

Research Article

Identification of Non-albicans *Candida* Yeasts Associated with Vulvovaginal Candidiasis in Tanzania Using a Combination of Multiplex PCR and DNA Sequence Divergence of the 26S LSU rDNA

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Abstract: Vulvovaginal candidiasis (VVC) is caused by various species of the yeast genus *Candida* which may require different drugs for therapy. The objective of this study was to rapidly identify non-albicans *Candida* species among yeast isolates from women attending a clinic in Temeke, Dar es Salaam, Tanzania. Initial screening for *Candida albicans* was done using a nested PCR approach in a previous study. Eight non-albicans yeast isolates remained and were subjected to multiplex PCR targeting the ITS1-5.8S-ITS2 rDNA region. One isolate with ambiguous Multiplex PCR results was subjected to further PCR of the D1/D2 domain of the 26S rDNA region followed by sequencing. Three isolates were identified as *C. glabrata* and four were identified as *C. tropicalis* by Multiplex PCR. The identity of the isolate unresolved by Multiplex PCR was confirmed by Blastn searches to be *C. parapsilosis* and a neighbor joining phylogenetic tree reconstruction positioned this isolate in the same clade with *C. parapsilosis* isolated elsewhere. Multiplex PCR is a fast and reliable method for identification of pathogenic *Candida* species. Women being attended for recurrent vulvovaginal candidiasis in Tanzania can easily benefit by getting specific treatments especially when antifungal resistant species like *C. glabrata* are suspected.

Keywords: Non-albicans *Candida*, Vaginal candidiasis, Multiplex PCR, D1/D2 Sequence

INTRODUCTION

Vulvovaginal Candidiasis (VVC) is a disease caused by abnormal growth of *Candida* yeasts in the mucosa of the female genital tract and it is a frequently diagnosed ailment in daily practice of gynecology [1]. Most of VVC is caused by *Candida albicans* (80 to 90%), while non-albicans *Candida* yeasts including *Candida tropicalis*, *Candida glabrata*, *Candida krusei* and *Candida parapsilosis* account for 10 to 20% [2, 3]. In the last decade, non-albicans *Candida* species have increasingly been considered as potential agents for clinical infections, partly due to a rise in the number of immunocompromised patients [4, 5]. Hence, rapid and correct identification of *Candida* isolates has become more relevant. Furthermore, the virulence and antifungal drug susceptibility of *Candida* isolates differ according to the species, with *Candida glabrata* and *Candida krusei* being innately resistant to commonly prescribed antifungal drugs e.g. fluconazole [6, 7]. Failure to correctly diagnose and treat appropriately has led to prolonged distress in women who end up suffering from recurrent vulvovaginal candidiasis defined as four or more proven infections per year [1].

Various commercial systems that can identify clinically important *Candida* species have been developed but most of them base their identification criteria on phenotypic characteristics which are very unstable and sometimes may not be trusted [8]. Moreover, these techniques are laborious and time consuming [9]. PCR based methods for rapid identification of *Candida* species have been described. These include restriction enzyme analysis (REA) of total genomic DNA (DNA fingerprinting) [10], and of PCR products (PCR-RFLP) [11], Nested PCR [12], randomly amplified polymorphic DNA (RAPD-PCR) [13], multiplex PCR [8, 14-16] and DNA sequencing [17-20].

Multiplex PCR involves simultaneous amplification of more than one locus in the same reaction; which makes it is a rapid and convenient screening assay in both clinical and research laboratories [15]. It is highly sensitive and specific and has the potential to produce considerable savings of time, test sample and effort within the laboratory without compromising its utility [15, 16]. By targeting the internal transcribed spacer regions (ITS 1 & ITS 2)

and the intervening 5.8S region with different combinations of universal and species specific primers in a multiplex PCR, up to eleven of the most commonly isolated *Candida* species have previously been correctly identified [8].

