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Distribution and Antifungal Susceptibility of*Candida* Species Isolated from Clinical Specimens at the University Teaching Hospital, Lusaka, Zambia

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ABSTRACT:-Background:*Candida* species have emerged as successful pathogens worldwide and are associated with immunocompromised patients. Additionally, there is increasing resistance of *Candida* species to antifungal agents, and this has greatly contributed to the high morbidity and mortality amongst affected patients. In Zambia, little is known about the distribution of *Candida* species and their antifungal susceptibility patterns. Speciation of *Candida* species is important as knowledge of the infecting species is important for guiding therapy. The objective of this study was, therefore, to characterise *Candida* species isolated from different clinical specimens at the University Teaching Hospital in Lusaka.

Methods: This was a cross-sectional study involving the identification of 96 *Candida*species from various clinical specimens, and determination of their antifungal susceptibility patterns. Identification of the isolates was achieved by the use of the API 20C AUX kit, followed by DNA sequencing of the Internal Transcribed Spacer region of the ribosomal DNA, whilst the agar-based E-test, using fluconazole, amphotericin B, flucytosine, and caspofungin, was used for antifungal susceptibility testing.

Results: Data obtained showed that *Candida albicans* were the predominant species (66.7%), followed by *C. lusitaniae* (12.2%), *C. glabrata* (6.7%), *C. tropicalis* (5.6%), *C. parapsilosis* (3.3%), *C. quilliermondii* (3.3%), *C.pelliculosa* (1.1%) and *C. keyr* (1.1%). Most of the *Candida* species exhibited high levels of resistance to fluconazole and amphotericin B, but were sensitive to caspofungin and flucytosine. *C. albicans* was resistant to fluconazole (18.3%,) with an MIC_{90} of 256μg/ml and amphotericin B (10%) with MIC_{90} of 1.5µg/ml. *C. glabrata* was the most resistant species against amphotericin B (66.6%) with an MIC_{90} of 2µg/ml.*C. albicans* and most of the non-albicans species exhibited multi-drug resistance.

Conclusion: This study demonstrated that identification of *Candida* species to species level and susceptibility testing are important for accurate treatment of *Candida* infections.

Key Words: *Candida* species, Antifungal susceptibility, Multi-drug resistance, Zambia.

I. INTRODUCTION

Fungal infections caused by yeasts of the *Candida* genus are a major problem especially in immunocompromised patients or those hospitalised with underlying disease [1,2]. *Candida* species are frequent colonizers of the skin and mucous membranes of animals and their dissemination in nature is widespread [3,4] There are over 350 heterogeneous *Candida* species but only a few have been implicated in human disease [5].

Although the majority of *Candida* infections are attributable to *Candida albicans*, there has been an increase in the rate of infections caused by non-albicans in various parts of the world [6,7,8]. Among these species *C. glabrata* has emerged as one of the most important opportunistic pathogens to infect a variety of human body sites [9,10], while *C.parapsilosis* often represents the second most commonly isolated *Candida* species from blood cultures in many parts of the world [11,12]. Other species are rarely encountered in clinical samples although there have been several reports describing infections caused by uncommon *Candida* species [13].

Identification of *Candida* species from clinical specimens has become increasingly important as a result of increasing numbers of immune-suppressed patients, increasing use of indwelling medical devices, immunosuppressive therapy and broad-spectrum antibiotics [9,14]. Many clinical laboratories, however, do not speciate the *Candida* species isolated from clinical samples unless specifically requested. Thus, local changes or trends in infection causing species are difficult to determine. In addition the intensive and long term use of antifungal drugs lead to a decline in susceptibility and resistance patterns of *Candida* species [15]. It should also be noted that *Candida* species differ in their antifungal susceptibility and virulence factors [16,17]

The objective of this study was to identify and determine the antifungal susceptibility patterns of *Candida* species isolated from various clinical specimens at the University Teaching Hospital, a tertiary referral hospital in Zambia.

