Prevalence and Antifungal Susceptibility Pattern of Candida Isolates from Women with Recurrent Vulvovaginal Candidiasis in Ghana - A Review of Laboratory Reports.

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Abstract

Vulvovaginal candidiasis (VVC) is a common infection among women of childbearing age, and few of these women experience recurrent vulvovaginal candidiasis (RVVC). The study was aimed at determining the virulent factors, and antifungal susceptibility of the *Candida* species isolated from women with RVVC attending the Nkawie Government Hospital, Ashanti-Region, Ghana. Over a 6-month period (October 2016 to March 2017), a total of 288 women with RVVC were evaluated. Isolation of the yeast was performed after the inoculation of the vaginal specimens onto Sabouraud Dextrose Agar (SDA), and incubated for 24-48 hours at 37°C. The isolates were identified by standardized conventional methods. The enzymatic activities of esterase, phospholipase, haemolysis and biofilm production were evaluated for the identification of the yeast isolates. Susceptibility to antifungal agents was determined by using the Kirby-Bauer disk diffusion method. Azole resistant isolates were further tested for *ERG11* gene which encodes the enzyme (*cytochrome P450 lanosterol 14- α -demethylase*) by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Vaginal swabs cultures of 200 women (64.4) from 288 samples yielded *Candida* species. *Candida albicans* was the commonest isolated specie (33.0%), followed by *Candida glabrata* (29.5 %), *Candida tropicalis* (23.0%), and *Candida krusei* (15.5%). Hemolysin production, phospholipase enzyme activity, and biofilms formation were found in 84.5%, 83%, 77.5%.of the isolates respectively. Most phospholipase producing *Candida* isolates also formed biofilms. All *Candida spp* isolated were susceptible to itraconazole while majority of them were resistant to voriconazole. *ERG11* genes were detected in 11.1% of Azole resistant *Candida* species. There is a significant increase in the rate of antifungal resistance among the *Candida* isolates to fluconazole and voriconazole. There is need for continuous surveillance as well as antifungal susceptibility testing on the *Candida spp* to guide therapy. A larger epidemiological study is also advocated to determining the degree of spread of *ERG11* genes.

Introduction

Vulvovaginal candidiasis (VVC) is a common infection caused by *Candida* species among women of childbearing age, and 5–8% of these women experience recurrent vulvovaginal candidiasis (RVVC). Symptoms include vaginal itching or soreness, dyspareunia, discomfort when urinating and in some cases abnormal vaginal discharge (Melo *et al.*, 2019). Even though most cases of VVC are mild, some women can develop severe infections that lead to redness, swelling and cracks in the vaginal wall (Melo *et al.*, 2019). It has been estimated RVVC affects about 138 million women annually with a global prevalence of 3871 per 100, 000 women while 372 million women are affected over life time (Denning *et al.*, 2018). The 25–34 year group has the highest prevalence (9%) according to Denning *et al.* (2018). The risk of acquiring RVVC depends on many host-related and behavioral factors like age, pregnancy, diabetes mellitus, therapeutic immune suppression, locality and social economic status (Donders and Sobel 2017). In a retrospective analysis of pertinent data compiled by the Microbiology and Infection Control Unit of the Latifa Hospital, in Dubai, the incidence of vaginal candidiasis significantly increased from 10.76% in 2005 to 17.61% in 2011, with average prevalence of 13.88% (Hamad *et al.*, 2014).

Different strains of *Candida* have been isolated from 21% of women who reported abnormal vaginal discharge at the Gynecological Department of the Komfo Anokye Teaching Hospital (KATH) in Kumasi, Ghana (Feglo and Narkwa 2012). Similar results were confirmed by Konadu et al. (2019), who reported prevalence of 36.5% of VVC among pregnant women attending antenatal clinic in the middle belt of Ghana.

Candida species are commensals in various ecological niches of the human body (Rajilić-Stojanović and de Vos, 2014), by co-existing with the normal bacterial flora in human hosts. The growth of *Candida* species is continually being restricted by the innate immune system and complex bacterial microbiome dynamics. (Ilkit and Guzel, 2011) Some of these bacteria produce molecules which inhibit the growth of the yeast cells (Maheronnaghsh et al., 2019). *Candida* can become a pathogen when the normal bacterial flora are disturbed leading to opportunistic infections in the immunocompromised host when the organism overgrows, and causes infection (Viegas et al., 2019). Pathogenicity is enhanced as a result of the activation of the virulent factors in the organism (Liken and Kaufman, 2018), which the fungus uses to establish itself, and interact directly with the host cells (Chen and Huang, 2018). *Candida* species use nutrient acquisition, stress adaptation and immune evasion mechanisms as well as polymorphism, biofilm formation, and extracellular hydrolytic enzymes to overcome normal bacteria flora. Others include the host's immune system and subsequent invasion of the host tissues (Desai, 2018).