In the yeasts, exclusively used for Phylogenetic inferences are the variable domains (D1/D2) at the 5'-end of the large subunit (26S) ribosomal DNA (LSU rDNA) [18, 21]. Normally strains of the same species have identical D1/D2 sequences or no more than 1% mismatch [17]. Kurtzman and Robnett [17] studied approximately 500 ascomycetous yeast species for divergence in the D1/D2 region of LSU rDNA and were able to identify correctly most of the species, including those of *Candida*. In a follow up study, Fell *et al.* [22] sequenced the D1/D2 region of 337 strains of basidiomycetous yeast species and yeast-like fungi and were able to correctly identify the majority of the species. In recent years several workers have reported yeasts identified by sequence analysis of the D1/D2 domains and available databases include all described yeast species [17, 18, 21].

In Tanzania, few studies have attempted to identify *Candida* species associated with VVC using molecular techniques. One study applied a RAPD-PCR technique to supplement results by biochemical tests [23]. In a previous study [24], eight vaginal yeast isolates were screened out of thirty isolates as non-*Candida albicans* yeasts. However, the identity of the isolates was not determined. The present study focused on rapid identification of these yeasts by multiplex PCR and DNA sequence analysis methods.

MATERIALS AND METHODS

Source of microorganisms:

Eight non-*Candida albicans* vaginal yeast isolates were used in this study. The isolates were described in a previous study that screened for *Candida albicans* among thirty vaginal yeast isolates [24]. The characteristics of the women who provided these samples have been published [23]. The yeast cultures were coded Cy1 to Cy30. Only isolate Cy1, Cy14, Cy15, Cy16, Cy20, Cy28, Cy29 and Cy30 were further analyzed in the current study. The yeast cultures were maintained on Sabouraud Dextrose Agar "SDA" (Difco, USA) (4% dextrose, 1% peptone, 1.5% agar; pH 5.6) supplemented with 50 µg/ml of chloramphenicol (Sigma, USA) at 37°C for 48 hrs and sub-cultured monthly. Prior to DNA isolation, the yeasts were cultivated in 25 ml of YEPD broth (1% Yeast extract, 2% Peptone, and 2% Dextrose) supplemented with ampicillin (200 mg l⁻¹) and incubated overnight at 37°C with shaking at 170 rpm.

DNA extraction:

The protocol used was developed and optimized in the laboratory at the department of Molecular Biology and Biotechnology, University of Dar es Salaam. Briefly, yeast cells were collected by centrifugation of YEPD broth culture in 1.5 ml eppendorf tubes at 14,000 rpm for 5 minutes, and re-suspended in 75 µl of yeast DNA extraction buffer [2% Triton X-100, 1% SDS, 100 mmol l⁻¹ NaCl, 10 mmol l⁻¹ Tris (pH 8), 1 mmol l⁻¹ EDTA and sterile distilled water to make up to 100 ml] and grinded with a mini-pestle until a homogeneous mixture was formed. The mixture was incubated in a water-bath at 60°C for 20 min after which 40 µl of 7.5 mmol l⁻¹ potassium acetate was added, mixed well and incubated on ice for 20 min. After centrifugation for 4 min at 14,000 rpm, an equal volume of chloroform was added to the supernatant and vortexed for 10 sec and further centrifuged for 4 min at 14,000 rpm. Two volumes of pre-chilled isopropanol were added to the upper phase in fresh tubes, mixed well without vortexing, and incubated at -20°C for at least 15 min. DNA was pelleted by centrifugation at 16,000 rpm for 10 min, dried in air for 7 min and washed once with 100 µl of cold 75% ethanol and re-centrifuged for 5 min at 14,000 rpm and left to dry in air for 10 min. All centrifugations were done at 4°C. DNA was dissolved in 30 µl of TE buffer [10 mmol l⁻¹ Tris-HCl (pH 7.0 - 8.0) and 1 mmol l⁻¹ EDTA (pH 8.0)] and 5 µl of the DNA sample was loaded on 0.9 % agarose gel prepared in TAE buffer (40 mmol l⁻¹ Tris acetate, 1 mmol l⁻¹ EDTA), stained with ethidium bromide (1 µg ml⁻¹), electrophoresed and visualized on UV transilluminator. DNA quantitation was performed by spectrophotometric measurements of optical density (OD) at 260 nm according to standard methods [25]. DNA was stored at -20°C until use.