II. MATERIAL AND METHODS

Study site

This was a laboratory-based cross-sectional study on 96*Candida* species isolated from 1456 clinical specimenthat included urine, sputum, high vaginal swab (HVS) and blood submitted to the Microbiology Laboratory from July, 2013 to September, 2014 at the University Teaching Hospital, Lusaka. Zambia.

III. ISOLATION OF *CANDIDA* SPECIES

Candida isolates were isolated from routine clinical specimens that included urine, sputum, HVS, and blood in the Microbiology Laboratory at University Teaching Hospital. The isolates were sub-cultured on Sabouraud s' Dextrose Agar (Mast Diagnostics, Merseyside, UK) labelled and stored in vials containing 50% glycerol at -70^{o}C for further analysis.

Phenotypic Identification of *Candida* species.The isolates were identified by API 20 C AUX kit (bioMerieuxSA, 69280 Marcy I'Etoile, France). The test was performed and interpreted according to the manufacturer's protocol.

IV. GENOTYPIC IDENTIFICATION OF *CANDIDA* SPECIES

1. Preparation of DNA Thermolysates

Genomic DNA used in this study was primarily prepared by thermo lysis of fresh *Candida* cells. Briefly, a loopful of yeast cells from Sabourand Dextrose Agar (Mast Diagnostics, Merseyside, UK) were transferred into a micro centrifuge tube containing 400µl of 1x TE buffer (10mM Tris-HCl [pH 8.0], 0.1mM EDTA [pH 8.0]) and boiled at 100^{o}C for15min. After cooling, the DNA thermolysate was then stored at -20^{o}C until required.

2. PCR Amplification of the Internal Transcribed Spacer Region

The sequence were first amplified by PCR in a final volume of 50µl containing 10µl of the *Candida*thermolysate and 40µl PCR master mix: 200µM of each dNTP, 2µM of each primer, ITS1-F(5'-TCCGTAGGTGAACCTGCGG-3') and ITS1-R (5'-GCTGCGTTCTTCATCGATGC-3'), 1x Mastermix (50mM KCl, 10mM Tris-HCl [pH 8.3], 1.5mM $MgCl_2$, 0.001 [wt/vol] gelatine, and 1.25U of *Taq*polymerase [Fermentas Life Sciences, Glen Burnie, MD, USA]). The PCR reaction was set for 10min at 95°C and then subjected to 35 cycles of amplification for 45 seconds at 95°C, 45 seconds at 55°C, 1 min at 72°C, followed by a 10 min extension step at 72°C on a Gene Amp 2700 PCR thermal cycler (Applied Biosystems, CA, USA). The presence of the ITS1 DNA sequence was detected by electrophoresis of 5µl of the PCR amplicons on a 1.5% SeaKem LE agarose gel **(**Lonza, Rockland, ME, USA) containing 0.5µg/ml ethidium bromide. A 50bp DNA ladder (Thermo Scientific, Handover MD, USA) was used as a molecular weight standard, and all gels were visualised using the BiotopBiosens SC-645 Gel Documentation System (Biotech Co. Ltd, Shanghai, China).

