Research Article ISSN 2639-9458

Microbiology & Infectious Diseases

RAPD Typing, Antibiotic Resistance Profiling and Genetic Diversity of *Candida* Isolates.

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Received: 25 Sep 2022; **Accepted:** 04 Nov 2022; **Published:** 09 Nov 2022

Citation: Jha V, Giri R, Koli J, et al. RAPD Typing, Antibiotic Resistance Profiling and Genetic Diversity of *Candida* Isolates. Microbiol Infect Dis. 2022; 6(4): 1-8.

ABSTRACT

Candida species is a leading cause of systemic mycosis in hospitalized patients, as well as morbidity and mortality globally. The purpose of this study was to observe the genetic diversity of Candida species among different soils located in different regions of Mumbai Suburban and their drug resistance pattern. A total of 86 soil samples were screened by using Candida Differential Agar and 19 isolates were obtained. Molecular analysis of these isolates revealed that 14 Candida albicans were the most common species, followed by 3 Candida tropicalis and 2 Candida krusei. The drug resistance pattern was studied by performing an Antifungal Susceptibility Test (AST) and determining Minimum Inhibitory concentration (MIC). The isolates did not show much variation in their resistance profile and were all resistant to antifungal drugs except, Amphotericin B. Candida tropicalis was highly resistant to Amphotericin B withstanding a concentration of 500µg/ml. Random amplified polymorphic DNA of Candida species showed that these species were highly similar to each other except for Candida tropicalis which showed a divergence while the other species were in the pair according to dendrogram analysis. The findings of the current study revealed genetic diversity and a correlation between antifungal susceptibility profiles and the genotype groups of Candida species isolated from different soil samples. We also found that Amphotericin B and Voriconazole were the most effective antifungal drugs against different Candida species.

Keywords

Genetic diversity, Molecular Analysis, Antifungal susceptibility, Amphotericin B, Voriconazole, *Candida albicans*, *Candida krusei*, RAPD-PCR.

Abbreviations

PCR: Polymerase chain reaction, TAE: Tris-acetate-EDTA, CLSI: Clinical and Laboratory Standard Institute, IV: Invasive Candidiasis, RAPD: Random amplified polymorphic DNA, AST: Antifungal Susceptibility Test and MIC: Minimum Inhibitory concentration.

Introduction

Out of 1.5 million fungal species, around 300 are known to be virulent toward humans. Fungal pathogens cause invasive, respiratory, skin, and genitourinary tract infections [1]. Overall, this class of pathogen infects a large population per year and if untreated can become a deadly pathogen [2]. *Candida* species, which is responsible for the both invasive and superficial infection, is one of the fungal pathogens [3]. *Candida albicans* reside on the body as a commensal but can cause an opportunistic infection when the host is immunocompromised. Other species of *Candida* include *Candida glabrata, C. parapsilosis, C. krusei, C. tropicalis, C. dubliniensis* and *C. lusitaniae.* [4]**.** Non-albicans *Candida* species like *C. tropicalis* and *C. krusei* have been isolated from natural environments including freshwater [5].

Surgery, hospitalisation in an intensive care unit, and certain illnesses like hematologic malignancy, solid cancer, prematurity,

cardiac disease, trauma, neurologic disease, gastrointestinal disease, organ transplant, pulmonary disease, vascular disease, and HIV infection all increase the risk of invasive infection in the host [6]. Invasive candidiasis (IC) is a fatal infection and around 42% of the cases are reported [7]. Antifungal drugs like Azoles and Polyenes act on different parts of the fungal cell. Azoles damage the ergosterol biosynthesis which is an important part of fungal membrane whereas Polyenes bind to ergosterol and produce pores that facilitate intracellular ion leakage in the fungal cell membrane [8]**.** The increased usage of antifungal medication has caused the species to develop drug resistance [9].

Due to commensal colonisation of mucosal surfaces, antibodies to *Candida* species may be present in healthy individuals, therefore it might be difficult to diagnose Candidiasis via antibody detection [10]. Furthermore, antigens produced by various *Candida* species are frequently eliminated from circulation rendering the antigen-based diagnostic procedures ineffective frequently [6]. Blood cultures of up to 50% of autopsy-proven patients with deep-seated Candidiasis may be negative or only positive during late infection, making microbiological validation even more difficult [11].

