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Diversity of Pathogenic Candida Species Colonizing Women with and without Candida Vaginitis in Dar es Salaam Region, Tanzania

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Abstract

The aim of this study was to establish phenotypic and genetic diversities of *Candida* species isolated from women with recurrent *Candida vaginitis*. A total of 3000 samples were cultured from 3000 women with and without complaints of vaginal discharges and pruritis vulvae. Growth characteristics on Sabouraud's dextrose agar and on conidia enhancing media (Corn meal agar) resulted in isolation of 5440 distinct colonies of *Candida* species. They were then analyzed by germ tube test to separate *C. albicans* from non-albicans Candida species. For confirmation, 300 doubtful isolates were then tested on API 20C AUX and by polymerase chain reaction.

The results showed *Candida albicans* species to be dominant 3661 (67.29%) while non-albicans Candida species presented only 1779 (32.71%) which included *C. glabrata* 435(8.0%), *C. tropicalis* 762(14.0%), *C. krusei* 163(3.0%), *C. famata* 109(2.0%), *C. parapsilosis* 131(2.41%), C. *guilliermondii* and C. lusitaniae each 27(0.5%) respectively, *C. incospicua* 13 (0.24%), *C. norvegensis* 82(1.5%), *C. colliculosa* 16 (0.3%) and *C. magnoliae* 15 (0.27%). On genotypic diversity, the principle coordinate analysis of the PCR results for the genetic relatedness and genetic distances revealed the distribution of 200 isolates tested as: *C. albicans* 90(36%), *C. glabrata* 40(20%), *C. krusei* 20 (10%), *C. tropicalis* 20 (10%), *C. famata* 10(5%), *C. parapsilosis* 5(2.5%), *C. norvegensis* 6(3%), *C. lusitaniae* 4(2%), and *C. guilliermondii* 5(2.5%).

From this study it shows that a combination of biochemical methods and PCR technology may identify most of the isolates, including the germ tube negative isolates. Both symptomatic and asymptomatic women possessed similar species and genotypic diversities. However, in symptomatic women, a single host can be colonized with multiple species or multiple

genotypes of the same species. In order to establish if the same species behave differently in symptomatic and asymptomatic individuals, this study recommend further investigation on virulent factors.

Keywords: Diversity, Pathogen, *Candida, Vaginits,* Pruritis

1. Introduction

The genus *Candida* has diverse species that are common residents of soil and of the mucosal surfaces of human gastrointestinal tract, genito-urinary tract and the mouth, and are capable of causing oral thrush or vaginal thrush. The most common vaginal isolate include *C. albicans* with a prevalence of 70-90% and less frequently non-albicans *Candida* species such as *C. tropicalis, C. glabrata (Torulopsis glabrata), C. kefyr (C. pseudotropicalis), C. krusei, C. famata, C. parapsilosis*, and *C. lusitaniae* (Xu *et al*., 1999; Abu-Elteen, 2001; Chong *et al*., 2003; Namkinga *et al.,* 2005). These different *Candida* species have been associated with differences in the morphotype and virulence factors such as germ tube and mycelia formation, proteinase secretion, the changes in vaginal pH, phenotypic switching and ability to cause vaginal candidiasis (Sobel *et al.,* 1981; Sobel, 1985; Hunter *et al.,* 1989; Cutler, 1991; De Bernardis, *et al*., 1993; Cassone *et al*., 1995; Hube, 1996; Samaranayake *et al.,* 2003; Namkinga *et al.,* 2005). However, a significant increase in non-albicans Candida species and formation of virulence factors have been reported to be associated with recurrent Candida vaginitis (Sobel, 1992; Sobel, 1998; Chong, *et al*., 2003; Namkinga, *et al.,* 2005).

The incidence of Candida infections has dramatically increased in recent years as a result of a large increase in HIV/AIDS cases, thus an ever-expanding population with immuno-compromise due to mucosal or cutaneous barrier disruption, defects in the number and function of neutrophils or in cell-mediated immunity, metabolic dysfunction, organ transplantation and extremes of age (<1 year and > 70 years). The expanded use of immunosuppressive chemotherapies, and transplantation further increases the risk for both common and uncommon *Candida* species. In addition, as our aging population becomes increasingly mobile, environmental exposures to a variety of endemic fungal pathogens become more common and sometimes, may further increase the risk of fungal diseases (Xu *et al.,* 1999; Samaranayake *et al.,* 2003; Pfaller and Diekema, 2004; Walsh *et al.,* 2004; Nucci, and Marr, *2005;* Chu *et al.,* 2006; Pfaller and Diekema, 2007). Other factors that have been associated with VC include: extensive use of broad spectrum antibiotics (Nasibwa *et al.,* 1994), oral contraceptives (Baeten *et al*., 2001) and to a lesser extent corticosteroids and other immunosuppressive drugs (Baeten *et al*., 2001). Also pregnancy and uncontrolled diabetes (Sobel, 1992; de Leon *et al*., 2002), promiscuity (Foxman, 1992; Ginter *et al*., 1992; Spinillo *et al.,* 1993; Marin *et al*., 2000), poor hygiene (Marin *et al*., 2000), iron deficiency anaemia (Higgs and Wells, 1972) and allergies from condom usage (Eckert *et al.*, 1998). The prevalence of VC is low in pre-pubertal girls (Fischer, 2001) and in post-menopausal women (Barhan and Ezenagu, 1997), emphasizing the influence of hormones through Ph changes (Dennerstein, 1998; Baeten *et al*., 2001). VC in postmenopausal women has been associated with hormonal replacement therapy, uncontrolled diabetes, and immunosuppression caused by medication or disease (Nwokolo and Boag, 2000).

