# Molecular Diversity of *Candida albicans* Isolated from High Vaginal Swab of Patients Attending Health Care Facilities in Nasarawa State, Nigeria

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## ABSTRACT

*Candida albicans* (*C. albicans*) has been a major causative agent of candidiasis. This study, looked at the molecular diversity of *C. albicans* associated with vaginal infections in Nasarawa State, Nigeria. A total of one thousand two hundred (1200) High Vaginal Swabs (HVS) samples were collected across the senatorial zones in Nasarawa State namely; Nasarawa North (NN), Nasarawa South (NS) and Nasarawa West (NW). The *C. albicans* were isolated, identified and confirmed by methods as described by Clinical and Laboratory Standards Institute (CLSI) including; RapID Yeast Plus System (R8311007) kits, genomic DNA was extracted using the quick Protocle (ZR Fungal DNA miniPrep) DNA extraction kit and Restriction Fragment Length Polymorphism (RFLP) analysis; Eight oligonucleotides i.e. Primers1(5'-GGGGGTTAGG-3'), 2(5'-GGTGTAGTGT-3'), 3(5'-GTATTGGGGT-3'), 4(5'-GGTTCTGGCA-3'), 5(5'-AGGTCACTGA-3'), 6(5'-AAGGATCAGA-3'),7(5'-CACATGCTTG-3'), and 8 (5'-TAGfATCAGA-3') were used, the forward (ITS1) and reverse (ITS2: 5'-GCTGCGTTCTTCATCGATGC-3') primers and the forward (ITS3: 5'-GCATCGATGAAGAACGCAGC-3') and ITS4 primers were used for amplification of ITS1 and ITS2 regions, respectively. The amplicons of each region for individual yeast isolate was mixed and subjected to agarose gel electrophoresis. Species identification was based on the unique pattern for each species. Out of 1200 swab samples obtained and analyzed across the Senatorial zones, a total of 144 (12%) were *C. albicans* positive. In relation to Senatorial zone, each zone had; Nasarawa North (NN) 3.8%, Nasarawa South (NS) 4.2% and Nasarawa West (NW) 4.0%. the RFLP analysis showed that all of the isolates had molecular band weight of 50bp-100bp, with some having other molecular band weights. Eight strains of *C. albicans* strains A, B, C, D, E, F, G and H were identified. In addition, the distribution of *C. albicans* strains A and E as while as B and H were 50.0% and 25.0% respectively.

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## INTRODUCTION

*Candida albicans* is a common commensal fungus in general population (Giovanni *et al.,* 2015; Agada *et al*., 2017)) causing (40%50%) of vulvovaginal candidiasis (VVC) (Umeh & Emelugo, 2011; Yang *et al.* 2015). Genital infection of *C. albicans* is sexually transmitted (Lisboa *et al.*, 2010; Nnadi *et al*., 2012; Nsofor *et al*., 2016). Due to phenotypic resemblance and unavailability of modern tools, this pathogen is readily misidentified in clinical laboratory (Tietz *et al.* 2001; Yang *et al.* 2015). Emerging *Candida albicans* have been detected in cases of candidiasis by using molecular identification (Li *et al*., 2008; Criseo *et al.*, 2015). Precise identification uses molecular techniques such as HWP1 gene amplification or internal transcribed spacer region 2 (ITS2) (Feng *et al.* 2014; Sharma *et al.* 2014; Criseo *et al.* 2015;). Furthermore, *C. albicans* differs in pathogenicity, adherence ability, and biofilm formation (Romeo and Criseo, 2011; Borman *et al.* 2008), necessitating the need to differentiate them in clinical laboratory. Available molecular typing methods include, Restriction Fragment Length Polymorphisms (RFLP), Pulsed-Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), and Internal Transcribed Spacer (ITS) sequencing (Shruti 2014; Giovanni *et al.* 2015; Yang *et al.* 2015). The identification of *C. albicans* as a causative agent of VVC will make the treatment for VVC very specific; and minimize possible treatment failure or recurrent infections due to mis-diagnosis.