For a very long time, sterile blood cultures have been the gold standard for identifying invasive Candidiasis [12]. In addition to culture-based testing, non-culture diagnostic approaches such as antibody, antigen, and polymerase chain reaction (PCR) have recently been brought into clinical practice [13]. These techniques can diagnose and treat a considerably greater proportion of individuals with invasive Candidiasis. The number of molecular typing approaches aimed at differentiating *Candida sp*. from various origins have been proposed. Duplex PCR, Restriction fragment length polymorphisms (RFLP), Randomly amplified polymorphic DNA (RAPD), and microsatellites are among the most well-known employed methods [14]. These traits, together with dendrograms of genetic relatedness among *C. albicans* isolates, have aided lineage investigations during progressive infective episodes or asymptomatic carriage. RAPD has also been used to investigate infections caused by identical or similar strains, formation of resistance strains during antifungal therapy, yeast colonisation patterns in various clinical circumstances and strain microevolution within species [15].

The objective of our research is to investigate and determine the species prevalence of *Candida* sp. by performing biochemical tests and determining drug resistance patterns from various non-clinical samples collected in various parts of Mumbai Suburban. The nucleotide polymorphism of the organisms was also studied using RAPD. Finally, using sequence analysis and multiple sequence alignment-based distance calculations, a phylogenetic relationship among the drug resistant strains was constructed.

Materials and Methods

Isolation and Biochemical Characterization of *Candida* Species

The collected samples comprised of soil adjacent to urinals, sewage water, beach water, and hospital soil from various regions of Mumbai. Microscopical analysis was the first step in the identification of *Candida*, which was followed by growth on Candida Differential Agar (CDA) and PCR [16]. The samples were initially plated on Sabouraud Dextrose Agar (SDA) plates and incubated for 24-72 hours at 37°C. Germ tube staining was followed by a microscopic examination of the colonies. Candida Differential Agar was used to culture colonies that have been identified microscopically as *Candida* and the plates were incubated at 37°C for 48 hours. Post 24-72 hours of incubation, the colonies were observed and the colony characteristics were recorded.

Identification Using Polymerase Chain Reaction for Detection of ITS

The genomic DNA of *Candida* species was extracted with the use of CTAB buffer for cell lysis according to Anerao et al. [17]. Two primers ITS1 (forward) 5-'CTTGGTCATTTAGAGGAAGTAA'-3 and ITS2 (reverse) 3-'GCTGCGTTCTTCATCGATGC'-5 were used to amplify the Internal Transcribed Spacer (ITS) segments in order to identify *Candida* spp. A 25µL reaction mixture consisting of 1µL of DNA samples, 0.5µL of Taq polymerase, 2.5µL of forward and reverse primers, 0.5µL of DNTPs, 2.5µL of PCR Taq buffer, and up to 15.5µL of nuclease-free water was used for the target DNA amplification. There were 35 cycles of heating at 95°C for 60 seconds each, followed by 58°C for 60 seconds, 72°C for 60 seconds and then heating at 72°C for 7 minutes. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and detected using a UV transilluminator along with a 1000 bp molecular DNA ladder [18].

Phylogenetic Analysis

The evolutionary history was inferred using the Neighbor-Joining method [19]. The evolutionary distances were computed using the Tamura-Nei method [20] and are in the units of the number of base substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 19 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 560 positions in the final dataset. Evolutionary analyses were conducted using MEGA11 [21].

Antifungal Susceptibility Test

The disc diffusion method was used to determine the susceptibility of *Candida* isolates towards various antifungal agents [22]. Around two to four well-isolated colonies were inoculated into 4-5 mL Sabouraud dextrose broth and incubated at 37°C for 24 hours. The turbidity of the suspension was adjusted to match the turbidity of 0.5 McFarland turbidity standards (10^7 cells/mL). After adjusting the turbidity, a sterile cotton swab was dipped into the prepared suspension to remove excess fluid and rotated firmly against the inside of the tube. The suspension was then spread on the surface of Sabouraud's agar plates. A 10µL (1mg/mL) dose of the desired antifungal agent was added to sterile Whatman paper discs, which were then placed on the surface of the plate. To ensure contact and

antifungal diffusion, the discs were firmly pressed against the agar surface. The plates were inverted and incubated for 24-48 hours at 37°C [23]. According to CLSI interpretative criteria, the diameter of inhibition zones around each antifungal disc was measured in millimeters and the isolates were classified as susceptible, intermediate or resistant [24]. The antifungal drugs included Amphotericin B (1mg/mL) as a Polyene drug, first-generation Fluconazole (1mg/mL) and second generation Voriconazole (1mg/mL) as an Azoles, and Ketoconazole (1mg/mL) [16].