Multiplex PCR approach:

For multiplex PCR, seven species-specific forward primers and one universal reverse primer (Table 1) were adopted from Li *et al.* [14] and used for amplification of respective species-specific DNA fragments in the ITS1-5.8S-ITS2 region of the fungal rDNA (Fig. 1). The multiplex PCR was performed with 1 µl (100 ng) of template DNA in a total reaction volume of 25 µl consisting of 2 µl PCR Buffer, 1.5 µl of 25 mmol l⁻¹ MgCl₂, 0.5 µl of deoxyribonucleoside triphosphates mixture (10 mmol l⁻¹ each), 0.5 µl of *Taq* DNA Polymerase (5U/µl), 1 µl of each of the eight primers (20 pmol µl⁻¹ each) and 11.5 µl of sterile distilled water. The following PCR conditions were used: Initial denaturation step at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were then stored at 4°C until further analysis.

Table 1: Primers used for PCR and Cycle sequencing

Species	Primer name	Sequence (5'-3')	Primer pair	Product size
<i>PCR of ITS1-5.8S-ITS2 region</i>				
All Fungi	ITS4	TCCCTCCGCTTATTGATATGC	-	-
<i>C. glabrata</i>	CGL	CACGACTCGACACTTTCTAATT	CGL/ ITS4	632 bp
<i>C. krusei</i>	CK	GATTTAGTACTACACTGCGTGA	CK/ ITS4	475 bp
<i>C. albicans</i>	CA	TCAACTTGTACACCAGATTATT	CA/ ITS4	402 bp
<i>C. guilliermondii</i>	CGU	GTATTGGCATGGGTAGTACTG	CGU/ ITS4	185 bp
<i>C. tropicalis</i>	CT	AAGAATTTAACGTGGAAACTTA	CT/ ITS4	149 bp
<i>C. parapsilosis</i>	CP	GGCGGAGTATAAACTAATGGATAG	CP/ ITS4	126 bp
<i>C. lusitaniae</i>	CL	GTTAGGCGTTGCTCCGAAAT	CL/ ITS4	116 bp
<i>PCR of 26S rDNA D1/D2 domain region</i>				
All Fungi	NL-1 F NL-4 R	GCATATCAATAAGCGGAGGAAAAG GGTCCGTGTTTCAAGACGG	NL-1/NL-4	600 bp
<i>Sequencing of 26S rDNA D1/D2 domain region</i>				
	NL-2A NL-3A	CTTGTTTCGCTATCGGTCTC GAGACCGATAGCGAACAAG		