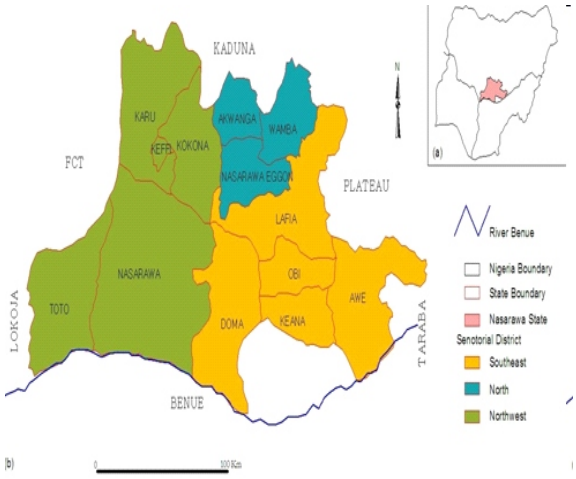


**Figure 1: Map of Nasarawa state showing the 13 LGAs divided into 3 senatorial zones.**


**Figure 2: Map of Nasarawa state showing the study areas.**

## METHODOLOGY

### Ethical Approval

The Ethical approval for this study was obtained from the Ethical Committee on Research of Infectious Diseases of the Dalhatu Araf Specialist Hospital Lafia, Nasarawa State. Also, consent was obtained from the female patients that presented themselves for medical treatment in the Health Centers (HC) of the State before sample collection. The approval was on the agreement that participants' anonymity will be maintained, good laboratory practice/quality / control ensured, and that every finding would be treated with utmost confidentiality and for the purpose of this research only. However, patients that desire to know the results of antifungal susceptibility testing would be given (verbally) free of charge.

### Demographic Data Collection

A well-structured questionnaire were used to collect relevant demographic, clinical and laboratory information of patients.

### Study Centers

Samples were collected from Primary, Secondary and Tertiary Healthcare centers in the three (3) senatorial zones in Nasarawa State, namely: Nasarawa North (NN), Nasarawa South (NS) and Nasarawa West (NW). In Nasarawa North, one (1) each of PHC and GH in Akwanga, Nasarawa Eggon and Wamba, In Nasarawa South, one (1) each of PHC and GH in Lafia, Doma and Obi were selected as study centers. In Nasarawa West, one (1) each of PHC and GH in Toto, Nasarawa, and Keffi were selected. The two (2) Tertiary Healthcare Centers- DASH Lafia and FMC Keffi, were also selected.

### Sample Size Determination and Sample Collection

The sample size was determined based on the prevalence rate of a study carried out by Sapkota *et al,* (2010) as follows:

$N=z^2p(1-p)/d^2$

Where N= patients to be sampled; Z= the standard normal deviation corresponding to a chosen level of confidence = 1.96; P= expected prevalence v (0.2); d= the degree of accuracy desired (2.5%) = 0.025

In our calculation, we used Z = 1.96, *P* = 0.2 and *d* = 0.025. This calculation resulted in a sample size of 1204. This sample size was reduced to 1200 samples to account for the clustered nature of the study design This total sample size was divided by the number of clusters (3 Senatorial Zones) included in the study to determine how many surveys should be administered at each Senatorial Zones. This method of dividing the sample equally among clusters was in accordance with "generic cluster sample" design methods previously described by the WHO Department of Vaccines and Biologicals (Sapkota *et al.,* 2010).

### Sample Collection

A total of One Thousand Two Hundred (1200) High Vaginal Swabs samples (HVS) were collected using sterile swab sticks from consented patients which attended the study centers from month of April To month of October, 2017. Sampling was assisted by specialists' medical doctors who were given the Consent Form to administer to patients.