3. DNA Sequencing

Prior to sequencing, the PCR products were first purified with the GeneJET PCR purification kit (Thermo Scientific, Hanover, MD, USA) by following the manufacturer's instructions. Forward and reverse linear amplification was performed in 10µl using 2µl of purified PCR product (about 20 to 200ng), 2µl BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems Foster City, CA, USA), 1µl BigDye Sequencing Buffer (Applied Biosystems Foster City, CA, USA) and 1µM of ITS1-F or ITS1-R primer. Linear amplification consisted of 25 cycles of denaturation at 96^{o}C for 10s, annealing at 60^{o}C for 30s and elongation at 72^{o}C for 60s using Gene Amp 2700 PCR thermal cycler (Applied Biosystems, CA, USA). Fluorescence-labelled DNA was purified using the ethanol precipitation method (Ausubel*et al*., 2001). Briefly, the entire extension were transferred into 80µl of freshly prepared precipitation solution (3µl of 3M sodium acetate [pH 4.6], 62.5µl of non-denatured 95% ethanol and 14.5µl deionised water), incubated for at least 1hr at room temperature and centrifuged at 14000 rpm for 20min. After carefully removing the supernatant, 250µl of 70% ethanol was added to the pellet, vortexed and the contents re-centrifuged at 14000 rpm for 8 min. The ethanol was carefully aspirated and the pellet air-dried for 15 mins at room temperature. The samples were analysed at the Inqaba Biotechnical Industries Sequencing Facility (Pretoria, South Africa) on an ABI PRISM 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA). The sequence reads were edited using RidomTraceEdit Software (Ridom Bioinformatics GmbH, Würzburg, Germany). The obtained sequences were compared with those available in GenBank by BLAST searches. Sequences that showed at least 98% sequence identity when compared to those in GenBank were preliminary considered as identified species [18].

Antifungal Susceptibility Testing

The antifungal susceptibility testing of isolates in this study was performed by the agar-based Epsilometer test **(**E-test) method. RPMI-1640 agar (Remel, Lenexa, USA) supplemented with 2% glucose and morpholinepropanesulphonic acid buffer (MOPS) (Remel, Lenexa, USA) in 130mm diameter plates was used. The plates were inoculated by dipping a sterile swab into a yeast cell suspension adjusted to 0.5 McFarland standard units (10^6 cells/ml) using a turbidometer (Oxoid Integrated Technologies Ltd, England). The standardised inoculum was then streaked across the surface of the agar in four directions. The plates were dried at ambient temperature for 15 minutes before applying the E-test strips. The *Candida* isolates were tested against the following antifungal agents: 0.016-256µg/ml fluconazole (LiofilChem Diagnostic Ltd, Italy), 0.002-32µg/ml flucytosine (LiofilChem Diagnostic Ltd, Italy), 0.002-32µg/ml amphotericin B (LiofilChem Diagnostic Ltd, Italy) and 0.02-32µg/ml for caspofungin (LiofilChem Diagnostic Ltd, Italy). These antifungal agents are used for the treatment of *Candida* infections in Zambia. The minimum inhibition concentrations (MIC) endpoints were determined after 24 and 48 hours of incubation at 35^{o}C. The resistance breakpoints for antifungal agents were as follows: fluconazole ≥64µg/ml, amphotericin B ≥1.0µg/ml, flucytosine ≥32µg/ml, and caspofungin ≥ 32µg/ml [19,20]

V. DATA ANALYSIS

The data was analysed using the Strata software version 12.1 (Strata, California, USA). Categorical variables were compared using t-test and this included susceptibility to fluconazole and amphotericin B. The tests were interpreted at 5% significance level (two-sided) and 95% Confidence Interval. A p-value of < 0.05 was taken as indication of statistical significance.

VI. ETHICS CONSIDERATIONS

Ethics approval for this study was granted by University of Zambia Biomedical and Research Ethics Committee. The ethics clearance certificate Number 004-05-14.