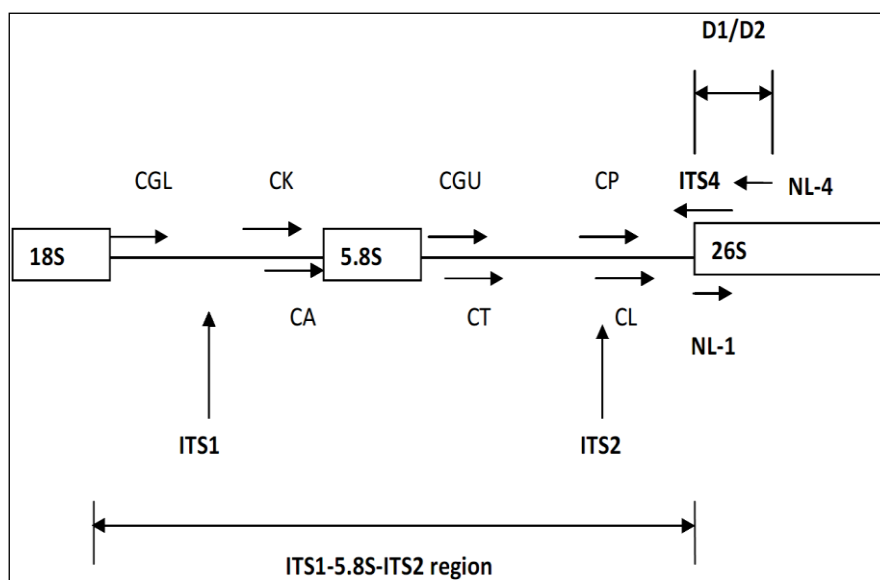


Fig.1: Genomic organization of rDNA showing the target area 5.8-ITS region and D1/D2 domain region.

Boxes indicate coding regions. Arrows labeled with CGL, CK, CA, CGU, CT, CP and CL represent forward species-specific primers for *Candida glabrata*, *Candida krusei*, *Candida albicans*, *Candida guilliermondii*, *Candida tropicalis*, *Candida parapsilosis* and *Candida lusitaniae*, respectively. ITS4, NL-1 and NL-4 are universal fungal primers [14, 17].

Analysis of PCR products:

For analysis, 5 µl of PCR products were mixed with 1 µl of loading dye (15% ficoll[®]400, 0.03% Bromophenol blue, 0.03% Xylene Cyano FF, 0.4% Orange G, 10 mmol l⁻¹ Tris-HCl [pH 7.5], 50 mmol l⁻¹ EDTA) and analyzed through ethidium bromide (1 µg ml⁻¹) stained 2% agarose gel electrophoresis in TAE buffer (40 mmol l⁻¹ Tris-acetate, 1 mmol l⁻¹ EDTA [pH 8.0]) at 70V for 1 hour and then visualized on the UV transilluminator and photographed.

DNA sequencing of 26S rDNA D1/D2 domain region

DNA from the yeast isolate Cy30 was further characterized by DNA sequencing as described by Kurtzman and Robnett [17].

PCR: The forward primer NL-1 and the reverse primer NL-4 (Table 1) were used for amplification of the approximately 600 bp D1/D2 domain of the 26S rDNA region (Fig. 1). PCR was performed with 1 µl (100 ng) of template DNA in a total reaction volume of 25 µl consisting of 2 µl PCR Buffer, 1.5 µl of 25 mmol l⁻¹ MgCl₂, 0.5 µl of deoxyribonucleoside triphosphates mixture (10 mmol l⁻¹ each), 0.5 µl of *Taq* DNA Polymerase (5U µl⁻¹), 1 µl of each of primer NL-1 and NL-4 (20 pmol µl⁻¹) and sterile distilled water to make up to 25 µl total volume. Amplification was performed for 36 PCR cycles each with annealing at 52°C for 1 min, extension at 72°C for 2 min, denaturation at 94°C for 1 min and a final extension of 8 min at 72°C as

described by Kurtzman and Robnett [17]. Analysis of PCR products was performed as for the multiplex PCR above.

Purification of PCR products: PCR products were purified using Montage® PCR Cleanup Kits according to manufacturer's instructions to eliminate primer dimers, primers, nucleotides, and extraneous bands.

Sequence analysis: After purification, sequencing was done by the dye terminator method using Amersham MegaBace Capillary and ABI 377 DNA sequencing platform at a commercial facility (Northwoods DNA Inc, Bemidji Minnesota, USA). Each sample was sequenced twice, using either forward primer or reverse primer but not both. The online nucleotide-nucleotide Basic Local Alignment Search Tool algorithm (Blastn) at the National Centre for Biotechnology Information (NCBI) Bethesda, Md

(<http://www.ncbi.nlm.nih.gov/BLAST/>) was used for GenBank searches and comparative sequence analysis for the DNA sequence data for isolate Cy30.