### Isolation of *Candida albicans*

Inoculation was carried out by stricken high vaginal swab sample on SDA media and incubate at 30°C for 3days for growth. In to a conical flask, 7.9g of SDA was weighed and 200ml of distilled water was added for broth preparation. The conical flask was covered using a stopper and swirled to ensure proper mixing of the dried ingredient and was filtered. After preparation of broth, test tubes and bijou bottles were sterilized and 5ml of the

broth were introduced in to each of them. Using sterilized wire loop, the test organisms were inoculated into each of the test tubes containing 5ml of broth and incubated for 24hours. The broth was standardized by comparing the turbidity to 0.5 Mcfarland standard. 0.5ml of the standardized broth culture was pour plated on a newly prepared SDA plate. Using a sterile wire loop each of the fungal isolate was separately inoculated onto the bijou bottles prepared SDA**.** The inoculated slants were incubated at room temperature for 3 days (Agada *et al*., 2017).

**Identification of *Candida albicans***

The isolates were confirmed by, colonial morphology, microscopic morphology and other biochemical characteristics.

**Colonial morphology (Macroscopy)**

After the incubation the slants were examined visually for important physical appearance (colour, texture, diffusible pigments)Colour: the slants were observed for colour of upside and downside.

Texture:the slants were observed for texture. Diffusible pigment: the reversed side of the slants was observed for diffusible pigments (Agada *et al*., 2017).

**Microscopy**

**Wet/Tease mount (Using lactose phenol cotton blue)**

On clean grease free slide was placed a drop of 95% ethanol. Using a sterile inoculating needle a small portion of the fungal growth was removed midway between the colony center and the edge. With the aid of two disserting needle, the yeast was teased gently such that it thinly spread out in the mounting medium. A drop of lactose phenol cotton blue was added and covered with a cover slip using × 40 objectives. Morphological characteristics of yeast such as budding were observed (Ochei & Kolhatkar, 2000).

**Gram staining**

A small amount of inoculum was taken (to ensure a sparsely single layer of yeast) from an isolated colony on Sabouraud Dextrose Agar (SDA) with a wire loop and emulsified in a drop of distilled water placed on a slide. The prepared smear was air-dried and heat-fixed by passing the slide through a flame a few times, without allowing the slide to become hot. It was then covered with crystal violet solution for 1 minute. The crystal violet stain was poured off, and the smear was rinsed with water and covered with Lugos iodine solution for another 1 minute. The solution was poured off and the slide was rinsed with water. Holding the slide in a tilted position, 95% ethanol was applied several times until no more colour appeared to flow. The slide was then rinsed with water and safranin was applied for 30 seconds as a counter stain. It was then washed, blotted gently and allowed to dry before examination microscopically using oil immersion objective (Ochei and Kolhatkar, 2000)..

**Germ Tube Test**

A small portion of 72hours old isolates of the yeast was suspended in human serum in a test tube. The sample procedure was repeated with known positive (*Candida albicans* and negative control (*Candida tropicalis).* All the test tubes were incubated at $37^0$C for 3hours. A drop of the yeast suspension was placed on a clean grease free slide. It was covered with cover glass and observed under the microscope for presence or absence of germ tubes (a filamentous extension from a yeast cell). Only *C. albicans* produces germ tubes within three hours (3 hrs) at 37°C and formation of chlamydospore. Germ tubes produced by *C. albicans* complex lack constriction (Ochei and Kolhatkar, 2000). Chlamydospores production was assessed by culturing yeast on cornmeal agar at 30°C for 5 days.

**RapID Identification of *Candida albicans***

The isolates collected were biochemically identified using one of the most widely used kit

methods, RapID Yeast Plus System (R8311007). Assimilation profiles were recorded according to the manufacturer's instructions. The isolates were stored at −80°C in Cryo-billes tubes (Laboratoire AES) until genotyping. Prior to molecular testing, isolates were subcultured on Candida ID medium to assess strain viability and purity.

**Selection of isolates for molecular analysis**

Isolates from patients who are positive for *C. albicans* attending health facilities for treatment of volvovaginal candidaisis in Nasarawa State, Nigeria, were obtained from information provided by patients, patients medical records and Physican /Doctor prescriptions in the various health facilities were selected for molecular analysis. Six (6) isolates were selected from senatorial zone. A total of eighteen (18) isolates were selected across the senatorial zones in the state.