VII. RESULTS

Out of the 96 culture isolates, 93.7% (90/96) were identified as *Candida* species, while 6.3% (6/96) were non-*Candida* species. These organisms were isolated from urine, sputum, HVS and blood. The predominant species was *C. albicans* (66.7%, 60/90), followed by *C. lusitaniae* (12.2%, 11/90), *C. glabrata* (6.7%, 6/90), *C. tropicalis* (5.6%, 5/90), *C. parapsilosis* (3.3%, 3/90), *C. guilliermondii* (3.3%, 3/90) *C. pelliculosa* (1.1%, 1/90) and *C. kefyr* (1.1%, 1/90) (Figure 1).Urine was the most common source of *Candida* species, accounting for 41.1% (37/90) of the isolates, followed by sputum (23.3%, 21/90), HVS (22.2%, 20/90) and blood (13.3%, 12/90) (Table 1).To confirm the identity of the *Candida* species identified in the fore-going section, PCR and DNA sequencing of the ITS1genomic region of the isolates was carried out (Figure 2). This also included the identification of the 6 unidentified species isolated from the different specimens. Using DNA sequencing it was noted that two isolates of *C. albicans* were misidentified as either *C. famata* or *C. krusei*, while one isolate of *C. tropicalis* was misidentified as *C. parapsilosis* by phenotypic method (Table 2). The six unidentified species were identified as being *Pichia kudrianvzevii* isolated from urine, *Cryptococcus neoformans* isolated from blood, *Saccharomyces cerevisiae* from blood, *Trichosporumjaponicum* from urine, and two species of *Cyberlindnerafabianii* from blood (Table 3).

C. albicans was resistant to fluconazole (18.3%, 11/60), amphotericin B (10%, 6/60), flucytosine (8.3%, 5/60) and caspofungin (3.3%, 2/60). *C.lusitaniae* showed resistance to fluconazole (22.2%), and amphotericin B (22.2%). Both *C. glabrata* and *C. tropicalis* strains were resistant to amphotericin B by 66.6% and 30%, respectively. *C. parapsilosis* was only resistant to flucytosine (33.3%), while *C.quilliermondii*was resistant to fluconazole, amphotericin B, flucytosine and caspofungin by 33.3%. *C. pelliculosa* and *C. kefyr*strains were sensitive to all the antifungal agents tested (Table 3). The lowest MIC_{50} observed for flucytosine was 0.004µg/ml for *C. lusitaniae* and *C. glabrata* strains. The highest MIC_{50} observed for fluconazole was 256µg/ml for *C. quilliermondii*. The MIC_{50} for *C. albicans*, the most isolated species, were 2µg/ml for fluconazole, 0.75µg/ml for amphotericin B, 0.016µg/ml for flucytosine, and 0.25µg/ml for caspofungin. The lowest MIC_{90} observed for flucytosine was 0.004µg/ml against *C. glabrata*, and the highest MIC_{90} observed for fluconazole was 256µg/ml against *C. albicans*, *C. tropicalis* and *C. quilliermondii.* The MIC_{90} for fluconazole against *C. lusitaniae* and *C. glabrata* were 96µg/ml and 16µg/ml, respectively (Table 3).
Most of the drug resistant *Candida* species (79.3%, 23/29) were from HIV- positive than from HIV-negative (20.7%) patients (Figure 2).*C. albicans* was the most drug-resistant strain in both HIV-positive (56.5%) and HIV-negative(50%) patients (Figure 3).The Mean MICs of antifungal agents on *Candid*a species from HIV-positive and HIV-negative participants were 52.6 and 27.9, respectively, for fluconazole ($p = 0.17$). Those for amphotericin B were 1.66 for HIV-positive and 2.39 for HIV-negative individuals ($p = 0.51$), while those for

flucytosine were 3.73 for HIV-positive and 1.58 for HIV-negative individuals (p = 0.24). The Mean MICs for caspofungin was 2.11 for HIV-positive and 1.97 for HIV-negative individuals (p = 0.92) (Table 4).
Multi-drug resistance to a combination of four and two antifungal agents was observed in 5 *Candida* isolates. Multi-drug resistant was defined as isolates being resistant to more than two classes of antifungal agents. *C. albicans showed* 40% MDR pattern to fluconazole, amphotericin B, flucytocine and caspofungin, while, *C. lusitaniae* and *C. quilliermondii* showed 20% MDR pattern to all antifungal agents tested (Table 5).

