SPECIES DISTRIBUTION AND ANTIFUNGAL SENSITIVITY PATTERNS OF VAGINAL YEASTS

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ABSTRACT

Objectives: To identify yeast isolates in vaginal specimens to species level and determine their antifungal susceptibility patterns.
Design: Cross-sectional laboratory-based study.
Setting: The Aga Khan University Hospital (AKUH), Nairobi.
Subjects: Yeast isolates from high vaginal swabs presented to the laboratory for culture and sensitivity were identified to species level using the API Candida system and subjected to broth microdilution susceptibility testing.
Main outcome measures: Frequency tables and graphs were used to summarise the data. Susceptibility data was analysed by the non-parametric Fisher's exact test. $P < 0.05$ was considered statistically significant.
Results: A total of 101 yeasts were studied. *C. albicans* was the prominent species (69.3%) followed by *C. glabrata* (12.9%), *C. famata* (5.0%), *C. krusei* (3.0%), *Trichosporon species* (3.0%) and *S. cerevisiae* (3.0%), *C. parapsilosis* (1.0%). Three (3.0%) of the isolates had profiles that fell between *C. glabrata* and *C. famata*. The percentages of *C. albicans* susceptible to flucytosine, amphotericin B, fluconazole and itraconazole were 94.3, 92.9, 100 and 90 respectively; that of non-albicans isolates were 93.5, 80.6, 77.4 and 29 respectively. There was no significant difference ($p > 0.05$) between the susceptibility of *C. albicans* and non-albicans isolates to flucytosine and amphotericin B, however there was a significant difference ($p < 0.05$) to fluconazole and itraconazole.
Conclusions: *C. albicans* was the predominant cause of vulvovaginal candidiasis in this study, and demonstrated good susceptibility to antifungal agents tested. A significant number of non-albicans yeasts were identified; these demonstrated reduced susceptibility to all drugs, in particular to the azoles which are commonly used for treatment of vaginal candidiasis. The isolation of non-albicans yeasts may have clinical implication given their reduced susceptibility to antifungals.

INTRODUCTION

Vulvovaginitis is the one of the most common gynaecological condition seen in women worldwide. The three common vaginal infections include vulvovaginal candidiasis (VVC), bacterial vaginosis, and *Trichomonas vaginalis* infection; there is a paucity of data on the global and national burden of these infections.

Vulvovaginal candidiasis frequently results from an overgrowth of the normal flora *Candida* species in the vagina. *Candida* is usually present in the lower female genital tract in small amounts, being part of the normal flora in 10-20% of healthy asymptomatic women (1). The over growth of the yeast and development of vulvovaginitis is usually prevented by the balance created by bacteria such as *Lactobacillus acidophilus*. Any condition that disrupts this balance of the normal flora predisposes to VVC. These include antibiotic use, oral contraceptives and contraceptive devices, high oestrogen levels and immunocompromised states such as diabetes mellitus and HIV.

C. albicans is responsible for most cases of VVC (2, 3); the rest are caused by non-albican species of Candida which include *C. glabrata*, *C. krusei*, and *C. tropicalis*. Non-albican species of *Candida* have demonstrated decreased susceptibility to

commonly used antifungal agents (5). *C.krusei* has intrinsic resistance while *C. glabrata* has decreased susceptibility to fluconazole. *C. albicans* has also been shown to be developing resistance to the azole group of antifungal agents. This makes identification of yeasts to species level and antifungal susceptibility testing necessary in order to appropriately select the agent to use. Antifungal susceptibility testing may be used to predict clinical response, or at least to forecast treatment failure, and consequently can be used to develop local antibiograms to aid in empirical selection of antifungals; in testing of isolates to aid in selection of long-term therapy, and in testing of isolates from recurrent mucosal disease to aid in selection of alternative regimens.

Species identification and antifungal drug sensitivity profiles are not routinely carried out in the management of vaginal discharge among women seeking medical care in health facilities in Kenya. Therefore the present species distribution patterns and status of fungal resistance to conventional antifungal drugs among women in Kenya is largely unknown. This cross-sectional laboratory-based study was therefore designed to determine the species of yeasts causing vaginal infection and the current antifungal susceptibility patterns in the study set up. Documentation of a significant level of non-albicans species of *Candida* and their susceptibility patterns would pave the way for larger studies that would inform on any policy changes required in the of vaginal candidiasis.