Despite all the above-mentioned putative and precipitating factors, there are, however, cases of VC without a recognizable predisposing factor (Sobel, 1985). In these women, the exact mechanism of pathogenesis is unclear. It has been postulated that allergy (Moraes, 1998), certain blood groups (Chaim *et al.,* 1997), use of commercially available solutions for vulvo-perineal cleaning or vaginal douching (Spinillo *et al*., 1993) could be the cause for the occurrence of VC in such women. Although *C. albicans* is the most frequent species isolated, other species such as *C. tropicalis*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis* and *C. glabrata* have increasingly been recognized as pathogens with a wide distribution (Hazen, 1995, Fidel *et al.,* 1999). The significant increase in the frequency of candidiasis has promoted the study and development of a variety of molecular based techniques aiming at the replacement of the traditional methods used for the identification and typing of *Candida* clinical isolates.

The purpose of this investigation was to identify and characterize the diverse species of *Candida* from women with a history of symptomatic vaginal candidiasis in HIV infected individuals, pregnant women and in symptomless healthy carriers of Candida, by using biochemical and molecular methods. To the best knowledge of this study, this is the first study in Tanzania, which comprehensively characterized a large number of vaginal *Candida* species over an extended period of time (2006-2010), using multiple methods.

2. Materials and Methods

A total of 3000 high vaginal swabs were collected from women with and without symptoms of vaginal Candidiasis who visited HIV and gynecological clinics from Private and Government hospitals of Dar Es Salaam Region, Tanzania. The inclusion criterion of patients in this study was women from different races and a wide age range (13-50 years) with complaints of vaginal discharges and/or pruritis vulvae also health carriers of Candida were included. Women with vaginal discharge (thick cudy white-yellowish), vulva erythema, burning and severe vulva itching were considered to have vaginal candidiasis (VC) (Denning, 1995, Namkinga *et al.,* 2005).

All specimens were processed in the Department of Molecular biology and biotechnology, University of Dar Es Salaam, Tanzania. Specimens from transport media were immediately cultured on Sabouraud's dextrose agar (Oxoid, Hampshire. UK). The wet mounts (from specimens) were prepared and examined microscopically by an experienced technician for the presence of yeast cells and trichomonas vaginalis. Gram stained smears were also prepared. Provisional detection of *Candida species* was based on colonial morphology on Sabouraud dextrose agar (SDA) media containing 0.005% chloramphenicol and 0.05% cycloheximide. Women were told to come after two weeks for their results. Symptomatic treatments were given to all women with VC and were told to come after seven days of treatment for evaluation. Indicated treatments were anti-fungal vaginal pesarries/ creams commonly found in Tanzania markets (clotrimazole, miconazole ketoconazole, nystatin), and anti-fungal oral tablets (ketoconazole, fluconazole, itraconazole) and for the individuals where the doctor feels that they have mixed infections, antibiotics were indicated. During the second visit, the subjects were interviewed again and high vaginal swabs taken for laboratory analysis. For those with the disappearance of symptoms or discomforts were told to come for check-up on monthly

basis for three months consecutively. For cases where symptoms persist or reoccur for three months consecutively despite of treatment with different anti-fungal agents were considered as recurrent cases. Recurrent cases were maintained again for three more months to find out the reasons for their recurrence in each visit.

Morphologically distinct yeast colonies from each culture were transferred and stored on Sabouraud's Dextrose agar slants for species identification and subsequent DNA fingerprinting. Yeast species were identified by growth on SDA to characterize the yeasts forms, and on conidia enhancing media; Corn meal agar (Oxoid, Hampshire. UK) inorder to characterize the mycelia forms. Germ tube test performed on horse serum (Oxoid, Hampshire. England) was used to separate *Candida albicans* from non-albicans *Candida* species. Biochemical testing with the Analytical Profile Index (API 20C) Candida (BioMerieux- SA, France) was used to speciate Candida based on sugar fermentation/assimilation. A few randomly selected isolates and the doubtful ones (total n=540), were further subjected to identification by API 20C AUX kits (BioMerieux- SA, France). Out of these 540 isolates, some few more (n=200) isolates were identified by RAPD fingerprinting. Stock cultures of *Candida albicans* (ATCC 32354), *C. glabrata* (ATCC 2001), *C. albicans* (HG 392), C. guilliermondii (ATCC 6260), were provided from Nijmegen University, Netherlands and were used as control.

DNA was amplified on a Techne-Thermocycler (Techne-Genius model FGENO2TD, Germany) according to the conditions described by Liu *et al* (1996). Thermocycling was achieved according to the following program: Initial denaturation at 94°C for 3minutes followed by 45 cycles of: 1 min at 95°C denaturation, 1 min at 35°C annealing and 2 min at 72°C elongation. The reaction ended with a 10 min extension at 72°C. The reaction mix contained 2µl 10X reaction buffer [16 mM $(NH_4)_2SO_4$, 70 mM Tris-HCI pH (8.8), 0.1% Tween 20]; 2 µl dNTP (2mM); 0.20 µl of Taq DNA polymerase; 2 µl MgC12 (25 mM), 1 µl primer (1.0 µg ml-1) [For primers used, see Table 3.3] and 1 µl template DNA (ca. 10-20 ng). Each reaction was carried out in triplicate. Twentyfive nanograms of each of the *Candida albicans* (ATCC 32354), *Candida glabrata (*ATCC 2001), *Candida albicans* (HG 392) and *Candida guilliermondii* (ATCC 6260) were used as a positive control for each sample run.