The azole drugs which include miconazole, clotrimazole, fluconazole, and itraconazole are the commonest antifungal agents that are usually prescribed for treating VVC (Choukri et al., 2014). These imidazole and triazole compounds inhibit the production of the enzymes needed for the demethylation step in the synthesis of ergosterol, a sterol found in the plasma membrane of fungi, and it is known to maintain the integrity of the fungal cell wall (Whaley et al., 2017). Azole drugs are cheap and safe to use; however, the frequent use of these drugs can lead to the emergence of resistant strains (Wiederhold, 2017). The mechanism of resistance is the failure of the drugs to cross the yeast cell membrane due to over-expression and alteration of the target enzyme gene *ERG11* that is involved in ergosterol production (Morschhäuser, 2016). Also, it has been found that the up-regulation of drug efflux pump genes such as *CDR1* and *CDR2* in some species of *Candida* also can results in the development of resistance to azole drugs. Missense mutations in the *ERG11* gene can also lead to the alteration of the drug target site and result in drug resistant in *Candida* species (Whaley et al., 2017). Studies have documented the aetiology, pathogenesis, immunity and development of vaccines for *Candida* species that have been causing RVVC in women in different parts of the world (Sobel, 2002; Cassone, 2015). However, in Ghana there are paucity of studies on the aetiology of RVVC especially infections caused by *Candida* species (Apea-Kubi et al., 2005; Abuquah, 2012; Essel et al., 2014). Even in those studies the virulent factors associated with the *Candida* species were not reported. This study was aimed to determining the prevalence of *Candida* species, their virulence factors, antifungal susceptibility pattern, and molecular characteristics of the isolates from women with RVVC attending the Nkawie Government Hospital, Ashanti-Region, Ghana.

Materials And Methods

Study design and sample collection

It was a cross sectional study carried out from October 2016 to March 2017 among women diagnosed with RVVC at the Out-Patient's Department (OPD), Ante-Natal Clinic (ANC) and the Anti-Retroviral Therapy (ART) Unit of the Nkawie-Toase Government Hospital, Ashanti Region, Ghana. High vaginal samples (HVS) were collected from women who presented with history of RVVC by a trained midwife after duly signed informed consent. The samples were placed into the tubes containing Amies transport medium and transported immediately to the laboratory for analysis.

Wet film preparation and Gram staining

The swabs were processed immediately upon arrival in the laboratory. The tube was shaken to dislodge materials from the swab into the saline to form a suspension. A drop of the saline suspension was placed on a clean grease free microscopic glass slide with cover slip, and then examined under the light microscope for budding yeast cells (Fig. 1). Cell counts of greater than 5 per high per field (> 5 /HPF) and hyphae or pseudohyphae were taken as indicative of an active infection. The swabs were also subjected to Gram staining for the presence of budding yeast cells and pseudohyphae or hyphae (Fig. 2).

Germ-tube test

Colonies of the *Candida* isolates were speciated using modification of the germ tube test as described by Matare et al. (2017). Briefly, triplicates sets of test tubes containing 0.5-1.0 ml of pooled human serum were inoculated with 2–3 colonies of each isolate. The tubes were incubated at 37°C for 3 hours, after which a drop of each suspension was placed on labeled microscopically for examination. The presence of germ tubes was used as presumptive identification of *C. albicans*.

Isolation of and identification of *Candida* species

The vaginal samples were cultured on Sabouraud Dextrose Agar (SDA), and incubated for 24–48 hours at 37°C to isolate the *Candida* strains. Isolated strains on the SDA were inoculated on CHROMAgar™ *Candida* (HiMedia, Laboratories, India) and incubated at 37°C for 48 hours for presumptive differentiations and identification of *Candida albicans* from non-*albicans Candida*. Growths with various colours were employed in the identification of *Candida* species (Fig. 3).

Determination of hemolytic activity

Sheep blood Sabouraud dextrose agar (SB-SDA) plates (HiMedia, Laboratories, India) were inoculated by placing 10 µl of *Candida* suspensions in saline on the plates using the spot inoculation method. The inoculated plates were incubated at 37°C with 5% carbon dioxide (CO₂) jar for 48 hours. Hemolytic activity was indicated by the presence of a distinct translucent halo around the colony (Fig. 4).

Determination of phospholipase enzyme

Phospholipase enzyme production in the yeast isolates was determined by inoculating 10 µl drop of the yeast isolates on egg yolk agar. The agar plates were left opened on the laboratory bench for 5 min for the fluid to dry. The plates were then covered and incubated at 37°C for 3 days. Formations of zones of precipitation around the colonies represented phospholipase enzyme production by the isolate as presented in Fig. 5.