VIII. DISCUSSION

In recent years, *Candida* infections in hospitalised patients have been on the increase [21,22]. This increase has been associated with surgical interventions, intensive care treatment, extreme of age, metabolic disorders, neutrophil dysfunction, and immunodeficiency states among others [23]. This calls for accurate identification of *Candida* species to select the most effective therapeutic strategies to control invasive fungal infections [24,25]. Data presented in this study demonstrate the occurrence of a variety of culturable fungal species obtained from different clinical specimens, which were mainly *Candida* species and a few non-*Candida* species. Among the *Candida* species, the predominant isolate was *C. albicans*, followed by *C. lusitaniae*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. pelliculosa* and *C. keyr*. The non-*Candida* species included *Pichia kudrianvzevii*, *Cryptococcus neoformans, Saccharomyces cerevisiae, Trichosporumjaponicum* and *Cyberlindnerafabianii.* An important observation in our study is that the commonly isolated *Candida* species was *C. albicans*, and this corroborates with findings in other studies worldwide [26,27,28]. The predominance of this species may be attributed to virulence, conferring upon its enhanced capacity for colonisation and pathogenic activity for humans [29,30].*C. albicans* is the predominant species regardless of the immune status of the patient [31,32]. Another significant finding was that a number of non-albicans species were also isolated which shows their importance as pathogens in clinical specimens. This was consistent with Tanzanian and Iranian studies which also showed that *C. albicans* was a dominant species, while non-albicans *Candida* species were present in lower proportions [21,33].Phenotypic methods employed in this study misidentified three *Candida* isolates, and failed to identify the non-*Candida* fungal isolates: *Pichia kudrianvzevii*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, *Trichosporumjaponicum* and *Cyberlindnerafabianii.* However, with the aid of molecular methods using the ITS region, the isolates were correctly identified.

The antifungal susceptibility testing is an important tool in the management of *Candida* infection because it promotes accurate administration of antifungal agents, and as an aid in drug development as well as a means of tracking the development of antifungal resistance in epidemiologic studies [34,35,36]. High resistance to fluconazole and amphotericin B, with high MIC levels, was observed with *C. albicans*, *C. lusitaniae* and *C. glabrata,* which were the most, isolated species in this study. There was a significant difference in the resistance pattern of *C. albicans* between fluconazole and amphotericin B (p = 0.001). Similar observations were made in an Iranian study in which *C. albicans* isolates were resistant to fluconazole, and usually have a poor response to treatment [37].The isolates of *C. glabrata* were the most resistant of all the *Candida* species against amphotericin B. This was consistent with a South African study which showed *C. glabrata* with high resistance to amphotericin B, and typically exhibited reduced susceptibility to amphotericin B [38].

The isolates of *C. guilliermondii* and *C. parapsilosis* showed relatively higher caspofungin MICs compared to other *Candida* species in this study. This was in keeping with studies conducted in South Africa and Tanzania where high caspofungin MICs were reported against *C. guilliermondii* and *C. parapsilosis* [33,39].Comparing the Mean MIC values in this study, *Candida* isolates from HIV-positive patients showed higher values of resistance than isolates from HIV- negative patients. These results corroborate with findings in South African studies where drug resistance was reportedly high in HIV-positive than HIV-negative patients [40].The MDR observed in five isolates of *Candida* isolates, with *C. albicans*, being the predominant resistant, mostly to fluconazole and amphotericin B. In fact all the three species of *C. albicans* that showed an MDR pattern were isolated from HIV-positive patients. These findings on MDR pose serious clinical challenges as they seem to suggest the emergence of MDR *Candida* strains in hospitalised patients at the University Teaching Hospital in Lusaka.

IX. CONCLUSION

This study demonstrated that *C.albicans* was the predominant *Candida* species causing infection at the University Teaching Hospital, and corroborates findings from other parts of the world. *C. lusitaniae* and *C. glabrata* were the most common non-albicans species isolated. Most of the organisms were isolated from HIV-positive patients, and were the most drug resistant. *C. albicans, C.lusitaniae* and *C. glabrata* were the most resistant species to all the antifungal agents tested. Caspofungin was the most effective drug compared to fluconazole and amphotericin B, two of the most widely used antifungal agents at the University Teaching Hospital. This study was the first to characterise *Candida* species using both phenotypic and molecular methods, and determine the antifungal susceptibility patterns in Zambia.