A total of 11 primers (Table 1), were tested in the assay. The presence and absence of each RAPD band was analyzed by visual inspection of gel photographs (Figure 1).

Table 1. List of 10-mer primers (Operon Technologies Inc. Alameda Canada) used in this study

Sn	Primer	Sequence
1	OPB-01	5'-GTTTCGCTCC-3'
2	OPA-08	5'-GTGACGTAGG-3'
3	OPU-15	5′-ACGGGCCAGT-3′
4	OPB-07	5'-GGTGACGCAG-3'
5	OPAX-20	5'-GGTCCCTGAC-3'
6	OPC-16	5'-CACACTCCAG-3'
7	OPAW 15	5'-CCAGTCCCAA-3'
8	OPAW-08	5'-CTGTCTGTGG-3'
9	OPB-10	5'-CTGCTGGGAC-3'
10	OPA-06	5'-GGTCCCTGAC-3'
11	OPB-09	5'-TGGGGGACTC-3'

The sizes of RAPD products were estimated by comparison with a 1 kb ladder in positions 1 and 22 of the PCR gel. Bands of similar molecular weight were scored as '1' when present or '0' when absent for all accessions studied and for all the primers assessed. Data management was performed on an IBM compatible PC with the DDAT program. In addition Excel (Microsoft) and NTSYS programs (Rohlf, 1989) were used for data processing and analysis. Genetic distances between two or more genotypes were defined as quotient between un-matches and the sum of matches and un-matches. This coefficient of association measures dissimilarities and corresponds to the complement (1-SC) of the Jaccard coefficient (SC) (Goodman, 1973). Cluster analysis was performed based on these dissimilarity coefficients, using UPGMA program as a clustering method.

A cophenetic matrix was computed from the obtained tree matrix and compared with the original dissimilarity matrix in order to measure the goodness of fit (Rohlf, 1989).

Social and demographic indicators from patients were evaluated using standard structured questionnaire. The study was ethically approved by the College Research and Publications Committee of the Muhimbili University College of Health Sciences, (MU/PGS/AEC/III/126 of November 11, 1997)

3. Results

A total of 3000 women with VC, aged between 13 and 50, were enrolled during the study period. The social and demographic characteristics of the study sample are presented in Table 2.

Table 2. Social and demographic indicators for women with VC which contribute to Candida spp. Diversity in Dar es Salaam. Tanzania (n=3000)

Variable		**N**	**%**
Age group:	1. <20	512	17.07
	2. 21-30	1268	42.27
	3. 31-40	1076	35.87
	4. 41+	144	4.80
Educational level:	1. No formal education	280	9.33
	2. Primary	2142	71.40
	3. Secondary +	578	19.27
Occupation:	1. Not employed	1120	37.33
	2. Entrepreneurs	1479	49.30
	3. Employed	401	13.37
N. of sex partners:	1. ≤1	250	8.33
	2. 2-3	1366	45.53
	3. 4 +	1384	46.13
Marital status:	1. Single	550	18.33
	2. Married/cohabiting	1870	62.33
	3. Widow/separated	580	19.33
Pregnancy status:	1. Not pregnant	952	31.73

2. Pregnant	2048	68.27
Prior antibiotic use: 1. Not used	1054	35.13
2. Used	1946	64.87
Income (TShs): 1. <50000	2360	78.67
2. 50000 +	640	21.33
Age at first sex (years): 1. <20	2012	67.07
2. 20-24	851	28.37
3. 25 +	137	4.57
HIV infection: 1. HIV negative	960	32.00
2. HIV positive	1826	60.87
3. Not tested for HIV	214	7.13

Almost all women were Africans aged between 21 and 40 years were 78.14%. These participants were mainly married or cohabiting, had more than one sexual partners and an income of less than 75,000/- Tanzanian shillings (~ 50 USD) per month, and had first sexual intercourse before 20 years of age.