Determination of Minimum Inhibitory Concentration (MIC) of the Selected Antifungal Drugs against Isolated *Candida* Species

Minimum inhibitory concentration is the lowest concentration of antifungal agent that inhibits the growth of common *Candida* strains after 48 hours of incubation [25]. The MIC of antifungal drugs such as Fluconazole, Ketoconazole, Amphotericin B and Voriconazole was determined [26]. *C.albicans* isolates were grown for 24 hours in Sabouraud's broth and maintained at 10^8 cfu/mL. In a 96-well plate, 100µL antifungal agent, 100µL media, 10µL culture suspension were added as positive controls in the first row, and 100µL media, 10µL culture suspension were added as negative controls in the second row. In the third row of wells, 100µL 2X media, 100µL drug of interest were added and the suspension was serially diluted in a 1:1 ratio in the following wells. In each well, a 10µL culture was inoculated and incubated for 24hrs-48hrs [27]. Post incubation 2mg/mL Resazurin dye was added to each well and incubated for 30 minutes in order to detect growth due to the appearance of pink colour [28]. The lowest inhibitory concentration was observed in the well with the lowest drug concentration and no colour change [9].

Randomly Amplified Polymorphic DNA

The genomic DNA isolation was carried out according to Anerao et al. [17]. RAPD was performed using five random primers (Table 1 for reference). The reactions were carried out by adding 1µL of DNA sample, 2.5µL of RAPD Primer oligonucleotides, 0.5µL of Taq DNA Polymerase, 0.5µL of DNTPs, 2.5µL of PCR Taq buffer and water for a final volume of 25µL in each tube. The reactions were performed using a thermocycler. The thermocycler was programmed to include a pre-denaturation step at 95°C for five min, followed by 35 cycles consisting of 95°C for 30 sec, 46°C for 30 sec and 72°C for 30 sec. The PCR products were analysed by gel electrophoresis in 1% agarose gel (w/v) in 1X TAE buffer (pH= 8.3) at 150 V for 1hr. Amplicons in the gel were stained with ethidium bromide (0.5 mg mL^{-1}) and visualized under UV transilluminator [29].

Table 1: Random Primer Sequences for RAPD analysis.

Primer ID	Sequence
RP20	ACGATGAGCG
RP12	AACGCGTCGG
RP13	AAGCGACCTG
RP14	AATCGCGCTG
RP15	AATCGGGCTG

Dendrogram Analysis

Dendrogram analysis was performed with the help of RAPD matrix scoring [30]. The matrix scores were obtained by scoring the bands obtained on agarose gel on running the RAPD products by scoring 1 for a positive band and 0 for a negative band (no band). The scoring matrix data was uploaded to the online tool "DendroUPGMA" for dendogram analysis with a set parameters according to Ranade et al. [31].

Results and Discussion

Preliminary Fungal Strain Identification

86 Soil samples were collected from various locations in Mumbai Suburban which included soil samples from the vicinity of hospitals, beaches and urinal sites. The samples were diluted in 0.8% saline and plated on Sabouraud's dextrose agar, and incubated for 48 hours. Post incubation, 19 colonies thus obtained were streaked on Candida Differential agar for preliminary identification of *Candida* species. From the colonies obtained, 14 of the isolates exhibited fuzzy green colour colonies, normally exhibited by *C.albicans*, 3 isolates exhibited purple and 2 exhibited metallic blue coloured colonies, an exclusive feature of *C. krusei* and *C. tropicalis* [32]. Out of 19 isolates, 11 isolates showed formation of elongated germ tube and 3 exhibited formation of partial germ tube confirming the presence of 14 *C. albicans* and 5 Non-albicans Candida species.

Molecular Identification Using Genomic DNA

Molecular identification using genomic DNA was carried out for absolute confirmation of the strains. Genomic DNA from all 19 isolates were extracted and PCR amplification was conducted using primers for 5.8s RNA ITS1 sequence. The amplified product was sequenced which confirmed the observation of the preliminary identification of the isolates.