Biofilm production

After resuscitating the yeast by incubating in saline for 4 hours, 10 µl of the broth were added to Sabouraud dextrose hycynth broth (HiMedia Laboratories, India) contained in polystyrene tube and then incubated at 37°C for 24 hours without agitation. This was followed by aspirating gently the top fluid from the tube with a Pasteur pipette. Then the tube without the broth was washed three times with phosphate buffered saline (PBS) at a pH of 7.2 (HiMedia Laboratories, India). The tube was then half filled with 2% safranin stain, and incubated in the dark for 15 min, after which the excess stain was washed off with PBS. Visible film adherent on the wall of the polystyrene tube was taken as indicative for biofilm formation by the yeast isolate (Fig. 6).

Antifungal susceptibility testing of the yeast isolates

Antibiotic susceptibility pattern of the yeast isolates and the reference controls (ATCC strains) were determined using the Kirby-Bauer disk diffusion method. Turbidity standard of 0.5 MacFarland of each of the yeast suspension was seeded on the surface of a sterile RPMI-1640 agar (HiMedia Laboratories, India). The following six different antifungal paper disks (Axiom Laboratories, India) were tested: amphotericin B (20 µg), clotrimazole (10 µg), fluconazole (25 µg), miconazole (50 µg), itraconazole (10 µg) and voriconazole (30 µg). Controls were set up using *C. albicans* (ATCC 10231) and *C. albicans* (ATCC 60193). All the plates were incubated aerobically at 37 °C for 24–48 hours. Diameters of zones of inhibition around the antifungal disks (Fig. 7) were measured and compared with the Clinical and Laboratory Standard Institute (CLSI, 2009) breakpoints.

Genotypic identification of the *Candida* species

Genotypic identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used for the species identification. *C. albicans* (ATCC 10231) was used as a positive control in the PCR-RFLP analysis.

DNA extraction

DNA extraction was performed using the modifications of the methods of Elfeky et al. (2016). Briefly, fresh pure colonies of the *Candida* cells were dissolved in 500 µl, sterile distilled water in a sterile 1.5 ml/L

micro-centrifuge tube. The *Candida* cells were disrupted using tissue homogenizer (Qiagen) for 3 min followed by centrifugation at 13,000 rpm for 3 min. The sediment was then subjected to DNA extraction using Q1Aamp DNA Mini kit according to the manufacturer's instructions.

PCR-RFLP analysis

The *ERG11* genes which encode the enzyme (*cytochrome P450 lanosterol 14- α -demethylase*) from 27 azole resistant isolates and controls were amplified by the PCR method using primers with the following sequences: *ERG11-F*: (5'-CAAGAAGATCATAACTCAAT-3' and *ERG11-R*: (5'-AGAACACTGAATCGAAAG-3')'

The amplification was carried out using a gradient thermal cycler (Techne TC-512, Bibby Scientific Limited, USA) as previously described by Xiang et al. (2013). Each reaction contained 10 μ l of 2X Dream Tag Green Master mix, 1.5 μ l of genomic DNA (template), 2 μ l each of primer (10 μ mol/l), in addition to change the annealing temperature (53°C). Amplified PCR products were run on 2% agarose gel electrophoresis and visualized by UV transilluminator (Biometra T 3, Analytikjena, USA). RFLP analysis was performed as described by Elfeky et al. (2016).

Data analysis

Data obtained were entered into Microsoft Excel (2007) and presented in summary tables. *Chi-square* (χ^2) test was used for statistical analysis with SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). P-value < 0.05 was assigned for statistical significance.

Results

Two hundred and eighty-eight women were enrolled for the study. Their mean age was 28 ± 5.2 years (range 17–55 years). Major risk factors identified to be associated with RVVC were pregnancy and human immunodeficiency virus (HIV) infection (Table 1). Pregnancy was found not to be a major predisposing risk factor associated with *Candida* vaginal infections. Majority of the women however had HIV infection as an underlying illness that predisposes them to *Candida* infections and the predisposing risk factor responsible for acquisition of *Candida glabrata* (N = 33; 55.9%) *Candida tropicalis* (N = 36; 78.3%) and *Candida krusei* (N = 18; 62.1%) was HIV infection (Table 2). HIV infection and pregnancy were not predisposing risk factors to infections by *Candida albicans*. Comparative analysis of risk factors associated with *C. albicans* and non-*albicans Candida* depicted significant difference ($P = 0.002$) for HIV infection (Table 2)