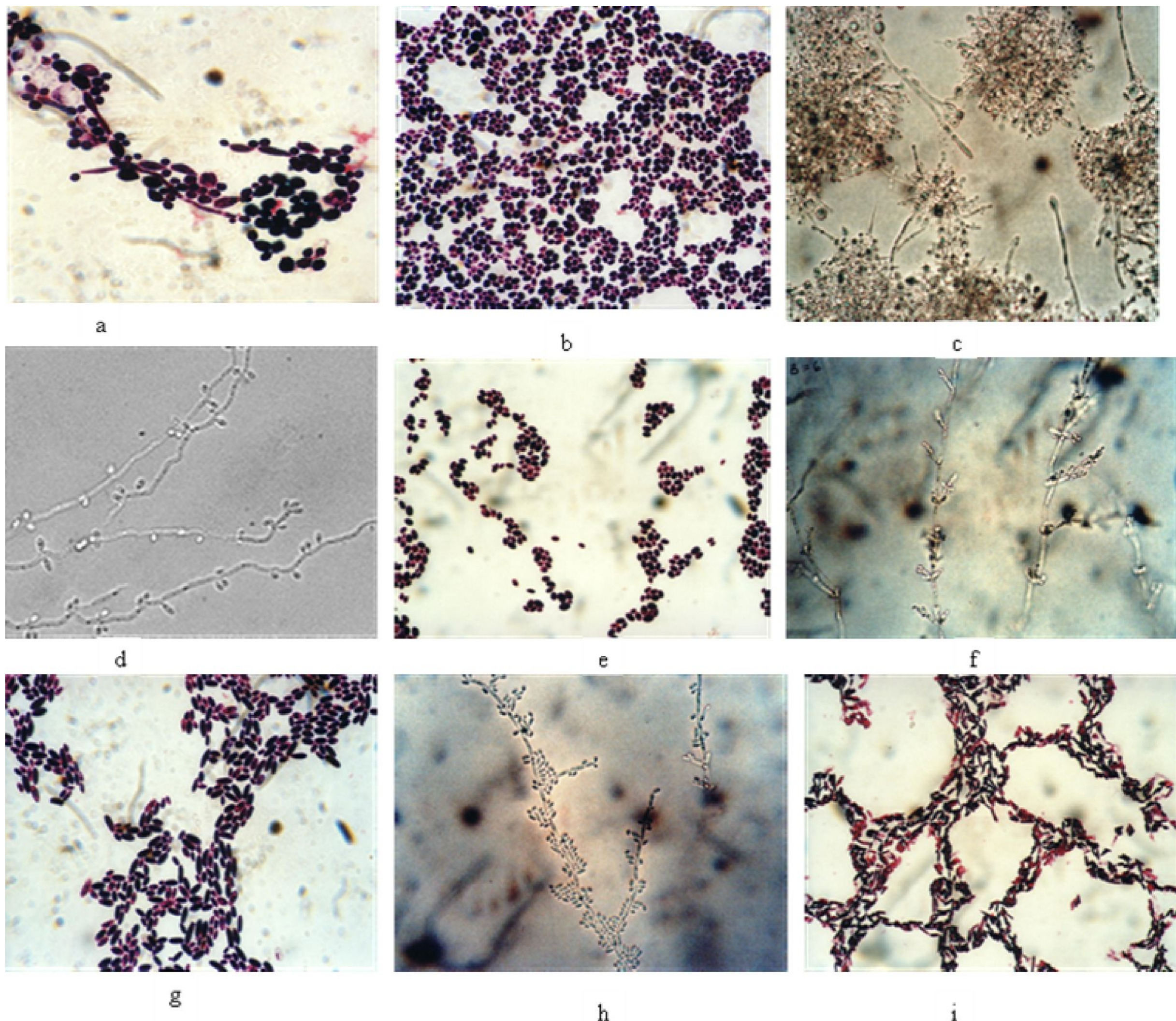

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Figure 1. Morphological feature of different Candida species (a) Gram stained fresh patient's specimen with mixed *Candida* species, bacteria and some epithelial cells on SDA; (b) *C. albicans* from SDA gram stained; (c) Mycelial form of *C. albicans* on com meal agar; (d) Mycelial form of *C. tropicalis* on corn meal agar; (e) Gram stained *C. glabrata* on SDA; (f) Mycelial form of *C. norvegensis* on corn meal agar; (g) *C. norvegensis* gram stained on SDA; (h) *C. krusei* on corn meal agar; (i) *C. krusei* gram stained on SDA. (all at x400)