MATERIALS AND METHODS

Study area: The study was carried out at the Microbiology laboratory of the Aga Khan University Hospital, a private university-affiliated hospital in Nairobi. The laboratory is well equipped and receives laboratory specimens from inpatients, outpatients from the casualty and outpatient clinics, as well as patients seeking medical care from the private doctors' clinics in the private wing.

Study population: All vaginal yeasts isolated at the Microbiology laboratory during the study period.

Sample size: The minimum desired number of samples to be analysed was 100.

Sampling: Consecutive sampling was used to select the vaginal yeasts for processing.

Inclusion criteria: Yeasts that demonstrated moderate or heavy growth on Sabouraud dextrose agar (SDA) were included in the study. In those with light growth, the gram stain from the original specimen was examined and only those with pus cells ≥2+ were included in the study.

Laboratory procedures: Suspected yeast colonies were examined in a drop of normal saline for budding cells, and confirmed by the Gram stain. The yeast isolates were identified to species level using API® Candida system (BioMerieux, Marcy-l 'Etoile, France). The API *Candida* strip consists of ten tubes containing dehydrated substrates, which enabled the performance of sugar acidification tests and enzymatic reactions. The reactions produced during incubation were revealed by spontaneous color changes which were transformed into a numerical profile using the interpretation table provided. The numerical profile obtained was compared to those given in the profile list in the package insert to get the yeast species.

The susceptibility of the 101 yeast isolates to four antimycotics was tested using the broth microdilution method using ATB® Fungus 2INT (BioMerieux, Marcy-l'Etoile, France). The kit contains the following drug dilution ranges: amphotericin B (AMB) 0.5-16mgl^{-1}, 5-flucytosine (5FC) 0.5-64 mgl^{-1}, fluconazole (FCA) 0.25-128 mgl^{-1} and itraconazole (ITR) 0.125-4 mgl^{-1}. The strip consists of 16 pairs of cupules. The first pair does not contain any antifungal agent and is used as a positive growth control. The next 15 pairs contain the four antifungal agents at several concentrations, enabling minimum inhibitory concentration (MIC) determination. The preparation of the inoculums, strip inoculation and incubation, and reading of the results were carried out according to the manufacturer's instructions. The growth score was read for each cupule and the MIC determined for each drug as per instructions. The MICs were interpreted into clinical guidelines, sensitive, intermediate and resistant, by using Clinical and Laboratory Standards Institute (CLSI) recommended breakpoints (in μgml^{-1}) for Candida species, provided in the manufacturer's instructions.

Data management: All data collected from the laboratory procedures were entered into MS Excel, verified and cleaned. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA). Frequency tables and graphs were used to summarize the data. The susceptibility data was analyzed by the non-parametric Fisher's exact test. P< 0.05 was considered statistically significant.

RESULTS

Species identification and distribution: A total of 101 yeast isolates were collected and tested between November 2006 and January 2007 (Table 1). *Candida* species accounted for 94.1 % of the isolates with *C.albicans* being the most common (73.7% of all *Candida* species) (Figure 1). Among the non-albicans species, *C.glabrata* (13.7% of all Candida species) and *C.famata* (5.3%) were the most commonly identified species. Three of

the non-albicans isolates had profiles that fell between *C. glabrata* and *C. famata*. Six isolates were identified as non-*Candida* yeasts (Figure 2).

Table 1

Species distribution of vaginal yeasts isolated from women with vaginal discharge at the AKUH

Specie	Isolates No.	(%)
C.albicans	70	69.3
C.glabrata	13	12.9
C.famata	5	5.0
C.krusei	3	3.0
C.parapsilosis	1	1.0
Unidentified Candida species*	3	3.0
Trichosporon species	3	3.0
Saccharomyces cerevisia	3	3.0

*Numerical profile could not differentiate between *C.glabrata* and *C.famata*

Figure 1

Percentage distribution of Candida species isolated from women with vaginal discharge at the AKUH
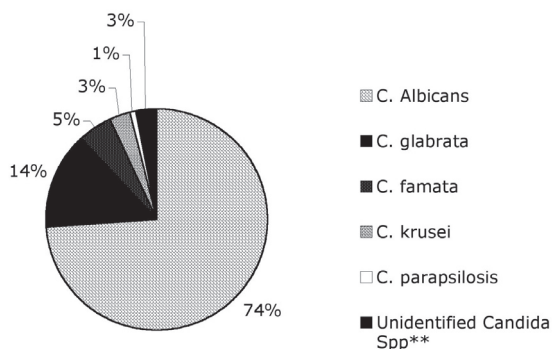
** Numerical profiles could not differentiate between *C. glabrata* and *C. famata*