**Extraction of *Candida* DNA**

*Candida albicans* were grown on Sabouraud dextrose agar for 24 hr. Cells were harvested, and genomic DdsNA was extracted using the quick Protocle (ZR Fungal DNA miniPrep) DNA extraction kit supplied by Iqaba Biotechnology, South Africa. One hundred mg of *Candida albicans* resuspended in 200µl of isotonic buffer was pipette into a ZR BashingBead Lysis tube, and seven hundred and fifty microlitre (750µl) of genomic lysis buffer was added. The samples were mixed by BashingBead for five minutes and were centrifuge in a microcentrifuge at 10000 x g for one minute. A 400 µl of the superntant was transferred to a Zymo-spin IV Spin Filter (orange top) in a Collection Tube and centrifuge at 7000 x g for 1 minute. 1200 µl of Fungal DNA Binding Buffer to the filtrate in the Collection Tube. 800 µl of the mixture to Zymo-Spin IC Column in a Collection Tube and centrifuged at 10000 x g for 1 minute. The flow through and collection tubes were discarded. The Zymo-spin columns were transfer to a new collection tube and two hundred microlitre (200µl) of DNA Pre-Wash Buffer was added and centrifuge at 10000 x g for one minute. Five hundred microlitre (500µl) of Fungal-DNA Wash Buffer was added to Zymo-Spin IC Column and centrifuged at 10000 x g for1 minute. The Zymo-Spin IC Column was transferred into a 1.5 micro centrifuge tube and one hundred microlitres (100µl) of DNA Elution Buffer was added to the spin column matrix and incubated at room temperature for five minutes and centrifuged at 10000 x g for 30s to elute the DNA (according to manufacturer's instruction).

**Polymerase Chain Reaction (PCR)**

PCR was performed according to standard procedures (Wahyuningsih *et al*, 2000), and all clinical samples were assayed three times in independent experiments. Oligonucleotide primers were derived from rRNA genes of fungi and were used for universal fungi PCR (Wahyuningsih *et al*, 2000). Forward primer ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA -3′) correspon to the 5.8S rRNA gene, and reverse primer ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) corresponds to the 28S rRNA gene of fungi. The biotinylated probe used for hybridization (5′-ATT GCT TGC GGC GGT AAC GTC C-3′) was designed to bind specifically to the ITS2 region of *C. albicans*, located between the 5.8S rRNA gene and the 28S rRNA gene (Wahyuningsih *et al*, 2000). Primers and the biotinylated probe was purchased from Inqaba Biotechnical Industries (Pty) Ltd (South Africa). PCR was performed in a total volume of 100 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, a 200 µM concentration of each deoxynucleoside triphosphate, a 0.5 µM concentration of each primer, 2.5 U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer), and 30 µl of extracted

specimens. Samples were placed in a Perkin-Elmer GeneAmp 2400 DNA thermal cycler. After an initial step of 5 min at 94°C, 35 cycles was performed for 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C. Finally, an additional extension was achieved for 7 min at 72°C, and samples were cooled to 4°C and kept at this temperature until further processing. For positive and negative controls, 30 µl containing 3 ng of purified *Candida* DNA or 30 µl of distilled water, respectively, were processed for DNA extraction with the Quick Protocle (ZR Fungal DNA miniPrep) DNA extraction kit. For visualization, 10 µl of the amplified product was electrophoresed for 30 min at 80 V in a vertical 8% polyacrylamide gel in TBE buffer (0.089 M Tris-HCl, 0.089 M boric acid, 0.002 M EDTA [pH 8.4]), stained for 15 min in 0.5 µg of ethidium bromide/ml, and photographed under ultraviolet illumination. To avoid sample contamination, the precautions suggested by Kwok and Higuchi (Wahyuningsih *et al*, 2000) was followed. Cross-contamination by aerosols was reduced by physical separation of laboratory rooms used for reagent preparation, sample processing, and DNA amplification. Other precautions included UV irradiation of microcentrifuge tubes, racks, surfaces of laboratory benches, and instruments. Laboratory procedures as autoclaving of buffers and distilled water, use of fresh lots of previously aliquoted reagents, combined use of positive-displacement pipetters and aerosol-resistant pipette tips, frequent changing of gloves, premixing of reagents, addition of DNA as the last step, and testing of negative controls, including omission of either the primer or the DNA template during PCR, were carried out. Appropriate negative controls which contained all of the reagents except the template DNA was included for each set of amplification

.

