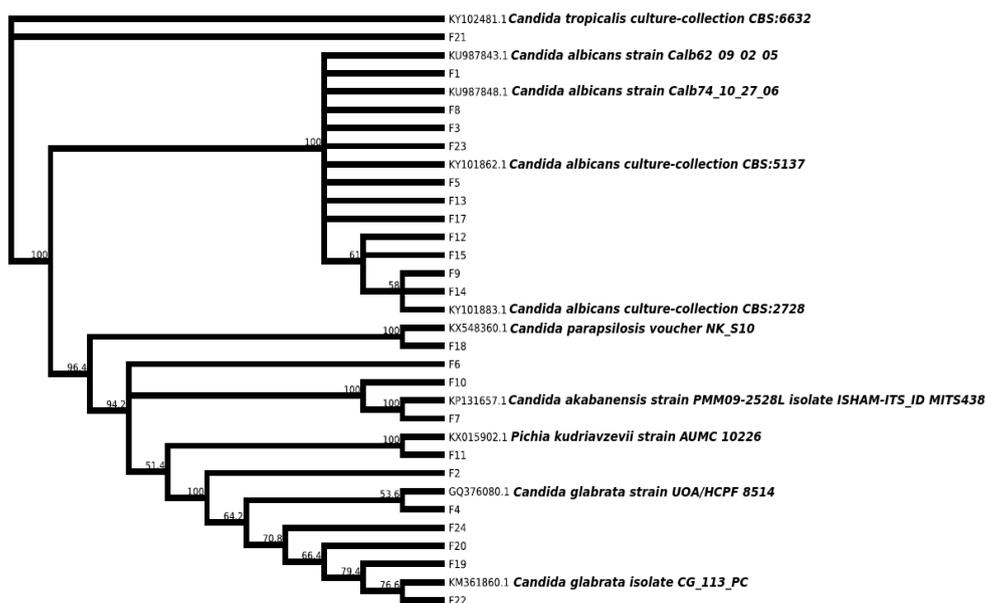


Figure 3: Phylogenetic tree of *Candida* species isolated.

DISCUSSION

Identification of *Candida species* conventionally is better achieved combining other techniques such as germ tube test, chlamyospore formation, chromogenic *Candida* test, sugar fermentation and assimilation. The discrepancies between molecular studies and Phenotypic studies (conventional) of *Candida* isolates included in this study can be attributed to the limitations of conventional techniques used in clinical laboratories which do not permit the identification of uncommon species of *Candida* (Mansour & Wingard,2012). Consequently, this amounts to overestimation of the incidence of emerged species to the detriment of the emerging cryptic species. Proper identification of *Candida* and other pathogenic yeast is critical for clinical management of patients (Hou et al., 2016). This research work was carried out to identify the non-albicans *Candida species* isolated from vaginal swabs. This is the first study done in Port Harcourt, Rivers State, Nigeria to

characterize *Candida species* isolated from High vaginal swab samples using molecular analysis of internal transcribed spacer (ITS). The ribosomal RNA gene complex sequencing which targets ITS regions is currently considered as 'gold standard' for identification of fungi (Zhang et al, 2014). Conventional approaches used in this study include; inoculation on a culture plates, wet preparation, and germ tube test.

The isolates from HVS were inoculated on SDA slants,(TITAN BIOTECH LTD, India), incubated at 37 degrees centigrade for 24hours. Seventy-two isolates showed the characteristic growth typical of *Candida albicans* as the growth yielded entire, creamy colonies with smell akin to breweries. Thus, it was difficult to differentiate *Candida* to species level looking at the cultural features only. This supports the opinion of Sumitra & Megha (2014), which reported that the speciation of *Candida species* conventionally is

appropriately done through combination of other techniques like germ tube tests, biochemical tests such as chromogenic *Candida* test, sugar fermentation tests, e.t.c. These procedures take long time and a lot of man power and results are not 100% reliable. Sabouraud dextrose medium was useful in detecting *Candida* when identification was inconclusive using Microscopy (Ugwa, 2015). The remaining 36(60%) did not grow. This shows that vulvovaginal candidiasis is also caused by organisms other than *Candida species*. This agrees with the work done by Ugwa in Kano (2015) which he reported that *Proteus vulgaris* was also isolated from HVS specimens.

Conjectural detection of *Candida albicans* is carried out by microscopic confirmation of germ tube test. In this study, the isolates from HVS specimens were incubated in pooled human sera for 3hours at 37 degrees centigrade. Out of 72 isolates, 24 (33.3%) showed short slender, tube like structures (germ tubes) when view under microscope. This indicates *Candida albicans*; 24(33.3%) showed no hyphal extension from a yeast cell, indicating non-albicans species and 24(33.3%) showed short hyphal extension with constriction at the point of origin (pseudo germ tube) indicating *Candida tropicalis*. Campbell et al (1998) reported that as *C.parapsilosis* and *C.tropicalis* exhibit germ tube when incubated in a serum under cultural conditions. Other studies like Sumitra and Megha (2014) reported that germ tube is a rapid technique to identify *C.albicans* and *C. dubliniensis* from other candida isolates. Tapiwa et al.,2017 also reported that isolates from cultures suspected to be *C. albicans* were confirmed by germ tube formation. Although germ tube test is generally accepted as a screening procedure for identifying *Candida albicans*, in this study, the assertion has been annulled because there are different strains of *Candida albicans* (Calb 62, Calb 74, CBS 5137 and CBS 2728) and not all these strains are responsive to the germ tube test. Only CBS 5137 and CBS 2728 strains were identified using germ tube test.

Molecular characterization carried out in this study showed that 33(45.8%) isolates of *Candida* were identified as *Candida albicans*, 3(4.2%) were *C.tropicalis*, 3(4.2%) were *C.krusei*. Others which were not identified with conventional techniques were molecularly identified and belong to species *C.glabrata*, *C.parapsilosis*, *C.akabanensis*, *C.krusei* (*Pichia kudriavzevii*), *C.tropicalis*, *C.albicans* and *Filobasidium uniguttulatum*. The *Candida albicans* isolated were of four different strains (Calb 62, Calb 74, CBS 5137 and CBS 2728). This study showed that Molecular characterization of *Candida* species is very discriminatory and remarkably showed conserved intra-specific patterns which are significantly different and specie-specific. Rapid and reliable characterization of species of *Candida* cannot be overemphasized considering species variation in susceptibility to antifungal drugs such as amphotericin B and azoles. It is

also important in epidemiological studies and proper statistical records.

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