Phylogenetic analysis

Multiple sequence alignment of KJ081743 with published D1/D2 26S rDNA sequences of eleven *Candida* species of medical importance and *Schizosaccharomyces pombe* (Table 2) was performed using the ClustalW alignment program [26]. Confidence limits for phylogenetic trees were estimated by bootstrap test (500 replicates) [27]. The evolutionary distances were computed using the Maximum Composite Likelihood method and the evolutionary history was inferred using the Neighbor-Joining method [28]. *Schizosaccharomyces pombe* was the designated outgroup. All analyses were conducted using MEGA version 6 [29].

Table 2: D1/D2 sequences of the yeast species used for phylogenetic analysis of isolate Cy30.

Species name	GenBank accession numbers
<i>Candida albicans</i>	U45776
<i>Candida dubliniensis</i>	U57685
<i>Candida famata</i>	U45808
<i>Candida glabrata</i>	U44808
<i>Candida guilliermondii</i>	U45709
<i>Candida kefyr</i>	U94924
<i>Candida krusei</i>	U76347
<i>Candida lipolytica</i>	AB197667
<i>Candida lusitaniae</i>	AJ539569
<i>Candida parapsilosis</i>	AB199906
<i>Candida tropicalis</i>	AY951983
<i>Schizosaccharomyces pombe</i>	AY048171.1

RESULTS

Multiplex PCR

All eight yeast isolates studied in this study including the type strain *Candida albicans* ATCC90028 produced a species-specific PCR product when amplified in a multiplex PCR system (Fig.2). In Fig. 2 (A), the amplicon sizes for DNA from isolates CY1, CY28 and CY29 approximated the 632 bp size expected from ITS4 & CGL primer pair amplification. These isolates were identified as *C. glabrata*. Similarly the amplicon size for DNA from the type strain *Candida albicans* (ATCC90028) was approximately 400 bp (Figs. 2 A&B) as expected for ITS4 & CA primer pair amplification [14].

In Fig. 2 (B), the amplicon size for DNA from isolates CY14, CY15, CY16 and CY20 was around 150 bp as expected for ITS4 & CT primer pair amplification [14]. These isolates were identified as *C. tropicalis*. However, although the amplicon size for DNA from isolate CY30 seemed to be around 125 bp which was the expected size for ITS4 & CP primer pair amplification, it was not easy to separate it visually on agarose gel from the 116 bp amplicon size expected for ITS4 & CL primer pair amplification as they differed by only 10 bp [14].