**Agarose gel electrophoresis**

The agarose gel was prepared by dissolving 1.0g of agarose in 100ml of buffer in a 250ml beaker and heat to boiling using hot plates until the agarose dissolved completely. 20ul Ethidium bromide was added to the dissolved agarose solution as dye and mixed. The gel was allowed to cool to about 50°C and was poured into a horizontal gel electrophoresis tank with the casting comb at the red band. The casting comb was then removed when the agarose gel had completely set and solidified. It was removed from the gel tank and transferred carefully into the electrophoresis tank and running buffer (TBE) was added until it covered the gel. Each sample was loaded into the respective gel wells using a micro-pipette after mixing with gel tracking dye-bromophenol (Wahyuningsih *et al*, 2000).

The electrophoresis tank was covered, the electrodes connected, the setup connected to a power pack (Bio-Rad) and set to run at 130v for 25 minutes. The gel was removed from the tank and the band patterns viewed under a trans-illuminator UV light and photographed using an electrophoresis gel documentation system (Bio-Rad).

**Molecular Typing of the Isolates using Restriction Fragment Length Polymorphism (RFLP) analyses**

RFLP was performed according to the method described by Mirhendi *et al*., (2010) to identify *Candida albicans*. To achieve species-specific discriminatory patterns, a 5µl aliquot of PCR products were digested with 5 U of *Bsr*G (Fermentas, Lithuania) in a final volume of 15 µl at 37°C for 2.5 hr. The restriction fragments were separated on 2% agarose gel electrophoresis in TBE buffer for about 2.5 h at 80 V and visualized by staining with 0.5 µg/ml of ethidium bromide. Fragment size polymorphism (FSP) of both ITS1 and ITS2 regions were used for

identification of the strains which were unknown species. Briefly, the forward (ITS1) and reverse (ITS2: 5'-GCTGCGTTCTTCATCGATGC-3') primers and the forward (ITS3: 5'-GCATCGATGAAGAACGCAGC-3') and ITS4 primers were used for amplification of ITS1 and ITS2 regions, respectively. The amplicons of each region for each individual yeast isolate was mixed and subjected to agarose gel electrophoresis. Species identification was based on the unique pattern for each species. Eight oligonucleotides i.e. primers 1 (5'-GGGGGTTAGG-3'), 2 (5'-GGTGTAGTGT-3'), 3 (5'-GTATTGGGGT-3'), 4 (5'-GGTTCTGGCA-3'), 5 (5'-AGGTCACTGA-3'), 6 (5'-AAGGATCAGA-3'), 7 (5'-CACATGCTTG-3'), and 8 (5'-TAGfATCAGA-3') were used in this study. Each amplification reaction was performed in a final volume of 25 µl containing 1 µl of genomic DNA, 1.25 U of *Taq* DNA polymerase, 0.3 mM of each four deoxynucleoside triphosphate,1.5 mM of $MgCl_2$, 0.4 µM of each individual primer and 2.5 µl of 10X PCR buffer. PCR was carried out with the following program: 1 cycle of 5 min at 94° C, followed by 35 cycles of 30s at 95°C, 30s at 30°C and 30s at 72°C and a final extension step at 72°C for 3 min with a final hold of 4.0°C at 00s. Amplified DNA fragments were run onto 2 % agarose gel electrophoresis in TBE buffer at 130V for 25 minutes. The products were detected by staining with ethidium bromide (0.5µg/ml) and photographed.