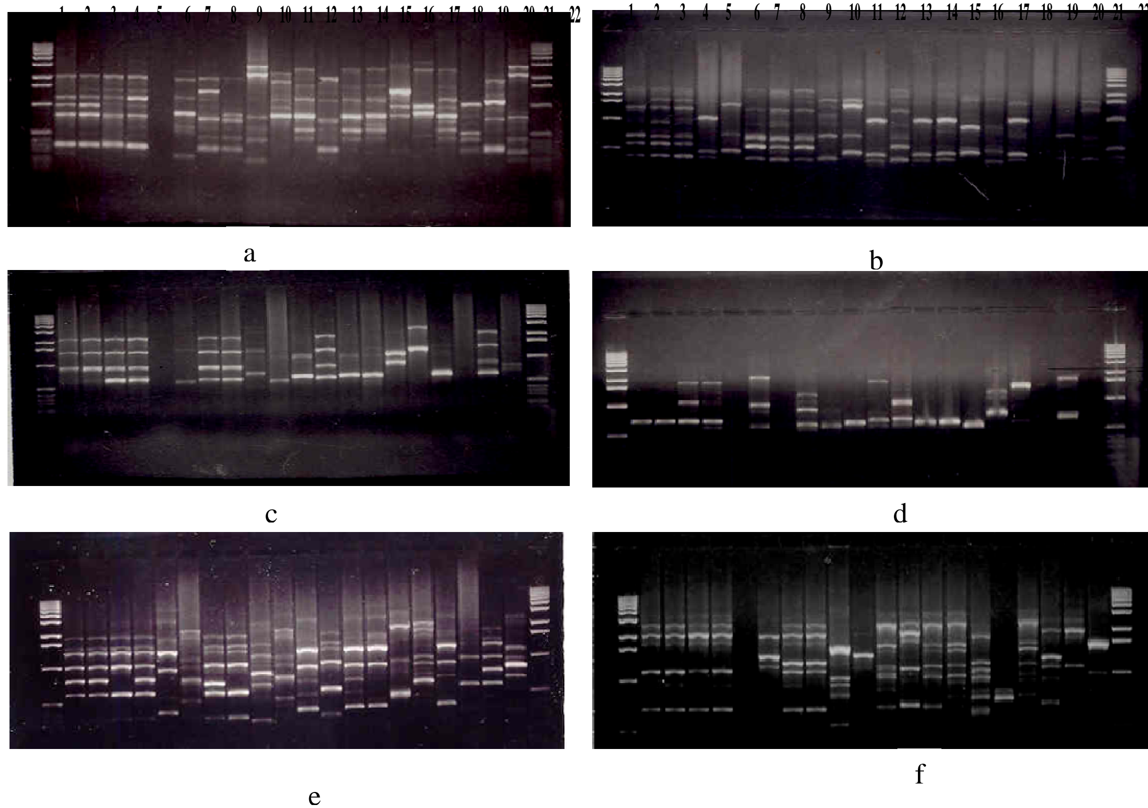

Figure 2. Some RAPD profiles of Candida species (from patients) using eleven different primers from visualized on 1.0% agarose gel; bands labeled 1-22 from left to right

Primers used in 1(a) OPA-08, in (b) OPAW-15, in (C) OPA-06, in (d) OPAX-20, (e) OPB-16, and (f) OPB-07. Arrangements from left to right is the same for all the gels; From left lane 1and 22 is 1kb ladder size marker, lane2= *C. albicans* ATCC 32354, *C. albicans* isolated from asymptomatic patients (lanes 3-5), and *C. albicans* isolated from symptomatic patients (lanes 8, 9, 13, 19 and 20), C. famata (lane6), C. glabrata (lane 12, 14, 15 and 18), *C. krusei* (lane 10 and 21), *C. tropicalis* (lane 7 and 11), *C. parapsilosis* (lane 17) and *C. lusitaniae* (lane 16).

Majority of women 1826 (60.87%) were HIV positive (Table2), with recurrences. They visited clinic with the same complaints more than four times a year. Laboratory results of this study showed that, out of 3000 specimens cultured from 3000 women with complaints of vaginal discharges and pruritis vulvae, 5440 Candida species were isolated by growth characteristics on Sabouraud's dextrose agar and on Corn meal agar.

Table 3. Comparison of results obtained by API Candida, API 20C AUX and PCR

Isolate #/or Amt.	**Germ tube** 2hrs 3hrs↑	**API Candida**	**API 20C AUX**	**RAPD RESULTS**
STD HG392	+ +	*C. albicans*	*C. albicans1* by 98%	*C. albicans*
STDATCC32354	+ +	*C. albicans*	*C. albicans1* by 99.9%	*C. albicans*
854	+ +	*C. albicans*	*C. albicans1*≅ ATCC 32354 for 50 tested	*C. albicans* ≅ATCC 32354 for 10 tested
527	- +	*C. albicans*	*C. albicans2* by 99.9% for 50 tested	*C.albicans* for 10 tested
448	+ +	*C. albicans*	C. albicans≅STD HG392 *for 50 tested*	C. albicans≅*STD HG392 for 10 tested*
109	- -	*C. famata*	*C. famata* by 99.9% for 20 tested	*C. famata* for 10 tested
716	- +	*C. tropicalis*	*C. trop*.(89.4)/ *C.lusitan*(11%) for 50 tested	*C. tropicalis* ≅N.46 for 5 tested
113	+ +	*C. albicans*	*C.albicans2/ C tropicalis* for 20 tested	*C. albicans* for 10 tested
164	- +	*C. albicans*	*C. alb*1(69%)/ *C. trop*(30%) for 20 tested	*C. albicans* ≅N.40 for 10 tested
157	+ +	*C. albicans*	*C. alb*1(98%)/ *C. trop*(30%) for 20 tested	*C. albicans* ≅N.40 for 10 tested
327	+ +	*Trichosporon*	*C. albicans2*(99.5%) for 50 tested	*C.albicans* for 10 tested
14	- -	*C. magnoliae*	*C. magnolia* for 10 tested	*C. magnoliae* for 5 tested
130	- -	*C. glabrata*	*C. glabrata*(99.4%) for 10 tested	*C.glabrata* for 10 tested
16	- -	*C. calliculosa*	*C. calliculosa*	*C. calliculosa* for 5 tested
27	- +	*C. lusitaniae*	*C.lusitaniae*(60%) for 10 tested	*C. lusitaniae* for 10 tested
131	- -	*C. parapsilosis*	*C. parapsilosis* for 10 tested	*C. parapsilosis ≅N212* for 10 tested
452	+ +	*C. albicans*	*C. albicans* for 10 tested	*C.albicans* for 10 tested
290	+ +	*C. albicans*	C.alb1(80) for 10 tested	*C. albicans ≅N14* for 5 tested