Phylogenetic analysis

Phylogenetic analysis based on nucleotide sequence data has been widely utilised to support and extend more traditional taxonomic classifications. The 5.8s RNA ITS1 sequence obtained for each isolate was aligned and analysed by MEGA XI. The distance between each isolate was computed and a phylogenetic tree was constructed by Neighbor-Joining method. It was found that 13 *Candida albicans* form a closely related clade (*C. albicans*) with similar sequences. While the other 2 *Candida albicans* (R11 and R14) showed less divergence to *Candida tropicalis* and *Candida krusei*. All *Candida* species were highly relative to each other Figure 1.

Antifungal Resistance Profiling

a. Kirby Bauer disc diffusion assay

The Antifungal resistance profiles were studied for the 19 *Candida* isolates obtained by using Kirby Bauer disc diffusion assay. All the isolates exhibited complete resistance towards Fluconazole and Voriconazole, mild sensitivity was observed for Amphotericin-B and Ketoconazole. Not much variation was observed with respect to the resistance pattern of different *Candida* species for any of the drugs (Table 2).

Figure 1: Neighbor-Joining method phylogeny reconstructed using 19-nucleotide sequences. Total of 560 positions were obtained in final dataset. The associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

Table 2: Observed Zone of inhibition towards 19 *Candida* isolates by drugs through Kirby Bauer Disc Diffusion Assay.

Name of organisms	Fluconazole Zone diameter(mm)	Ketconazole Zone diameter(mm)	Amphotericin B Zone diameter(mm)	Voriconazole Zone diameter(mm)
Candida albicans	Sensitive	Sensitive	Resistant	Resistant
Candida albicans	Resistant	Resistant	Resistant	Resistant
Candida albicans	Resistant	Sensitive	Resistant	Resistant
Candida albicans	Resistant	Sensitive	Resistant	Resistant
Candida albicans	Resistant	Sensitive	Resistant	Resistant
Candida albicans	Sensitive	Sensitive	Resistant	Sensitive
Candida albicans	Resistant	Resistant	Resistant	Resistant
Candida tropicalis	Resistant	Sensitive	Sensitive	Resistant
Candida krusei	Resistant	Sensitive	Resistant	Resistant
Candida krusei	Resistant	Sensitive	Resistant	Resistant
Candida albicans	Resistant	Sensitive	Sensitive	Resistant
Candida albicans	Sensitive	Sensitive	Sensitive	Sensitive
Candida albicans	Sensitive	Sensitive	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Sensitive	Sensitive	Sensitive	Sensitive
Candida albicans	Resistant	Sensitive	Sensitive	Resistant
Candida tropicalis	Resistant	Resistant	Resistant	Resistant
Candida tropicalis	Resistant	Sensitive	Resistant	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant

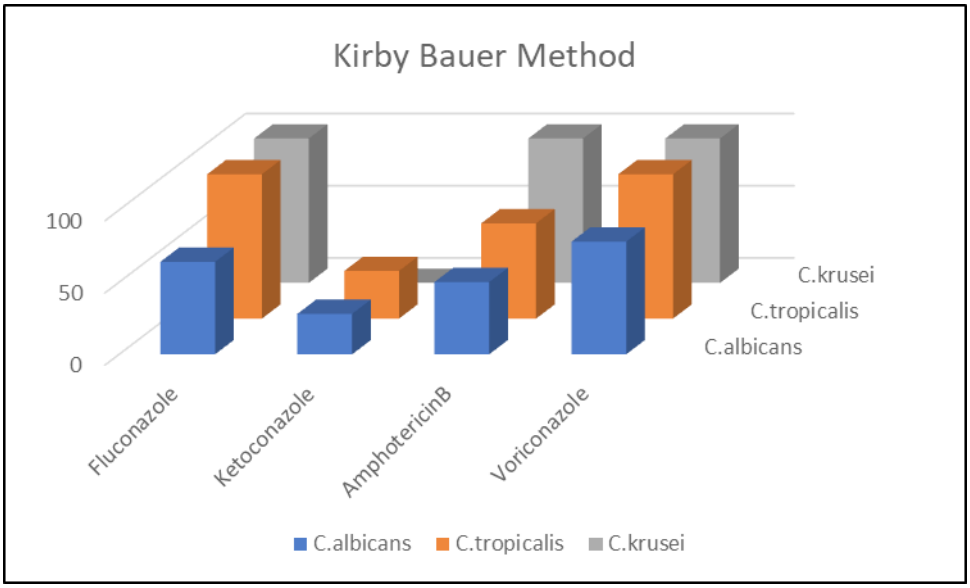

Figure 2: Percentage resistance of *Candida* species to different drugs obtained by performing Kirby Bauer method.