## RESULTS

**Table 1: *Candida albicans* isolated from women attending health facilities in Nasarawa State, Nigeria**

| Macroscopy | Microscopy/Morphology | Biochemical | Inference | No. (%) Prevalence |
|---|---|---|---|---|
| Smooth creamy Pasty-Coloured colonies | Oval shaped single budded cells. Pseudo hyphae and chlamydospores, Germ tube without constriction. | RapID Yeast Plus System (R8311007) | ***C**andida albicans* | 144 (12.0) |

**Table 2: *Candida albicans* strains isolated from women with suspected cases of vaginal candidiasis attending selected hospitals in the Senatorial zones of Nasarawa State, Nigeria**

| Senatorial zone | No. Resistant isolates | *C. albicans* observed | No. | (%) | *C.* | *albicans* | strains | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | A | B | C | D | E | F | G | H |
| NN | 6 | ABCD | 2(50.0) | 1(25.0) | 2(50.0) | 1(25.0) | | | | |
| NS | 6 | ABCEFG | 1(16.7) | 1(16.7) | 1(16.7) | | 1(16.7) | 1(16.7) | 1(16.7) | |
| NW | 6 | ABEH | 2(50.0) | 1(25.0) | | | 2(50.0) | | | 1(25.0) |

*NN =Nasarawa North; NS =Nasarawa South; NW =Nasarawa*

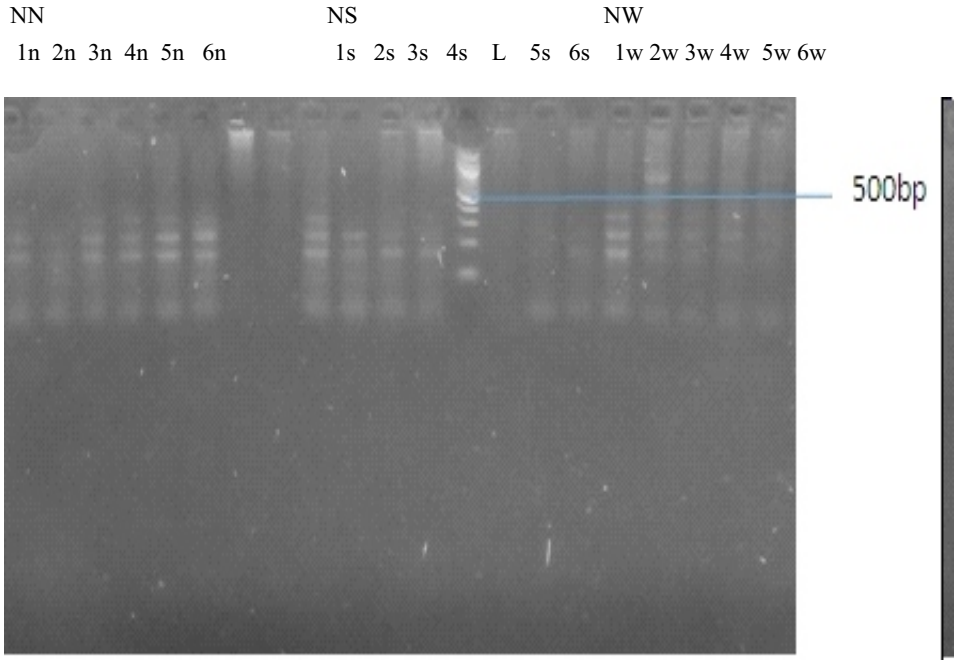


**Plate 1: *Candida albicans* isolates detected by BsrG (restriction enzyme) digestion**

*Key; NN = Nasarawa North Senatorial Zone : 1n= NN 14; 2n= NN 22; 3n= NN 32; 4n= NN 36; 5n= NN 37; 6n= NN 38. NS= Nasarawa South Senatorial Zone: 1s= NS 2; 2s= NS 4; 3s= NS 11; 4s= NS 12; 5s= NS 40; 16s= NS 42. NW = Nasarawa West Senatorial Zone: 1w= NW 6; 2w= NW 11; 3w= NW 27; 4w= NW 31; 5w= NW 36; 6w= NW 47. L= Molecular weight size marker*

## DISCUSSION

The emergence of resistant and rapidly fatal candidiasis, early and fast detection of *Candida* species and their molecular diversity are critical. Existing diagnostic methods using *Candida* blood culture, antigen, or antibody detection lack sensitivity and specificity (Wahyuningsih *et al*, 2000). DNA-based diagnostic tests not only are sensitive and specific but also have the potential to decrease the time taken for the laboratory identification of pathogens that are growing slowly or difficult to culture. In this study, 1200 HVS samples collected, 144 isolates of *Candida albicans* were obtained accounting for 12% which is less than 40% obtained by Rather (2005) and Barbic & Hukie (2010).