Figure 2

Percentage distribution of yeast species isolated from women with vaginal discharge at the AKUH
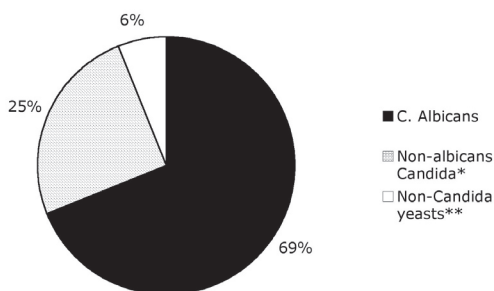
* *C. glabrata, C. Famata, C. krusei, C. Parapsilosis,* unidentified *Candida* species (*C.glabrata/ C.famata*)
** *Trichosporon* species, *S. cerevisiae*

Antifungal susceptibility testing: The 101 isolates were tested for susceptibility to flucytosine, amphotericin B, fluconazole and itraconazole. Overall, 55 of the 70 *C. albicans* (78.6%), six of the 25 non-albicans *Candida* strains (24%) and one of the six strains of non-candidal yeasts (16.7%) were susceptible to all tested antifungal agents. Resistance to one drug was demonstrated by 20% of the *C. albicans* strains (amphotericin B or fluconazole or itraconazole), 52% of the non-albicans *Candida* (flucytosine or itraconazole) and 66.7% of the non-candidal yeasts (amphotericin B or itraconazole). Of the *C.albicans* strains, 1.4% showed resistance to two of the tested agents (amphotericin B and itraconazole) while 8% of the non-albicans *Candida* and 16.7% of the non-candidal yeasts showed resistance to three of the antifungal agents (amphotericin B, fluconazole and itraconazole). No isolate tested was resistant to all tested antifungal agents. The percentage of resistant strains to each of the antifungal agents was as shown in Table 2.

Ninety five of the 101 yeast isolates (94.1%) were susceptible to flucytosine. One isolate (1.0%) showed intermediate susceptibility and five isolates (5.0%) were resistant. Sixty six of the 70 *C.albicans* strains (94.3%), 23 of the 25 non-albicans *Candida* (92%) and all the non-candidal yeasts were susceptible. The susceptibility patterns to flucytosine did not differ significantly between the *C. albicans* and the other yeast isolates (p=0.09) (Table 3). Of the 101 isolates, 90 (89.1%) were susceptible to amphotericin B and 11 (10.9%) resistant. Sixty five (92.9%) of *C. albicans* strains, 23 (92%) of the non-albicans *Candida* and two (33.3%) of the non-candidal yeasts were susceptible. There was no significant difference between the susceptibility patterns of C. *albicans* and the non-albican isolates (p=1.00) (Table 3).

Ninety four of the isolates (93.1%) were susceptible to fluconazole. Four isolates (4.0%) were susceptible-dose dependent while three (3.0%) were resistant. All *C. albicans* strains were susceptible; 19 (76%) of the non-albicans *Candida* and five (83.3%) of the non- *Candida* yeasts were also susceptible. One of the three *C. krusei* strains was susceptible; the other two were susceptible-dose dependent. This is however not an important finding clinically since the species must be considered clinically resistant to fluconazole as per CLSI recommendations (6). There was significant difference between the susceptibility patterns of *C. albicans* and the other yeast isolates to fluconazole (p=0.00) (Table 3).

Itraconazole demonstrated the lowest susceptibility overall with only 72 of the 101 isolates (71.3%) being susceptible, 23 isolates (22.8%) were resistant. *C.albicans* was the species with the highest susceptibility at 90%; the lowest was demonstrated by *C. parapsilosis* (0%), *C. glabrata* (15.4%) and *C. famata* (20%). There was significant difference between the susceptibility patterns of *C. albicans* and the other yeast isolates to itraconazole (p=0.00) (Table 3).