X. COMPETING INTERESTS

The author declare that they have no competing interests

Authors' contributions

KLS, CLS and GK conceived and designed the study, analysed the data, and drafted the manuscript. MTS, AK and JM performed microbiological works. JCLM and TK helped study design and draft of the manuscript. All authors reviewed and approved the final manuscript.

XI. ACKNOWLEDGEMENTS

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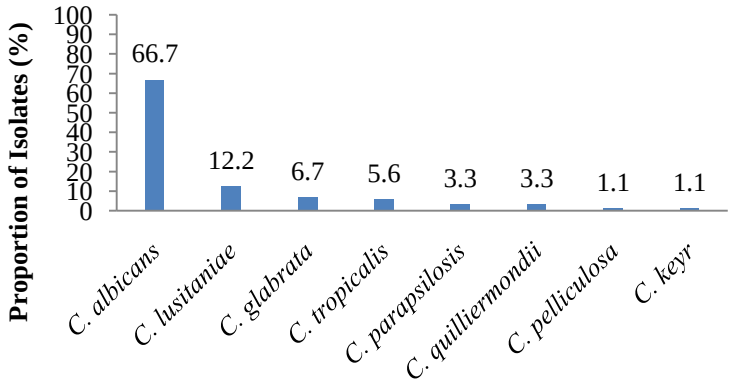

Figure 1: Diversity of *Candida* species isolated from different clinical specimens

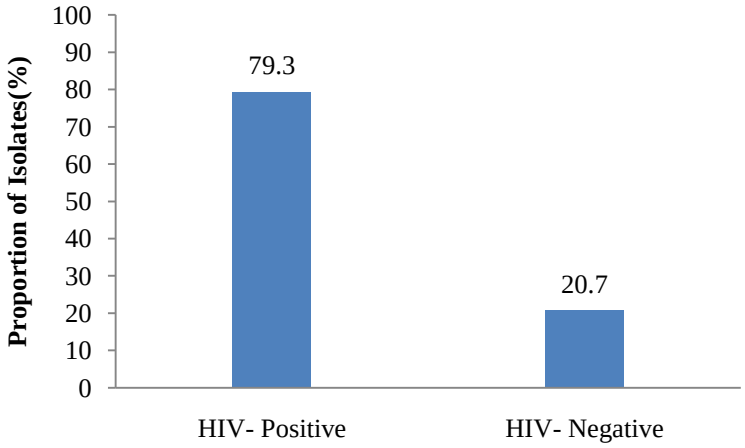

Figure 2: Comparative distribution of resistant *Candid*a species from HIV- negative and HIV- positive patients