Table 1
Risk factors associated with *Candida* infections

Risk factor	<i>C. albicans</i> (n = 66) %	<i>C. glabrata</i> (n = 59) %	<i>C. tropicalis</i> (n = 46) %	<i>C. krusei</i> (n = 29) %
Pregnancy				
Yes	25 (37.9)	21 (35.6)	11 (23.9)	6 (20.7)
No	41 (62.1)	38 (64.4)	35 (76.1)	23 (79.3)
HIV				
Yes	27 (40.9)	33 (55.9)	36 (78.3)	18 (62.1)
No	39 (59.1)	26 (44.1)	10 (21.7)	11 (37.9)
Total number of isolates (n = 200); (%), Percentage				

Table 2
Comparative analysis of risk factors associated with *Candida albicans* and non-*albicans Candida*

Risk factor	<i>C. albicans</i> (n = 66) %	Non- <i>albicans Candida</i> (n = 134) %	<i>p-value</i>
Pregnancy			
Yes	25 (37.9)	39 (28.4)	0.197
HIV			
Yes	27 (40.9)	87 (64.9)	0.002
Total number of isolates (n = 200); (%), Percentage			

For the symptoms associated with *C. albicans* and non-*albicans Candida*, all the women who had RVVC (N = 200) had discharges. All the women had malodourous vagina discharges accompanied by vulva itching and burning sensation. Comparative analysis of the remaining three (3) symptoms; itching, burning sensation and odor associated with *C. albicans* and non-*albicans Candida* showed significant differences ($P = 0.0005$ and $P = 0.043$) for itching and burning sensation respectively (Table 3).

Table 3
Comparative analysis of symptoms associated with *Candida albicans* and non-*albicans Candida*

Symptoms	<i>C. albicans</i> (n = 66) %	Non- <i>albicans Candida</i> (n = 134) %	<i>p-value</i>
Itching			
Yes	49 (74.2)	64 (47.8)	0.0005
Burning sensation			
Yes	59 (89.4)	130 (97.0)	0.043
Odour			
Yes	61 (92.4)	129 (96.3)	0.303
Total number of isolates (n = 200); (%), Percentage			

There were 200 (69.4%) vagina swabs that produced culture positive of *Candida* spp out of the 288 tested. The most common *Candida* species isolated was *C. albicans* with 33% (66/200) followed by non-*albicans Candida* species comprising of *Candida glabrata* 29.5% (59/200), *Candida tropicalis* 23% (46/200) and *Candida krusei* 15.5% (Table 4). None of the vagina swab samples from the women with RVVC at the Nkawie Government Hospital, Ashanti Region, Ghana yielded *Candida dubliniensis* and *Candida parapsilosis*. Antifungal susceptibility test results indicated that all the isolates (n = 200) were susceptible to itraconazole. The highest resistance proportion was against voriconazole where *C. tropicalis* recorded 79% followed by *C. albicans* (71%), *C. glabrata* (71%) and *C. krusei* (10%). High resistance proportions were recorded against fluconazole with *C. krusei* registering 93% and *C. glabrata* (78%), *C. tropicalis* (20%) and *C. albicans* (18%). Details of the resistance proportions are presented in Table 5.

Table 4
Distribution of *Candida* species in women with recurrent vulvovaginal candidiasis

Isolate	Number of Isolates N (%)	Recurrent Infections N (%2)	Non-Recurrent Infections N (%3)	P-value
<i>Candida albicans</i>	66 (33.0)	17 (25.8)	49 (74.2%)	< 0.01
<i>Candida glabrata</i>	59 (29.5)	41 (69.5)	18 (30.5%)	< 0.01
<i>Candida tropicalis</i>	46 (23.0)	12 (26.1)	34 (73.9%)	< 0.01
<i>Candida krusei</i>	29 (15.5)	15 (51.7)	14 (48.3%)	0.67
Total	200 (100)	85 (42.5)	115 (57.5%)	
N: Number of Isolates; (% ₁): Percentage of Isolate from the total number of <i>Candida</i> species;				
(% ₂): Percentage of Isolate found in recurrent infection; (% ₃): Percentage of Isolate found in non-recurrent infection.				

Table 5
The resistance patterns of the *Candida* species to the antifungal agents tested in the study.

Yeast species	Amp B (10 µg)	Cot (10 µg)	Flu (25 µg)	Mic (50 µg)	Itr (10 µg)	Vor (30 µg)
<i>Candida albicans</i>	42 (64%)	21 (32%)	12 (18%)	25 (38%)	0 (0)	47 (71%)
<i>Candida glabrata</i>	49 (83%)	38 (63%)	46 (78%)	32 (54%)	0 (0)	49 (83%)
<i>Candida krusei</i>	20 (69%)	14 (48%)	27 (93%)	6 (21%)	0 (0)	3 (10%)
<i>Candida tropicalis</i>	37 (80%)	21 (46%)	9 (20%)	13 (28%)	0 (0)	18 (79%)
Amp B-amphotericin B; Cot-cotrimoxazole; Flu-fluconazole; Mic-miconazole; Itr-itraconazole;						
Vor-voriconazole						