b. Minimum Inhibitory Concentration

The Minimum inhibitory concentration assay was performed using Amphotericin B, Fluconazole, Ketoconazole and Voriconazole followed by Resazurin viability assay for testing the viability of the isolates after treatment with the Antifungal agents. Except the *C.tropicalis* isolate, all the other isolates obtained were sensitive to Amphotericin B. All of the isolates showed resistance against all the 3 Azoles tested excluding *C.tropicalis* which was sensitive to Voriconazole (Table 3).

Table 3: Minimum Inhibitory Concentration detected by Resazurin Reduction Dye Test for 19 probable isolates of *Candida.*

Name of organisms	Fluconazole (µg/ml)	Ketoconazole (µg/ml)	Amphotericin-B (µg/ml)	Voriconazole (µg/ml)
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Sensitive
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida tropicalis	Resistant	Resistant	Resistant	Resistant
Candida krusei	Resistant	Resistant	Sensitive	Resistant
Candida krusei	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant
Candida tropicalis	Resistant	Resistant	Sensitive	Resistant
Candida tropicalis	Resistant	Resistant	Resistant	Resistant
Candida albicans	Resistant	Resistant	Sensitive	Resistant

Genome Fingerprinting

To analyse genetic diversity among *Candida* species RAPD was performed. Random Amplified Polymorphic DNA analysis of the genomic DNA of all isolates was carried out by 5 different random primers as mentioned in Table 2. The PCR product obtained was separated in 2% Agarose gel that showed the polymorphism pattern of each isolate. The amplified product was obtained with primer RP20 for almost all organisms except for R4(*Candida albicans)* and R9 (*Candida krusei)*, R14 *(Candida albicans)*. Other primers resulted in less or no product amplification for all organisms. By observing the position of each polymorphic band, a matrix scoring was provided to each organism with respect to the primers used. With the help of matrix score the dendrogram was created and the relationship between each isolate was established (Figure 2).

Dendrogram Analysis

The dendrogram was constructed on unweighted pair group method of analysis (UPGMA), by Pearson co-efficient method using http://genomes.urv.cat/UPGMA/ software. This revealed the genetic correlation among isolates based on a matrix constructed from polymorphic patterns displayed by 19 isolates in RAPD. It was found that R1 (*Candida albicans*) and R2 (*Candida albicans*) showed 100% similarity but were left out from the main cluster. R17 (*Candida tropicalis*) and R18 (*Candida tropicalis*) resistant to Amphotericin B showed similarity of only 12% while remaining formed a main cluster with high similarity index. The Cophenetic Correlation Coefficient (CP) was found to be 0.728.

Conclusion

Candida species are the most common fungal pathogens causing various invasive infections. Changes in the *Candida* sp. distribution and its antifungal resistance profile may affect the treatment recommended for the non-clinical candida infections. As observed, the prevalence of *Candida albicans* was average in different soil samples collected from various locations of Mumbai Suburban and was highest in urinal soils. The isolates did not show major variation in their resistance profile and were significantly resistant to Anti-candida drugs especially Azoles.

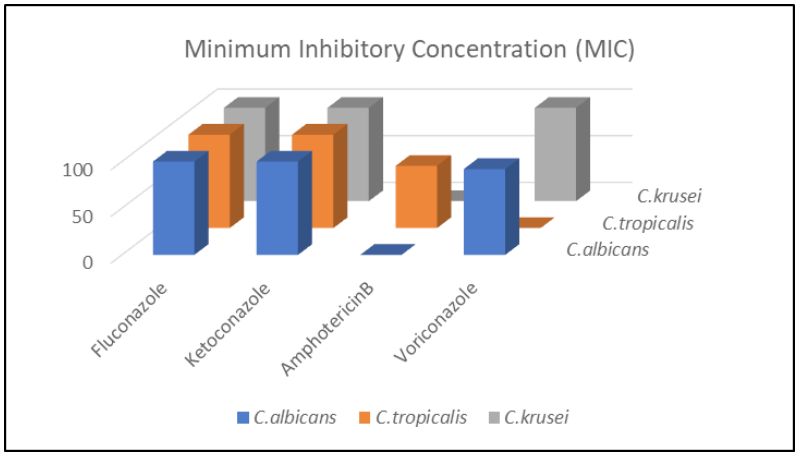

Figure 3: Percentage resistance of *Candida* species to different drugs obtained by performing Minimum Inhibitory Concentration of Drugs.