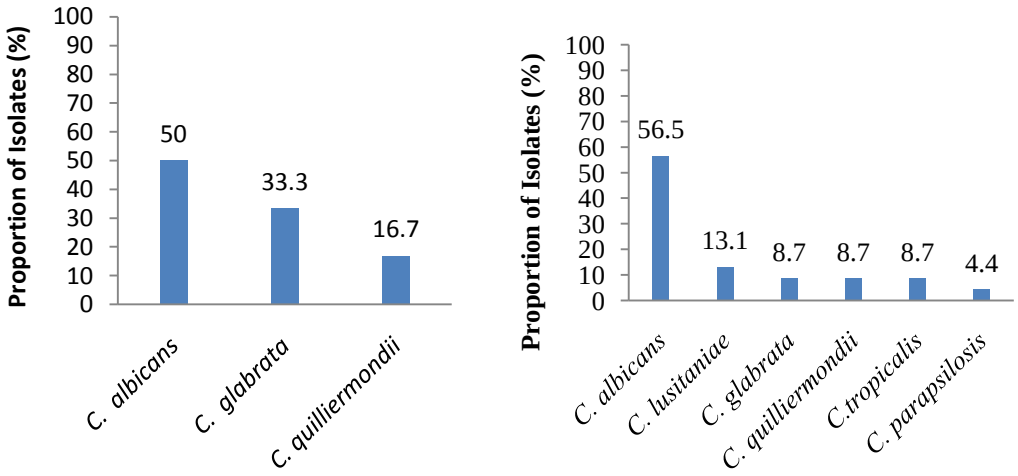

Figure 3: Distribution of drug-resistant *Candida*species from A) HIV-positive and B) HIV-negative patients

Table 1: Distribution of *Candida* species by specimens

	Types of specimen				
Species	Urine(%)	Sputum(%)	HVS(%)	Blood(%)	Total %
C. albicans	22 (59.5)	18 (85.7)	18 (90)	2 (16.7)	60 (66.7)

C. lusitaniae	4 (10.8)	0 (0)	0 (0)	7 (58.3)	11 (12.2)
C. glabrata	3 (8.1)	1 (4.8)	2 (10)	0 (0)	6 (6.7)
C. tropicalis	2 (5.4)	1 (4.8)	0 (0)	2 (16.7)	5 (5.6)
C. parapsilosis	3 (8.1)	0 (0)	0 (0)	0 (0)	3 (3.3)
C. quilliermondii	2 (5.4)	1 (4.8)	0 (0)	0 (0)	3 (3.3)
C. pelliculosa	0 (0)	0 (0)	0 (0)	1 (8.3)	1 (1.1)
C. keyr	1(2.7)	0 (0)	0 (0)	0 (0)	1 (1.1)
Total %	37 (41.1)	21 (23.3)	20 (22.2)	12 (13.3)	90/96 (93.7)

Table 2: Concordance between genotypic and phenotypic identification methods for *Candida* species and other fungal species

Organism identified	**Genotypic (n)**	**Phenotypic (n)**	**Misidentified**
C. albicans	60	58	2
C. lusitaniae	11	11	0
C. glabrata	6	6	0
C. tropicalis	5	4	1
C. parapsilosis	3	4	1
C. quilliermondii	3	3	0
C. pelliculosa	1	1	0
C. keyr	1	1	0
C. famata	0	1	1
C. krusei	0	1	1
Cryptococcus neoformans	1	1	0
Saccharomyces cerevisae	1	1	0
Cyberlindnerafabianii	2	ND	NA
Pichia kudrianvzevii	1	ND	NA
Trichosporonjaponicum	1	ND	NA

Abbreviation: ND, Not detected: NA, Not applicable.

Table 3: *In vitro* antifungal susceptibility by MIC (µg/ml) using E - test method.

Species (no.of isolates)	Antifungal agents	Range	MIC_{50}	MIC_{90}	No. of resistant isolates (%)
C. albicans (60)	Fluconazole	0.5 – 256	2.0	256	11 (18.3%)
	Amphotericin B	0.5 - 32	1.0	1.5	6 (10%)
	Flucytosine	0.004 - 32	0.016	0.25	5 (8.3%)
	Caspofungin	0.125 - 32	0.25	0.75	2 (3.3%)
C. lusitaniae (09)	Fluconazole	0.75 - 96	1.0	96	2 (22.2%)
	Amphotericin B	0.75 - 3	1.0	1.5	2 (22.2%)
	Flucytosine	0.003 - 32	0.004	6.0	1(11.1%)
	Caspofungin	0.25 - 1	0.25	1	0.00
C. glabrata (06)	Fluconazole	1 - 16	6	16	0.00
	Amphotericin B	1 - 2	1.5	2	4 (66.6%)
	Flucytosine	0.002 - 0.004	0.004	0.004	0.00
	Caspofungin	0.19 - 0.5	0.25	0.5	0.00
C. tropicalis (05)	Fluconazole	1 - 256	1	256	1 (20%)
	Amphotericin B	0.75 - 2	1	2	1 (20%)
	Flucytosine	0.006 - 0.016	0.016	0.016	0.00
	Caspofungin	0.125 - 1	0.125	1	0.00