ERG11 genes were amplified and detected in only two *C. albicans* and one *C. tropicalis* isolates investigated (Fig. 8). Hemolytic activity was the virulence factor found to be mostly expressed by all the *Candida* species recovered from the women with RVVC. About 90.9% of *C. albicans* were found to produce phospholipase whilst 95.5% formed biofilms and 97% produced hemolysin. With *C. glabrata*, 89.8% of the isolates produced phospholipase, 78% formed biofilms and 93.2% produced hemolysin. There was high production of hemolysins among the isolates of *C. krusei* (93.5%) and *C. tropicalis*

(93.1%). Almost all *Candida* isolates which produced phospholipase were found to form biofilms (Table 6).

Table 6
Virulence factors associated with the *Candida* isolates.

Yeast species	Phospholipase	Biofilm	Hemolysin
<i>Candida albicans</i>	60 (90.9%)	63 (95.5%)	64 (97.0%)
<i>Candida glabrata</i>	53 (89.8%)	46 (78.0%)	55 (93.2%)
<i>Candida krusei</i>	34 (73.9%)	31 (67.4%)	43 (93.5%)
<i>Candida tropicalis</i>	19 (65.5%)	15 (51.7%)	7 (93.1%)

TAXANOMY: Not applicable

Discussion

Inadequate laboratory support in the sub-Saharan African has hampered the laboratory diagnosis involving culture, isolation and identification of various *Candida* species. This present study attempted to identify the most prevalent *Candida* species causing RVVC. Previous study conducted in Ghana reported *C. albicans* followed by *C. glabrata* as the most common isolated species (Adjapong et al., 2014). Good Laboratory Practices could improve the data on the aetiology of *Candida* infection among women in Africa.

VVC can be managed with either topical or oral antifungal agents. It has been reported that a single oral dose of fluconazole can lead to total cure of VVC (Pappas et al., 2004). In Ghana over-the-counter acquisition of drugs and other pharmaceuticals renders many drugs potential for abuse. Most abused drugs are the topical clotrimazole creams and vaginal tablets. They are easy to administer and are safe and effective, so are usually the first options for most women when they get VVC. In majority of the cases fluconazole is the antifungal agent used after failed attempts with clotrimazole. Nevertheless fluconazole is the drug used for first-line treatment of VVC in most parts of the world (Fan et al., 2008; Matheson and Mazza, 2017).

Due to the overuse without proper laboratory diagnosis and medical prescription of both orthodox, herbal medicines and other natural products in the treatment of different forms of vaginal infections in Ghana, there has been increasing number of *Candida* species causing infections. Just as the *Candida* species are varied in the aetiology of their infections, so also drug is resistance among *Candida* species vary to commonly prescribed antifungals in Africa (Africa and dos Santos Abrantes, 2016). Majority of the studies have attributed the resistance of the yeast, to yeast varieties, immunosuppression and age of the host (Africa and dos Santos Abrantes, 2016) in addition to intrinsic resistance among *Candida* strains. None of *Candida* isolates in our present study was resistant to itraconazole, a situation which may be attributable to less availability of itraconazole on the Ghanaian market and also hardly prescribed.

Resistance to itraconazole has been previously reported in the United Kingdom (UK) among *C. dubliniensis* and *C. albicans* isolates (Venkateswarlu et al., 1996; Whaley et al., 2017; Tsitsopoulou et al., 2018). Fluconazole resistance was demonstrated in 18% of *C. albicans*, and 93% among *C. krusei* isolates from this present study. This high proportion of *C. krusei* isolates resistance to fluconazole is similar to values obtained elsewhere (Massa et al., 2018) and the results were attributed to over dependence on fluconazole for treatment of *Candida* vaginitis infections (Revie et al., 2018). In this study, it was discovered that 64–83% of the *Candida* species isolated from the women with RVVC in the Nkawie Government Hospital, Ashanti Region, Ghana were resistant to amphotericin B. This pattern of *Candida* resistance to amphotericin B is unexpected because the drug is usually reserved for treating serious systemic fungal infections. Amphotericin B resistance has also been observed in 10–15% of *C. krusei* fungaemia (Dudiuk et al., 2019). Reports of emergence of resistance in some of the strains of *Candida* could be worrisome especially in resource limited settings like Africa. The highest and widest range of resistance (10–83%) of the *Candida* isolates to the antifungal agents investigated in the study was observed for voriconazole. Overexpression of multidrug resistance genes is responsible for the reduced susceptible proportions of *Candida* strains to voriconazole. These have been identified in fluconazole and voriconazole-resistant strains of *C. parapsilosis* too (Balkan et al., 2019; Hu et al., 2019).