Table 2

In vitro susceptibilities of 101 vaginal yeasts to four antifungal agents as determined by MIC_{90}

Drug and breakpoint		Species		
		C. albicans (0=70)	Non-albicans *Candida* (n=25)	Non- candidal yeasts (n=6)
Flucytosine (MIC >16)	MIC_{90}	2	4	4
	% resistant	5.7	4	0
Amphotericin B	MIC_{90}	0.5	1	16
(MIC ≥2)	% resistant	7.1	8	66.7
Fluconazole	MIC_{90}	1	32	128
(MIC > 32)	% resistant	0	8	16.7
Itraconazole	MIC_{90}	0.125	4	4
(MIC > 0.5)	% resistant	10	56	33.3

MIC_{90} and MIC in μgm^{-1}

Table 3

Antifungal sensitivity patterns of vaginal yeasts isolated from women with vaginal discharge at the AKUH to amphotericin B, flucytosine, fluconazole and itraconazole

Variable (Drug)	Species		Statistical tests Fisher Exact test
	C. albicans	Non albicans	
Amphotericin[B]			
Sensitive[a]	92.9%	80.6%	X^2= 3.30: 1df
Resistant[B]	7.1%	19.4%	P>0.05 (0.09)
Flucytosine			
Sensitive[c]	94.3%	93.5%	X^2= 0.02: 1df
Intermediate[d] & resistant	5.7%	6.5%	P>0.05 (1.00)
Fluconazole			
Sensitive[f]	100.0%	77.4%	X^2= 16.98: 1df
S-DD[g] & resistant[h]	0.0%	22.6%	P<0.05 (0.00) significant
Itraconazole			
Sensitive[i]	90.0%	29.0%	X^2= 39.02: 1df
S-DD[j] & Resistant[K]	10.0%	71.0%	P<0.05 (0.00) significant

S-DD= Susceptible-dose dependent

[a]$MIC<2\mu gml^{-1}$, [b]$MIC≥2\mu gml^{-1}$, [c]$MIC≤4\mu gml^{-1}$, [d]$MIC=8\text{-}16\mu gml^{-1}$, [e]$MIC≥32\mu gml^{-1}$, [f]$MIC≤8\mu gml^{-1}$, [g]$MIC=16\text{-}32\mu gmr^{-1}$, [h]$MIC^2≥64\mu gmr^{-1}$, [i]$MIC≤0.125MMI^{-1}$ [j]$MIC=0.25\text{-}0.5\mu gml^{-1}$, [k]$MIC≥1\mu gml^{l}$

DISCUSSION

Most (69.3%) of the species isolated in this study were *C.albicans* and a significant percentage (30.7%) were non-albicans yeasts (Table 1). The non-albicans species isolated were mainly *C.glabrata* (12.9%) and *C. famata* (5.0%). Only two species of non-*candida*. yeasts were isolated: *Trichosporon* species and *S.cerevisiae*. *C.albicans* was the predominant species isolated in other studies (7-14) which concurs with the findings in this study. The distribution of the non-albicans species, however, differed in different studies. The most commonly isolated non-*albicans* species differed as *C.glabrata* (7-13) and *C. parapsilosis* (14). The second most common non-albicans species differed in the different studies as *C.glabrata* (14), *C. tropicalis* (8,10,11) *C. parapsilosis* (12), *C. famata* (9) and *C.krusei* (7).

Comparing the findings in the current study with those of some of the studies which demonstrated *C.albicans* in 40.47-82.90% of isolates, *C. glabrata* in 5.6-30.09%, *C.famata* in 0.08-2.20%, *C.krusei* in 1.57-11.17%, *C.parapsilosis* in 0.49-11.30%, *C.tropicalis* 2.43-7.8%, unidentified *Candida* species in 0.90-7.54%, *Trichosporon* species in 0.29-0.89% and *S.cerevisiae* in 0.15-0.70% of isolates. Most of the findings in this study were within these ranges except for *C.famata*, *Trichosporon* species and *S. cerevisiae* which were more frequent in this study. *C.tropicalis* was also isolated in these studies but was not found in the current study (7-14).

In the current study, flucytosine showed an overall susceptibility of 94.1%; 5.0% of the isolates were resistant (Table 2). The tested strains demonstrated a 0 - 5.7% resistance except for *C.krusei* which had a resistance of 33.3%. The high resistance rate in this species may be explained by the low rate of isolation of *C. krusei* (three isolates). Nawrot *et al* (13) found a low frequency (0-3%) of resistance of tested strains while Richter *et al* (12) found an overall resistance of 3% with resistance of tested strains in the range 0-100%. The 100% resistance was found in the strains isolated in small numbers in that study (12) *C.lusitaniae* and *Trichosporon* species which had one isolate each. A study on isolates of *Cryptococcus neoformans* from clinical sources from Nairobi found a 21.2% resistance to flucytosine (15). This is a drug for treatment of *cryptococcosis* and it has been recommended for use in combination with amphotericin B. It was not established whether that is actually being done in the current study. It was also not established whether the patients with flucytosine-resistant isolates had previously been on treatment with the drug.