50	- -	*C. krusei*	-	*C. krusei ≅N51* for 10 tested
46	- +	*C. albicans*	*C. tropicalis* for 25 tested	*C. tropicalis ≅ N12* for 5 tested
13	- -	*C. inconspicua*	*C. incospicua* for 5 tested	*C. incospicua* for 10 tested
47	- -	*C. krusei*	*C. krusei/ C. inconsp./ Geotrichum spp.* for 5 tested	*C. krusei≅N45* for 10 tested
159	- -	**C. glabrata**	*C. glabrata(99.4%)* ≅ ATCC 2001 for 25 tested	*C. glabrata≅* ATCC 2001 for 10 tested
60	- +	*C.albicans/C. tropicalis*	*C.collic*(54)/C.magnol(45)/C.trop(30) for 5 tested	*C. albicans* for 5 tested
31	- -	*C. glabrata*	*C. glabrata*(99.4%) for 5 tested	*C. glabrata ≅ N136* for 5 tested
89	- +	C. albicans/C. tropicalis	*C.magnol(45)/C.trop(60)* for 10 tested	*C. albicans* for 5 tested
20	+ +	*C. albicans*	*C.alb*1(80.9)/C.alb2(13)/*C.tropicalis*(5) for 5 tested	*C. albicans* for 5 tested
5	- -	*C. glabrata*	-	*C. glabrata*≠similarity
27	- -	C. *guilliermondii*	C. *guilliermondii* for 5 tested	C. *guilliermondii* ≅ ATCC 6260 for 10 tested
110	- -	*C. glabrata*	*C. glabrata*(91.5%) for 5 tested	*C.glabrata* for 10 tested
11	+ +	*C. albicans*	*C. alb1*(98)/*C. trop*(30) for 5 tested	*C.albicans* for 5 tested
74	- -	*C. norvegensis*	*C. norvegensis* for 10 tested	*C. norvegensis* for 10 tested
3	- +	*C. norvegensis*	*C. norvegensis/ Kloeckera* spp for 3 tested	*C. norvegensis* for 3tested
66	- -	*C. krusei*	*C. krusei/C. inconspi*cua for 5 tested	*C. krusei* for 5 tested
17	+ +	*C. albicans*	*C. alb2/ C. trop* for 5 tested	*C. albicans* for 5 tested
5	- -	*C. norvegensis/ C. parapsilosis*	*C. norveg(45)/C. paraps(30)/C. krusei* for 5 tested	*C. norvegensis* for all 5 tested
22	+ +	*C. albicans/C. parapsilosis*	C. parapsilosis/ C. norvegensis *for 5 tested*	*C. albicans* for 3 tested

The species identified using diverse methods were as follows; Germ tube test show that 3661 (67.29%) were Candida albicans. However, a considerable percentage 1779 (32.71%) was non-albicans Candida species, mainly C. glabrata 435(8.0%), C. tropicalis 762(14.0%), C.

krusei 163(3.0%), C. famata 109(2.0%), C. parapsilosis 131(2.41%), C. guilliermondii and C. lusitaniae each 27(0.5%) respectively, C. incospicua 13 (0.24%) C. norvegensis 82(1.5%), C. colliculosa 16 (0.3%) and C. magnoliae 14 (0.26%) (Table3).

Table 4. Identification of *Candida* isolated from women with VC using API-Candida (n=5440)

Activity In Cupule												**Species**	**No. (%)**
Glu	Gal	Sac	Tre	Raf	β Mal	α Amy	β Xyl	β Gur	Ure	β Nag	β Gal		
+	-	-	-	-	-	-	-	-	-	-	-	*C. krusei*	163 (3)
+	-	-	+	-	-	-	-	-	-	-	-	*C. glabrata*	435 (8)
+	-	-	-	-	+	+	-	-	-	+	-	*Trichosporon*	317 (5.8)
+	+	-	-	-	-	-	-	-	-	-	-	*C.krusei/C.parapsilosis*	90 (1.7)
+	+	-	-	-	+	+	-	-	-	+	-	*Trichosporon*	10(0.18)
+	-	+	+	-	-	-	-	-	-	-	-	*C. famata*	11(0.2)
+	-	+	+	+	-	-	-	-	-	-	-	*C. famata*	50(0.9)
+	+	+	-	-	-	-	-	-	-	-	-	*C. parapsilosis*	131(2.4)
+	+	+	-	-	-	+	-	-	-	-	-	*C. tropicalis*	120(2.2)
+	+	+	-	-	-	+	-	-	-	+	-	*C. albicans*	830(15.3)
+	+	+	+	-	-	+	-	-	-	-	-	*C.tropicalis/C. albicans*	76(1.4)
+	+	+	+	-	-	+	-	-	-	+	-	*C. albicans*	1878(34.5)
+	+	+	+	-	-	-	+	-	-	-	-	*C lusitaniae / C. famata/C. tropicalis/C. guilliermondii*	325(5.97)
+	+	+	+	+	-	+	-	-	-	+	-	*C. albicans*	189(3.37)
+	+	+	+	-	+	+	-	-	-	-	-	*C. tropicalis*	186(3.4)
+	+	+	+	-	+	+	-	-	-	+	-	*C. albicans*	764(14)
+	+	+	+	-	+	-	+	-	-	-	-	*C. lusitaniae*	20(0.36)
+	+	+	+	-	+	+	+	-	-	-	-	*C. tropicalis*	332(6.1)

All doubtful isolates were further tested with API 20C and on API 20C AUX (Table 3). The results showed *Candida albicans* species to be dominant 3661 isolates while non-albicans Candida species species presented only 1779 isolates which included mixed species of *C. glabrata, C. tropicalis, C. krusei, C. famata, C. parapsilosis,* C. *guilliermondii* and *C. lusitaniae* , *C. incospicua, C. norvegensis, C. colliculosa and C. magnoliae*.