There are different resistance mechanisms but resistance to azole antifungal drugs is mostly due to modifications in the target enzyme as a result of mutations (Balkan et al., 2019; Brilhante et al., 2019). Low access of azole drugs to the target site is also due to the over-expression of the *ERG11* gene. Resistance to azole antifungal agents is of much concern as these drugs are mostly dependent upon for the treatment of candidiasis. This is because of their safety and effectiveness against most *Candida* strains isolated from vulvovaginal infections (Felix et al., 2019). The detection of *ERG11* genes as observed in the present study is similar to previous findings in other parts of the world (Hou et al., 2019; Sardari et al., 2019). Apart from the over-expression and mutations in the *ERG11* gene, another major mechanism mostly exhibited by *C. glabrata* which confers resistance to azole antifungals is the up-regulation of multidrug efflux transporter genes such as *CDR* (Navarro-Rodríguez et al., 2019). Most *C. glabrata* strains therefore express *CDR1* and *CDR2* genes over *ERG11* genes and this may have accounted for the non-detection of *ERG11* in azole-resistant *C. glabrata* in this study (Whaley et al., 2017).

Virulent factors facilitate the ability of *Candida* species to cause infections (El-Houssaini et al., 2019). These aid the yeast to protect itself against antibodies and phagocytic activity of the host immune system (Oliver et al., 2019). About 95.5% of *C. albicans* produced the most biofilms followed by 78% *C. glabrata* produced biofilms, and then *C. krusei* (67.4%) and *C. tropicalis* (51.7%) as presented in Table 3. Biofilm formation increases the ability of *Candida* species to withstand host defenses and helps in establishing a reservoir for continuing and recurrent infections (Ishchuk et al., 2019). Infections from biofilm forming *Candida* species are therefore associated with higher morbidity, recurrence and then mortality rates increase in systemic infections.

Hemolysins are putative virulence factors contributing to the pathogenicity of *Candida* species by facilitating hyphal invasion (El-Houssaini et al., 2019). Hemolytic activity is therefore a factor used to

initiate an infection. It is used to break down cells and enables the yeast to proliferate to cause the irritation and oedema of the vulva which is often associated with *Candida* infections. The high hemolytic activities were observed for all the *Candida* species investigated (Table 6). *Candida* species produce hydrolases which play important roles in adherence, penetration, invasion and destruction of host tissues (El-Houssaini et al., 2019). *Candida* species are capable of producing exo-enzymes but the potency varies among species and depends on the site of infection (Naglik et al., 2019). Phospholipase is a hydrolytic enzyme which can act by damaging the host cell membrane and can facilitates the invasion by the yeast. This is made possible as the enzyme hydrolyzes phospholipids into fatty acids therefore exposing receptors for adhesion (Maheronnaghsh et al., 2019; Melo *et al.*, 2019). Majority of *C. albicans* (90.9%) produced phospholipases, and so did the other non-*albicans* *Candida* species isolated (Table 6). All the biofilm forming *Candida* isolates found in this study also had phospholipase activity. Studies have shown that enzyme activity, hydrophobicity and the ability for biofilm formation are important factors that are responsible for the pathogenicity of various *Candida* species (Sobel, 2016; Oliver et al., 2019). These virulent factors together with resistance mechanisms may act synergistically to be responsible for the recurrent infections in women from Nkawie Government Hospital, Ashanti Region, Ghana. This study had established that antifungal resistance was widespread among the isolates, though no isolate was resistant to itraconazole. Resistance proportion to voriconazole was high and was associated with *C. glabrata*. The most common *Candida* specie noted for women with recurrent vaginal discharge was *C. glabrata*. It is recommended that culture and antifungal sensitivity testing should be performed routinely on *Candida* isolates as it is done on many bacteria isolates from clinical specimens. This may help in the selection of appropriate antifungal agent(s) to stem recurrence among the women. A larger study to determine degree of spread of *ERG11* genes in Ghana is suggested.

Conclusions

From the present study, the most prominent isolated species in RVVC infections in Ghana were *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei*. Non-*albicans* *Candida* species were noted to have almost all the virulent factors as *C. albicans* for the establishment of an infection. However, non-*albicans* *Candida* species may not be as virulent as *C. albicans*. *C. glabrata* species do not have all virulent factors as compared to *C. albicans*. This is because the *C. glabrata* species were unable to form hyphae and pseudohyphae. Also all the *C. glabrata* species had relatively small yeast cell sizes compared to *C. albicans*. The antifungal susceptibility testing showed that there was a significant increase in the rate of resistance among the *Candida* isolates to fluconazole and voriconazole. It is therefore suggested that there is need for continuous surveillance as well as antifungal susceptibility testing on *Candida* spp and other microbial infections to guide therapy in Ghana. A larger epidemiological study is also advocated to determining the degree of spread of *ERG11* genes.