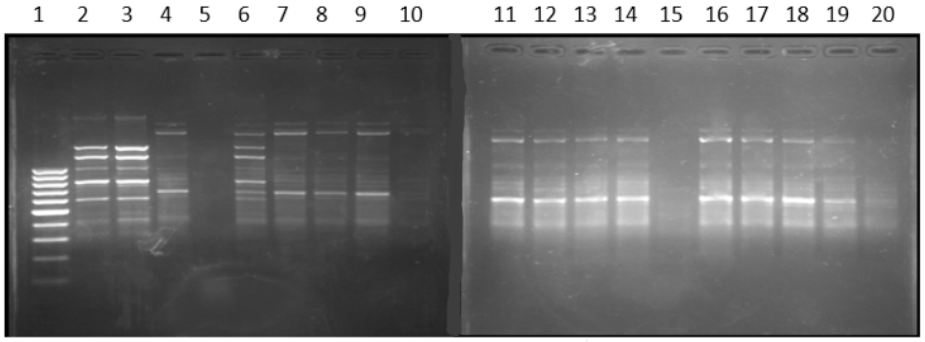

Figure 4: Rrepresentative of RAPD analysis of 19 *Candida* isolates using RP20 primer.

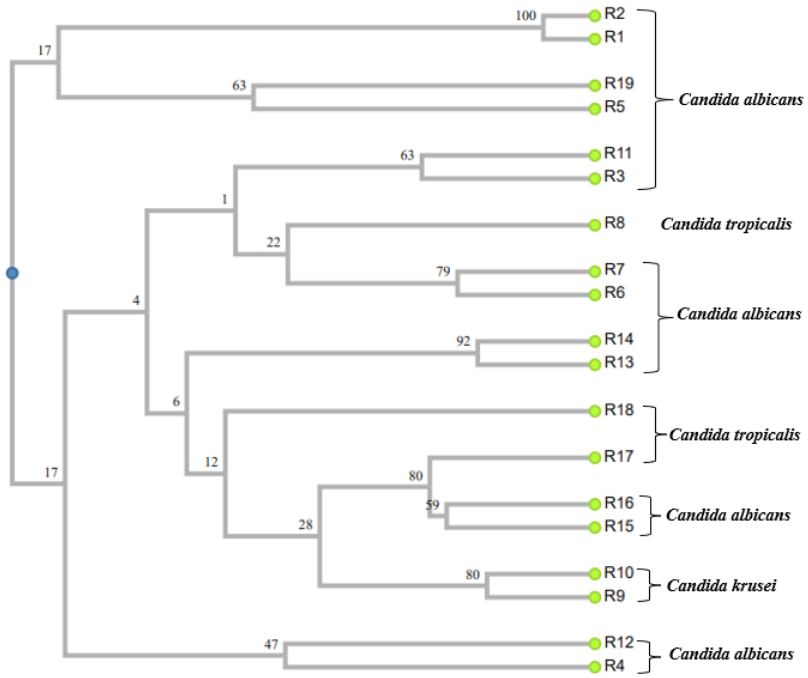

Figure 5: Dendrogram by Pearson co-efficient method for 19 isolates of *Candida spp. R1(C. albicans), R2(C.albicans), R3(C.albicans), R4(C.albicans), R5(C.albicans), R6(C.albicans), R7(C.albicans), R8(C.tropicalis), R9(C. krusei), R10(C.krusei), R11(C.albicans), R12(C.albicans), R13(C.albicans), R14(C.albicans), R15(C.albicans), R16(C.albicans), R17(C.tropicalis), R18(C.tropicalis), R19(C.albicans)*

Amphotericin-B showed inhibition against the isolates; thus, these resistance profiles provided concrete evidence against increasing MDR and CDR of *Candida albicans*. Using Random Amplified Polymorphic DNA, the polymorphism pattern of each *Candida* Species was observed. They exhibited high similarity index, and least phylogenetic divergence, pointing towards the genetic congruency amongst these obtained isolates. Lastly, for accurate species identification, standard antifungal susceptibility testing was found to be necessary, which in turn could aid in controlling the spread of resistant *Candida* strains.

Author Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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