On genotypic diversity, the Polymerase chain reaction results analysed by the principle coordinate, showed the genetic relatedness and genetic distances (Figure 3) which found all the isolates distributed in 20 groups out of 200 isolates tested

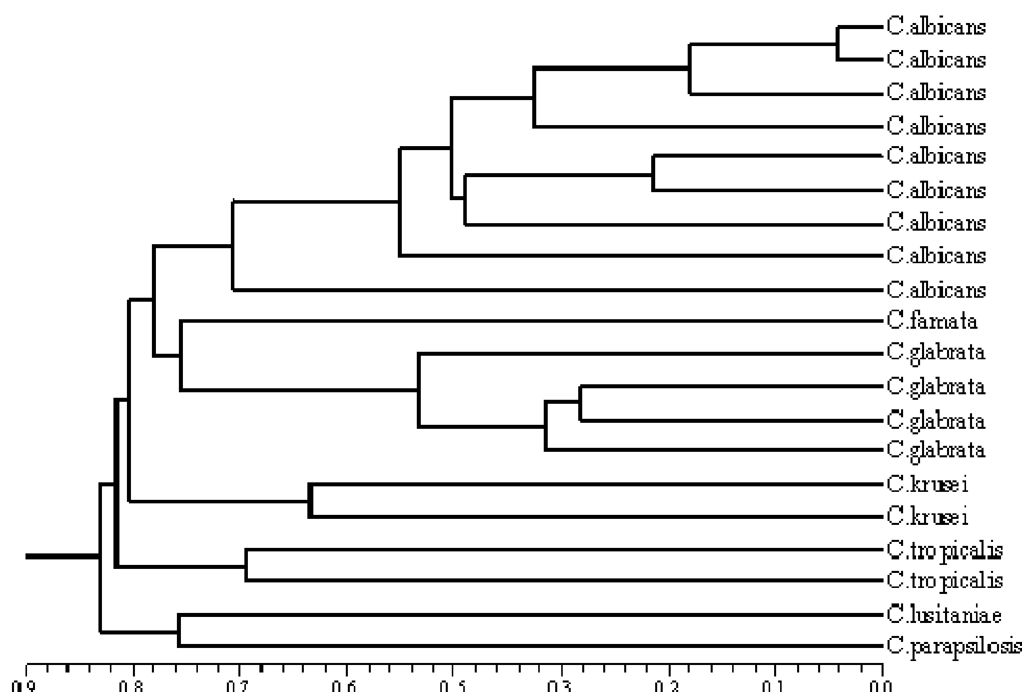

Figure 3. Phylogenetic analysis tree showing genetic relationships between the 20 *Candida* species, constructed using RAPD profiles of 11 primers in fig.2.

4. Discussion

This study comprehensively investigated *Candida* species occurring in women with vaginal candidiasis who were attending HIV and gynecological clinics in Private and Government hospitals of Dar Es Salaam Region, Tanzania. The study was based on several parameters, including Analytical Profile Index (API), colonial morphology on Sabouraud's dextrose agar and corn meal agar and RAPD PCR fingerprinting. On API 20C test, the predominant types of *Candida* isolated were *C albicans* 3121 (57.4%), *C tropicalis* 716 (13.2%), *C glabrata* 435 (8%), *C krusei* 163 (3%), *C. famata* 109 (2%), *C. parapsilosis* 131 (2.4), *C. lusitaniae* 27 (0.5), and *C. guilliermondii* 27 (0.5%), *C. norvegensis* 77(1.4%) together making a total of 88.4% of all isolates. This finding concur with that of Okonofua and Ako-Nai (1995); Okungbowa (2002); Okungbowa *et al.* (2003); Kam & Xu (2002) who also noted similar distribution of *C.albicans* being more abundant than other species. The other uncommon species (Table 3) accounted for less than 0.5% were *C. magnoliae 14* (0.26%) and *C. inconspicua* 13(0.23%). *C.*

glabrata never showed any changed on conidia enhancing media such as Corn meal agar.

In some cases, identification with the API Candida failed to discriminate between species and /or genera; e.g. *C. albicans* and C. *tropicalis* n=2 (Table 3&4) due to lack of reactions on raffinose, β maltosidase and N-acetyl-beta-glucosamine cupules. However, on subsequent testing the isolates were found to be germ tube positive and also formed chlamydospores on corn meal agar, features that are consistent with *C. albicans*. The difficulty in differentiating *C. krusei* from *C. parapsilosis* was due to variation in the assimilation of galactose and saccahrose (Table 3). The API Candida kit also had problems in differentiating some strains of *C. lusitaniae, C. famata, C. norvogensis* and *C. guilliermondii* due to variations of ß-maltosidase and raffinose reactivity, which are the key tests for their discrimination.

It is worthy mentioning that *C. albicans* had four API profiles due to variations in the assimilation of trehalose, raffinose and ß-maltosidase. *C. famata* had two API profiles due to variability in the assimilation of raffinose. The four isolates that were identified by API Candida as *Trichosporon*, had germ tube reaction and colonial morphological features on SDA, gram stain appearance and colonial morphology on corn meal agar that were consistent with *C. albicans*. This misidentification was attributed to the variability in assimilation of galactose, trehalose and ß-maltosidase.

There were five disagreements between API Candida and API 20C AUX, two of them did not meet the API 20C AUX identification criteria (% ID ‹ 80%). The three remaining discrepancies were: *Trichosporon* (API Candida) versus *C. albicans* (API 20C AUX), this was further tested with a germ tube test and production of chlamydospores on corn meal agar, the results confirmed to be *C. albicans* with PCR test. The second was *C. albicans* (API Candida) Vs *C norvegensis* (API 20C AUX), while the third *C. albicans* (API Candida) and *C. parapsilosis* (API 20C AUX), these isolates were later confirmed to be *C. albicans* by PCR test (Figure 3).