List Of Abbreviations

VVC-vulvovaginal candidiasis; RVVC-recurrent vulvovaginal candidiasis; *CDR*-multidrug efflux transporter genes; *ERG11*-genes which encode the enzyme (*cytochrome P450 lanosterol 14- α -demethylase*).

Declarations

Ethics approval and consent to participate:

The study was approved by the Joint Committee on Human Ethics, Research and Publication, School of Medicine and Dentistry, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana as well as from the Management of the Nkawie Government Hospital, Ghana. The objectives of the study were also explained to the study participants and those that were willing to take part were asked to sign or thumbprint a consent form before the samples were collected from them.

Adherence to national and international regulations:

Not applicable

Consent for publication:

Not applicable

Availability of data and materials:

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests:

None

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None

Author's contributions:

Conceptualization, Methodology & Formal Analysis, P.K.F, G.A.P. and R.K.B.; Writing – Original Draft Preparation, G.A.P., P.K.F. and S.A.F Writing – Review & Editing, G.A.P., P.K.F. and S.A.F. All authors read

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References

1. Abuquah HH (2012). Prevalence and antifungal susceptibility of *Candida* species isolated from women attending a gynaecological clinic in Kumasi, Ghana. *Journal of Science and Technology* 32(2): 39-45.
2. Adjapong G, Hale M, Garrill A (2014). An investigation of the distribution of *Candida* species in genitourinary candidiasis and pelvic inflammatory disease from three locations in Ghana. *African Journal of Microbiology Research* 8(6): 470-475.
3. Africa CWJ, dos Santos Abrantes PM (2016). *Candida* antifungal drug resistance in sub-Saharan African populations: A systematic review. *F1000 Research* 5: 2832.
4. Apea-Kubi KA, Sakyi B, Yamaguchi S, Ofori-Adjei D (2005). Bacterial vaginosis, *Candida albicans* and *Trichomonas vaginalis* infection in antenatal and gynaecological patients in Ghana. *Tropical Journal of Obstetrics and Gynaecology* 22(2): 108-112.
5. Balkan C, Ercan I, Isik E, Akdeniz ES, Balcioglu O, Kodedová M, et al (2019). Genome-wide elucidation of drug resistance mechanisms for systemically used antifungal drugs amphotericin B, caspofungin and voriconazole in the budding yeast. *Antimicrobial Agents and Chemotherapy* doi: 10.1128/AAC.02268-02218.
6. Brilhante RSN, de Alencar LP, Bandeira SP, Sales JA, de Jesus Evangelista AJ, Serpa R, et al (2019). Exposure of *Candida parapsilosis* complex to agricultural azoles: An overview of the role of environmental determinants for the development of resistance. *Science of The Total Environment* 650 (1): 1231-1238.
7. Cassone A (2015). Vulvovaginal *Candida albicans* infections: pathogenesis, immunity and vaccine prospects. *BJOG* 122 (6): 785-794.
8. Clinical and Laboratory Standards Institute (CLSI, 2009). Method for Antifungal Disk Diffusion Susceptibility Testing of Yeast. Approved Guideline 2nd ed., CLSI document M44-A2, Wayne, PA, CLSI.
9. Chen C, Huang X (2018). *Candida albicans* Commensalism and Human Diseases. In: Sun J., Dudeja P. (eds) *Mechanisms Underlying Host-Microbiome Interactions in Pathophysiology of Human*

Diseases. Physiology in Health and Disease. Springer, Boston, MA.