Resistance is defined as the following MIC in microgram per millilitre: Flu ≥ 64; AMB > 1.0; FC ≥ 32; CAS≥ 32 using interpretive breakpoint criteria of EUCAST and CLSI (EUCAST 6.1, 2013; MIC test strip, 2014). MIC_{50} and MIC_{90} - MIC value to inhibit 50% and 90% of the strains tested, respectively.

Table 3: *In vitro* antifungal susceptibility by MIC (µg/ml) using E - test method (*continued*).

Species (no. isolates)	Antifungal agents	Range	MIC_{50}	MIC_{90}	No. of resistant isolates (%)
C. parapsilosis (03)	Fluconazole	1 - 8	2	8	0.00
	Amphotericin B	0.75 - 1	0.75	1	0.00
	Flucytosine	0.006 - 32	0.016	32	1 (33.3%)
	Caspofungin	0.125 - 0.5	0.25	0.5	0.00
C. quilliermondii (03)	Fluconazole	2 - 256	256	256	1 (33.3%)
	Amphotericin B	1 - 32	0.75	32	1 (33.3%)
	Flucytosine	0.004 - 32	0.016	32	1 (33.3%)
	Caspofungin	0.25 - 32	0.75	32	1 (33.3%)
C. pelliculosa (01)	Fluconazole	3	-	-	0.00
	Amphotericin B	1	-	-	0.00
	Flucytosine	0.016	-	-	0.00
	Caspofungin	0.5	-	-	0.00
C. kefyr (01)	Fluconazole	8	-	-	0.00
	Amphotericin B	1	-	-	0.00
	Flucytosine	0.003	-	-	0.00
	Caspofungin	1.5	-	-	0.00

Resistance is defined as the following MIC in microgram per millilitre: Flu ≥ 64; AMB > 1.0; FC ≥ 32; CAS≥ 32 using interpretive breakpoint criteria of EUCAST and CLSI (EUCAST 6.1, 2013; MIC test strip, 2014). MIC_{50} and MIC_{90} - MIC value to inhibit 50% and 90% of the strains tested respectively.

Table 4: Mean MIC in µg/ml of antifungal agents on *Candida* species isolated from HIV- positive and HIV-negative Study participants (n= 96).

	Mean MIC (No. of isolates)			
Variables	**HIV + (n = 55)**	**HIV - (n = 41)**	**95% CI**	***p* – value**
Fluconazole	52.6 ± 97.6	27.9 ± 76.7	24.5 - 60.8	0.17
Amphotericin B	1.66 ± 4.2	2.39 ± 6.8	0.87 - 3.08	0.51
Flucytosine	3.73 ± 10.3	1.58 ± 6.8	1.01- 4.61	0.24
Caspofungin	2.11 ± 7.26	1.97 ± 6.9	0.62 – 3.49	0.92

P-values were determined using students t test. Values are expressed as mean ± SD and 95% Confidence Interval. Abbreviations: MIC, Minimum Inhibitory Concentration. Resistance Breakpoints:Fluconazole ≥64µg/ml, Amphotericin-B >1.0µg/ml, Flucytosine ≥32µg/ml, Caspofungin≥32µg/ml.

Table 5: Antifungal multi-drug resistance pattern of *Candida* isolates (n=5)

Species	Multi-resistance Pattern	Proportion of Isolation % (n)
C. albicans	FLU + AMB + FC + CAS	40 (2)
C. guilliermondii	FLU + AMB + FC + CAS	20 (1)
C. lusitaniae	FLU + AMB	20 (1)
C. albicans	AMB + FC	20 (1)

Abbreviation: FLU, Fluconazole; AMB, Amphotericin B; FC, Flucytosine; CAS, Caspofungin.