Amphotericin B, which is the drug of choice for cryptococcal meningitis, demonstrated an overall susceptibility of 89.1% in this study, with 10.9% of isolates being resistant (Table 2). The susceptibility testing of this drug is still a controversial area; CLSI breakpoints have not yet been defined. Though some studies use a breakpoint of $1 \mu gml^{-1}$, strains in this study with $MIC \geq 2\mu gml^{-1}$ were considered resistant according to the manufacturer's instructions. There is limited information on amphotericin B susceptibility in this country except for cryptococcal infections. Bii *et al* (15) found a 0% resistance of the drug to *C.neoformans* with a low MIC_{90} of $0.5\mu gml^{-1}$. In the current study, though the resistance was much higher, a similar MIC_{90} was found for *C.albicans* with a resistance of 7.1%; the other species had higher MIC_{90} values Table 2 of $1 \mu gml^{-1}$ for non-albicans *Candida* (8% resistant) and $16\mu gml^{-1}$ for non-candidal yeasts (66.7% resistant). Other studies (12,13,16) also demonstrated a low frequency of resistance to amphotericin B (0-1%). The use of amphotericin B for treatment of cryptococcal meningitis, which is an opportunistic infection that has increased especially in the wake of the HIV / AIDS

pandemic, may result in development of resistance in the normal flora *Candida* species found in the vagina and in the mouth and if these then cause endogenous infection will demonstrate resistance to the drug. The resistant strains can also circulate in the community and may have far-reaching implications especially in the event of systemic candidiasis.

Fluconazole demonstrated an overall susceptibility of 93.1% and 3.0% resistance in the current study (Table 2). Among the isolates in this study, *C. albicans* had a 100% susceptibility which is in agreement with the results obtained in the study by Pfaller *et al* (8) that demonstrated *C. albicans* susceptibility of approximately 94-100% though with some geographical differences. In the same study, the *C.albicans* isolates from South Africa had a susceptibility of 99.4% (0.3% resistant). In other studies, *C.albicans* demonstrated fluconazole susceptibility of 11 (84.3%), 13 (92%) and 14 (92.5%). *C.krusei* has intrinsic resistance to fluconazole while *C. glabrata* shows reduced susceptibility. The *C.glabrata* isolates in the current study demonstrated a susceptibility of 84.6% (7.7% S-DD, 7.7% resistant). The ARTEMIS Global Antifungal Surveillance study demonstrated *C.glabrata* susceptibility in the range 29.2-100%; the isolates from S. Africa had a susceptibility of 49.6% (21.1% resistant). The MIC_{90} of fluconazole for *C.albicans* in the current study (Table 2) was $1 \mu gml^{-1}$ (range: 0.25-8) which was similar to that obtained by Comert *et al* (range: 0.125-8) (14). The MIC_{90} for *C.glabrata* in the current study was $32\mu gml^{-1}$ (range: 0.25-128) which was much higher than that for the same species in the study by Comert *et al* of $8\mu gml^{-1}$ (range: 0.125-16). The elevated MIC_{90} for *C.glabrata* in this study may have been due to either the small number of *C.glabrata* isolates with a wide range of MIC distribution or to a rising resistance of the isolates in this study. Bii *et al* in the study on *C.neoformans* demonstrated a resistance to fluconazole of 11.2% with a MIC_{90} of $64\mu gml^{-1}$ (15). The MIC_{90} of *C.neoformans* in that study was higher than that of Candida in the current study hence *C. neoformans* demonstrated a higher resistance rate compared to *Candida*.

Itraconazole had the lowest overall susceptibility (71.3%) in this study with a resistance rate of 22.8% (Table 2). *C.albicans* was the species with the highest susceptibility (90%) to this drug with that of the other isolates ranging between 0% (for *C.parapsilosis* which had only one isolate) and 100%. The resistance rate for *C. albicans* was 10% which is in agreement with that found by Nawrot *et al* of 11% (13). Non-albicans *Candida* had a resistance rate of 50% in the current study which was higher than that found by Galle *et al* of 23.5%. The study by Bii showed itraconazole resistance rate of 6.3% (MIC_{90} $0.5\mu gml^{-1}$) which is much lower compared to that of *Candida* isolates in the current study. All the isolates resistant to fluconazole were

also found to be resistant to itraconazole supporting the possibility of cross-resistance between the azoles. This cross-resistance may be due to a common mode of action of the azole derivatives.