The observed difficulties of the API Candida and API 20 C AUX. kits in identifying germ tube negative rare isolates such as; *C. lusitaniae, C. krusei, C. parapsilosis, C. inconspicua* and *C. norvegensis* is in keeping with the findings of other studies (Verweji, 1999; Michel-Nguyen, 2000). These findings do indicate the need to expand the battery of reagents in both kits and possibly a change of testing algorithm for adequate identification of the rare germ tube negative species. Culturing germ tube negative isolates on Corn meal agar had produced features that aided speciation (Figures 1).

The genotypic identification of species by randomly amplified polymorphic DNA from crude single-colony lysates (Steffan *et al*., 1997) produced simple diagnostic fingerprints that are unique to isolates of *Candida* species. However, few isolates of *C. lusitaniae* or *C. norvegensis* and *C. parapsilosis* could not be adequately separated, see the phylogenetic tree as one cluster (Figure 3), this limitation may be solved by using more specific primers.

The species *Candida albicans*, considered as one of the most pathogenic, is the most frequently isolated yeast from the vagina of both symptomatic and asymptomatic individuals. However, *C. albicans* shows various mechanisms that are suggestive of virulence such as: capacity to take on various colonial forms, called adaptive variations; ability to form hyphae (germ tube

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formation and pseudohyphae); capacity to adhere to mucosal surfaces; produces hydrolytic enzymes; proteinases which hydrolyze peptides and phospholipases which hydrolyze phospholipids .

The findings in this study also indicate that, most of the patients were young, aged below 40 years (Table 2), with only primary education and having no employment/ or entrepreneurs and had first sexual intercourse before 20 years of age. Most are co-habitors /or married but with more than one partners. This combination of factors makes these women fall in the high risk group for HIV and other sexual transmitted infections. *C. albicans* in this study was observed to occur in almost all age groups, indicating that *C. albicans* is a resident flora in the vagina of every woman, and this is in keeping with the study done by Tanimowo *et al.,* (2012), Okungbowa *et al*., (2003).

The yeast microflora isolated from symptomatic women and those from asymptomatic women had similar species and genotypic diversities (Xu *et al.,* 1999, Boldo *et al*., 2003). However, it was noted from symptomatic women that, a single host can be colonized with multiple species or multiple genotypes of the same species, a trend that is in keeping with other studies Xu *et al.*, (1999), Kam & Xu (2002).

Among 1826 women with HIV infection in this study; 1713 (93.81%), were found to have recurrent vaginal candidiasis and were carrying species that are resistant to treatment especially with azoles. Several reasons might be the cause, for this trend to have refractory and recurrence vaginal candidiasis, including; increased incidences of vaginal candidiasis associated with non-albicans Candida species such as *C. glabrata, C. krusei, C. tropicalis, C. lusitaniae* etc, because 1672(93.98%) non-albicans Candida species from 1779 were isolated from HIV patients with recurrent vaginal candidiasis. This result is in keeping with studies done by; Hazen (1995), Sobel (1998), Sheehan *et al., (1999),* Abu-El-teen, (2001), Erika (2000).

Majority of HIV patients in Tanzania are given Fluconazole tablets for prophylaxis of vaginal thrush and cryptococcal meningitis, the compliance to therapy in such patients is not known, there might be uncontrolled consumption of antifungal preparations, which may have been resulted in secondary, resistance, i. e. increasing usage of short course antifungal therapies, inadequate treatment or poor patients compliance e.g. drug interruptions in patients with recurrent vaginal candidiasis had enabled the emergence of less common more resistant yeast strains as vaginal pathogens, this is in keeping with studies done elsewhere, (Jovanovic *et al., 1991,* Bjornson *et al., 1992, Rex et al., 1995,* Silverman *et al., 2001,* Segal & Minamoto, 2001).

Although this study revealed interesting results, it yet had some limitations. For instance the methods used to identify a large number of isolates were not discriminatory (Table 3&4) especially the API- identification results gave more than one species, only the PCR method was discriminatory, but due to financial constraints it was not possible to study species characteristics in a large sample size. This study therefore recommends further studies using molecular methods with specific primers that will discriminate candida species, also to study specific underlying factors in order to compare *Candida* species carriage in healthy individuals with that of patients. There is also a need to study the pathogenicity factors associated with

Candida species in order to find out, as to why the same species behave differently from asymptomatic to symptomatic persons.

5. Conclusion

In conclusion, the findings of this study do show that approximately half of women seeking primary health care for vaginal discharge and pruritis vulvae have VC, and also, a larger percentage (93.98%) of HIV patients are carrying non-albicans Candida species which are resistant to treatment leading to recurrences. This finding should be taken into account when considering management of women with vaginal discharge or pruritus vulvae. The results of this study also indicate that a combination of biochemical methods and RAPD technology are feasible for identification and differentiation of various pathogenic Candida isolates, including the germ tube negative isolates. The results also revealed that a single host with symptoms of vaginal candidiasis can harbor multiple Candida species (fig. 1a). Moreover, the significance of increased species diversity in vaginal Candida carriage must be investigated in relation to the development of candidiasis.

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