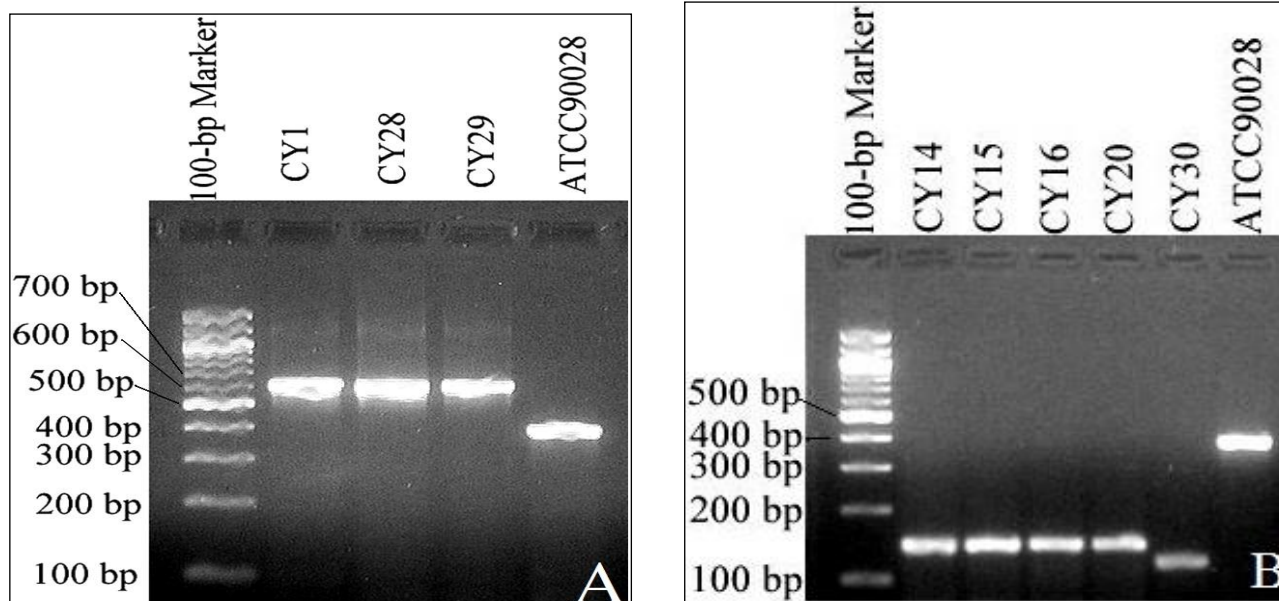


Figure 2 (A & B): Results of multiplex PCR using universal fungal primer ITS4 and species-specific forward primers, CL, CP, CT, CGU, CA, CK and CGL. Lanes show 100-bp molecular weight marker and PCR products for isolate CY1, CY14, CY15, CY16, CY20, CY28, CY29, CY30 and the type strain *Candida albicans* (ATCC90028).

PCR of D1/D2 domain of 26S rDNA

PCR amplification of the D1/D2 region of the 26S rDNA of isolate Cy30 produced a PCR product of approximately 600 bp. Blasting of the sequence of this PCR product resulted into highest identity matches (99%) to that of *Candida parapsilosis* AY894827. The sequence was given a unique identifier VMAK30 and submitted to Genbank where it was assigned a provisional accession number KJ081743.

Phylogenetic analysis

The optimal Neighbor-Joining Phylogenetic tree with the sum of branch length = 1.05433714 is shown (Fig. 3). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses clearly indicated that isolate Cy30 (KJ081743) is closely similar to *Candida parapsilosis* and the clade was well supported (Fig. 3).

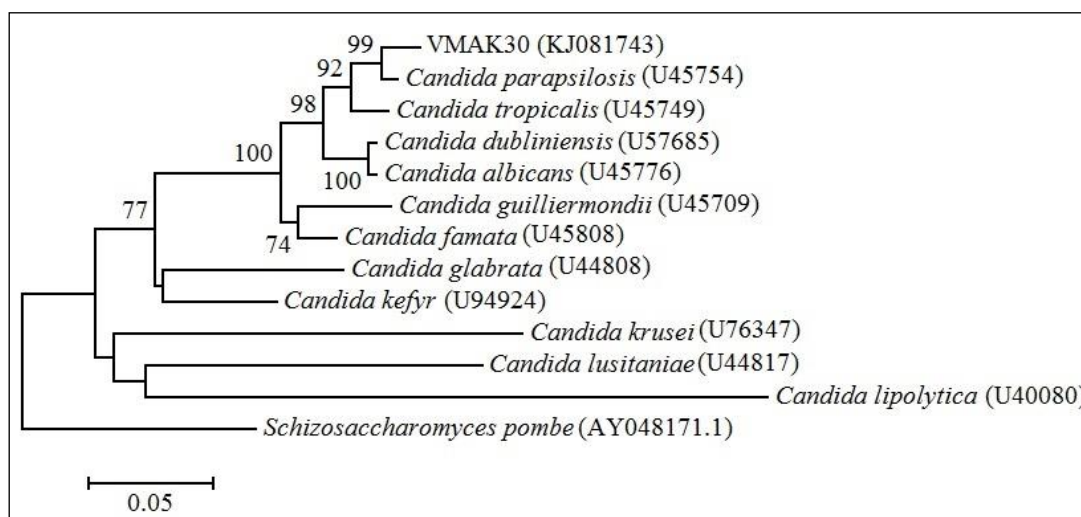


Fig. 3: Phylogenetic tree derived from neighbor-joining analysis of 26S rDNA domain D1/D2 depicting placement of isolate Cy30 (KJ081743) among medically important *Candida* species.