Isolates inhibited by fluconazole and itraconazole at concentrations of 16-32μgml^{-1} and 0.25-0.5μgml^{-1} respectively are placed in a category known as susceptible-dose dependent (S-DD). This category identifies yeast isolates with intermediate susceptibilities, between fully susceptible and fully resistant. Isolates in this category will respond to fluconazole if higher doses are administered and to itraconazole if higher plasma levels are achieved (5). The definition of fluconazole susceptible and resistant does not apply for *C.krusei* since the organism is considered to have intrinsic resistance to the drug (6).

Identification of yeast isolates to species-level may aid in guiding the clinician on therapeutic options. VVC may be classified into complicated and uncomplicated forms. The uncomplicated form is caused by *C.albicans,* which is usually susceptible to all antifungal agents, and responds to short-course oral or topical treatment (17). The complicated form is recurrent, caused by non-albicans species of *Candida* and requires treatment for $\geq$7 days. Patients with recurrent VVC are also more likely to acquire resistant *Candida* species due to frequent exposure to antifungal agents for the recurrent infections. Approximately 33% of RVVC is also known to be caused by non-albicans Candida species some of which are inherently less susceptible to common antifungal agents making them less useful in their treatment (18). The optimal treatment of non-albicans *Candida* species remains unknown[1] but they frequently respond to topical boric acid or topical flucytosine (17). Isolation of *C.krusei* in any specimen rules out the use of fluconazole for the treatment of the patient, even when the isolate tests susceptible *in-vitro*, since the species has intrinsic resistance to this antifungal agent while *C.glabrata* shows reduced susceptibility to the same agent, *C.lusitaniae* has been associated with amphotericin B resistance. Susceptibility testing is especially useful in infections due to non-albicans *Candida* species (17). In the setting of candidemia and deep infections susceptibility testing may be of benefit especially in cases where initial therapy has failed, the results may guide on suitable adjustment of therapy.

It is however important for the clinician to understand that *in-vitro* susceptibility results are supposed to be guidelines to the treatment of patients since they may not always predict the success of a particular treatment. However, when the infection is caused by a resistant isolate, the tests may predict the possibility of failure for a particular agent or the dosage. The clinician must also understand that *in-vivo* response to a drug is a combination of several factors: drug factors, host factors, site of the infection and fungal factors (5). Drug factors include the dosage (quantity, frequency and cumulative dose), pharmacokinetics (absorption, distribution, metabolism and excretion), pharmacodynamics and other concomitantly administered drugs (drug-drug interactions). Host factors include the immune system which works with the drug to clear the infection, the site and severity of the infection and patient compliance to the given treatment. Site of the infection factors include the source of the infection, drug penetration and the presence of a foreign body which acts as a foci for infection. Fungal factors include the species, cell type, the size of the micro-organism population, virulence factors, evasion of host inflammatory response and biofilm formation. All these factors have a role to play in the final resolution of the infection and should be taken into consideration when starting the patient on treatment.

The current study had limitations in time and cost. Due to these, a small sample size of 101 yeast isolates was analyzed. This resulted in some of the yeast species being isolated in very small numbers. The antifungal susceptibility testing was also to very few antifungal agents and none of the new agents in the market were tested for example voriconazole.

This study therefore paves the way for more studies with larger sample sizes, and in different parts of the country in order to understand the local epidemiology of yeast species and to study the local epidemiological susceptibility patterns. These would then be used to come up with local antibiograms that will aid clinicians in the selection of effective antifungal agents that can be used empirically. Larger studies to assess the clinical correlation of MIC and outcome are also required to enhance the understanding of the susceptibility testing in our set up. These studies would then form a baseline from which surveillance of antifungal drug resistance can be monitored both in hospitals and in the community.

In conclusion, this study observed that *C. albicans* was the prominent cause of vulvovaginal candidiasis in the study set up; non-albicans yeasts were a significant cause of vulvovaginal candidiasis. *C.albicans* still demonstrates susceptibility to fluconazole and itraconazole. There is a higher resistance demonstrated by non-albicans yeasts to fluconazole and itraconazole than by *C.albicans.*

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