DNAs from several bacteria, fungi, and viruses were amplified from serum samples of patients (Wahyuningsih *et al*, 2000). However, the procedures used to extract the genomic DNAs of these microorganisms were rather time consuming and complicated. Difficult and labor-intensive methods for extraction of *Candida* DNA, such as mechanical disruption *Candida* cells followed by protein digestion and DNA

purification with phenol-chloroform, were performed, addition of lysis buffer and chaotropic agents, such as guanidium thiocyanate, followed by DNA precipitation with sodium acetate and isopropanol, and the use of proteinase K and sodium dodecyl sulfate for protein denaturation and extraction of DNA with phenol-chloroform (Wahyuningsih *et al*, 2000). The Quick Protocle (ZR Fungal DNA miniPrep) DNA extraction kit procedure used in our study provides a standardized method and circumvents rather complicated extraction methods by application of ready-to-use columns for purification of genomic DNA. No hazardous reagents are needed anymore, and DNA extraction can be performed in about ten minutes. Quick Protocle (ZR Fungal DNA miniPrep) DNA extraction kit is more expensive than other extraction procedures, but it allows the processing of more samples per unit of time than other methods. Therefore, recommended this method as a first choice for DNA extraction.

RFLP was the first DNA-based marker for constructing genetic linkage maps; it is also one of the most widely used markers in AnGR assessments and breeding program development. By combining this method with PCR (PCR-RFLP), The main advantages of RFLPs include: (1) high reliability, because it is generated from specific sites via known restriction enzymes and the results are constant over time and location. (2) Co-dominance, which means investigators are able to distinguish heterozygotes from homozygotes.( 3) Selective neutrality refers to a situation in which different alleles of a certain gene confer equal fitness. PCR- RFLP fingerprints have been successfully used in defining genetic diversity among different species. (Wanjie *et al*., 2013). In our study, the RFLP analysis showed that all of the isolates had molecular band weight of 50bp-100bp, with some having other molecular band weights. This indicates that the *C. albicans* isolated in this study differs across the Senatorial zones of Nasarawa State . The presence of some isolates with the same molecular band weights among the three Senatorial zones of the State, implies the mobility of *C. albicans* in Nasarawa State, as people infected move or travel from one zone to the other in the State. The result of our study agree with the findings of Muriel *et al.*(2011), that *C. albicans* reference strain (SC5314) had a molecular band weight of 40bp-80bp with strain CBS $562^{T}$ having a molecular band weight of 56bp-90bp. According to the report of Bonfim-mendonca *et al.* (2012), strains are clinical isolates which tend to be genetically similar to each other if they were isolates from patients with same profile. Eight (8) strains of *C, albicans* were identified; strains A-H, which agrees with the findings of Bonfim-mendonca *et al.* (2013), by using epidemiological data, it is possible for the formation of groups with high similarity (90-100%). Also, the commonest *C. albicans* strains distributed in the Senatorial zones were strain A and B respectively. Confirming the mobility of these *C. albicans* strains from one senatorial zone of the state to another.

## CONCLUSION

The commonest *C. albicans* strains distributed in the Senatorial zones of Nasarawa State, were strain A and B respectively. The RFLP electrophoretogram, indicate high similarity between the isolates. RFLP analysis, also confirmed the distribution of these *Candida albicans* strains from one senatorial zone of the State to another. The cost implication may be high, but the overall benefit to the people cannot be overemphasized, as life can be saved through correct diagnosis.

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