Branch lengths are proportional to the number of nucleotide differences, and the numbers given on the branches are the frequencies with which a given branch appeared in 500 bootstrap replications. Frequencies under 70 are not given. The marker bar denotes 5% nucleotides relative to branch lengths.

DISCUSSION

A total of eight pure clinical yeast isolates were screened out as non-albicans *Candida* by a nested PCR method [24]. All eight non-albicans yeast isolates were included in this study. The multiplex PCR used was able to identify conclusively seven out of the eight isolates under the study. Three isolates were unambiguously identified as *C. glabrata* and four isolates as *C. tropicalis*. Resolving amplicon sizes differing by small base pair numbers as was obtained by primer sets for *C. parapsilosis* and *C. lusitaniae* could not be done for isolate Cy30 on agarose gel electrophoresis. However, the identity of the isolate Cy30 was conclusively determined by DNA sequence analysis of the D1/D2 domains of the 26S rDNA to be *C. parapsilosis*.

Multiplex PCR results obtained in this study were similar to results obtained in a study by Li *et al.* [14] who evaluated the use of this approach in identifying eight yeast species commonly found in positive blood cultures. Compared to the multiplex PCR approach using ITS1, ITS2, CA3 and CA4 primers [15, 30], the multiplex PCR system used in this study could be a better approach in two ways. First, the approach by Chang and colleagues could only separate *C. parapsilosis* (229 bp) from *C. tropicalis* (218 bp) by polyacrylamide gel electrophoresis (PAGE) [30]. Similarly, the Rad and colleagues study could only do so by use of a UVIssoft software, lacking in most laboratories in developing countries [15]. Secondly, in both cases, distinction between *C. albicans* (218 or 219 bp) and *C. tropicalis* (218 bp) depended on *C. albicans* specific primers (CA3 & CA4) included in the multiplex and parallel running of a species specific marker with amplicons for all seven common pathogenic yeast species. However, such a marker could have facilitated the identification of isolate CY30 in this study for which it was difficult to identify it as *C. parapsilosis* or *C. lusitaniae* (Fig. 2 B). This difficult in separating between *C. parapsilosis* and *C. lusitaniae* was reported before in Dar es Salaam, using RAPD-PCR [23].

The ultimate identification system for most of the clinically relevant *Candida* species is based on the analysis of sequence divergence in the D1/D2 domains of their 26S rDNA. This is because to date worldwide most species have already been described by this method and databases are accessible online for comparison [17, 18]. The method has been very successful in the identification of novel species [20] and has been accepted internationally and used to re-allocate *Candida* species to other genera once their teleomorphs are known [17, 21].

The results obtained in this study are very significant. Firstly, they confirm the previous conclusion that most women in Dar es Salaam suffer

from VVC caused by *Candida albicans*, *C. glabrata* and *C. tropicalis* [23]. Secondly they give an indication that the type of *Candida* species that are prevalent in Dar es Salaam women with VVC are similar to those affecting women elsewhere in the world [3, 31, 32]. Thirdly, the results indicate that there may be cases of complicated or recurrent VVC, because *C. glabrata* and *C. tropicalis* are frequently implicated in such cases than in uncomplicated VVC [1, 31]. And lastly, the issue of resistance to the best group of antifungal drugs for VVC, the azole family e.g. fluconazole must be expected to occur since *C. glabrata* is resistant or less susceptible to treatment with most azole drugs [7, 33].

CONCLUSION

The multiplex PCR system used in this study is recommended for routine use as it was able to identify seven of eight yeast isolates previously screened out as non-albicans *Candida* isolates by a nested PCR. Species specific markers when available may facilitate isolate identification. However, because of cost considerations, D1/D2 sequence analysis should be left for resolution of ambiguous results.

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