10. Choukri F, Benderdouche M, Sednaoui P (2014). *In vitro* susceptibility profile of 200 recent clinical isolates of *Candida* spp to topical antifungal treatments of vulvovaginal candidiasis, the imidazoles and nystatin agents. *Journal de Mycologie Medicale* 24(4): 303-307.
11. Denning DW, Kneale M, Sobel JD, Rautemaa-Richardson R (2018). Global burden of recurrent vulvovaginal candidiasis. A systematic review. *Lancet Infectious Diseases* 2018/18: e339-347.
12. Desai JV (2018). *Candida albicans* hyphae: From growth initiation to invasion. *Journal of Fungi* 4(1): 10.3390/jof4010010.
13. Donders GG, Sobel JD (2017). *Candida* vulvovaginitis: A store with a butterfly and a show window. *Mycoses* 60(2): 70-72.
14. Dudiuk C, Berrio I, Leonardelli F, Morales-Lopez S, Theill L, Macedo D, et al (2019) Antifungal activity and killing kinetics of anidulafungin, caspofungin and amphotericin B against *Candida auris*. *Journal of Antimicrobial Chemotherapy* 74(8): 2295-2302.
15. Elfeky DS, Gohar NN, El-Seidi EA, Ezzat MM, AboElew SH (2016) Species identification and antifungal susceptibility pattern of *Candida* isolates in cases of vulvovaginal candidiasis. *Alexandria Journal of Medicine* 52(3): 269-277.
16. El-Houssaini HH, Elnabawy OM, Nasser HA Elkhatib WF (2019) Correlation between antifungal resistance and virulence factors in *Candida albicans* recovered from vaginal specimens. *Microbial Pathogenesis* 128: 13-19.
17. Essel E, Amenga-Etego L, Quaye S (2014) A case study of the incidence and risk factors of vaginal candidiasis in a Girls Senoir High School in Bolgatanga, Ghana. *International Journal of Health Sciences and Research* 4(7): 212-217.
18. Fan SR, Liu X P, Li JW (2008) Clinical characteristics of vulvovaginal candidiasis and antifungal susceptibilities of *Candida* species isolates among patients in southern China from 2003 to 2006. *Journal of Obstetrics and Gynaecology Research* 34(4): 561-566.
19. Feglo P, Narkwa P (2012) Prevalence and antifungal susceptibility patterns of yeast isolates at the Komfo Anokye Teaching Hospital (KATH), Kumasi, Ghana. *British Microbiology Research Journal* 2(1): 10-22.
20. Felix TC, de Brito Röder DVD, dos Santos Pedroso R (2019) Alternative and complementary therapies for vulvovaginal candidiasis. *Folia Microbiologica* 64(2): 133-141.
21. Hamad M, Kazandji N, Awadallah S, Allam H (2014) Prevalence and epidemiological characteristics of vaginal candidiasis in UAE. *Mycoses* 57(3): 184-190.
22. Hou X, Lee A, Jiménez-Ortigosa C, Kordalewska M, Perlin DS, Zhao Y (2019) Rapid detection of *ERG11*-associated azole resistance and FKS-associated echinocandin resistance in *Candida auris*. *Antimicrobial Agents and Chemotherapy* 63(1): e01811-01818.
23. Hu M-J, Cosseboom S, Schnabel G (2019) *atrB*-associated fludioxonil resistance in *Botrytis fragariae* notlinked to mutations in transcription factor *mrr1*. *Phytopathology* 109(5): 839-846.

24. Ishchuk OP, Sterner O, Strevens H, Ellervik U, Manner S (2019) The use of polyhydroxylated carboxylic acids and lactones to diminish biofilm formation of the pathogenic yeast *Candida albicans*. RSC Advances 9(19): 10983-10989.
25. Konadu DG, Owusu-Ofori A, Yidana Z (2019) Prevalence of vulvovaginal candidiasis, bacterial vaginosis and *Trichomonas* in pregnant women attending antenatal clinic in the middle belt of Ghana. BMC Pregnancy Childbirth 19, 341 (2019): doi: 10.1186/s12884-019-2488-z.
26. Liken HB, Kaufman DA (2018) *Candida*. In *Neonatal Infections*, Springer.
27. Maheronnaghsh M, Fatahinia M, Dehghan P, Mahmoudabadi AZ, Kheirkhah M (2019) Comparison of virulence factors of different *Candida* species isolated from the oral cavity of cancer patients and normal individuals. Jundishapur Journal of Microbiology 12(5): 1-8.
28. Massa N, Cantamessa S, Novello G, Ranzato E, Martinotti S, Pavan M (2018) Antifungal activity of essential oils against azole-resistant and azole-susceptible vaginal *Candida glabrata* strains. Canadian Journal of Microbiology 64(10): 647-663.
29. Matare T, Nziramasanga P, Gwanzura L, Roberston V (2017) Experimental germ tube induction in *Candida albicans*: An evaluation of the effect of sodium bicarbonate on morphogenesis and comparison with pooled human serum. Biomed Research International 2017: doi: 10.1155/2017/1976273.
30. Matheson A, Mazza D (2017) Recurrent vulvovaginal candidiasis: A review of guideline recommendations. Australian and New Zealand Journal of Obstetrics and Gynecology 52 (2): 139-145.
31. Morschhäuser J (2016) The development of fluconazole resistance in *Candida albicans*—An example of microevolution of a fungal pathogen. Journal of Microbiology 54(3): 192-201.
32. Naglik JR, Gaffen SL, Hube B (2019) Candidalysin: Discovery and function in *Candida albicans* infections. Current Opinion in Microbiology 52: 100-109.
33. Navarro-Rodríguez P, Martín-Vicente A, López-Fernández L, Guarro J, Capilla J (2019) Expression of *ERG11* and efflux pump genes *CDR1*, *CDR2* and *SNQ2* in voriconazole susceptible and resistant *Candida glabrata* strains. Medical Mycology myz014: doi:10.1093/mmy/myz014.
34. Oliver JC, Ferreira CBRJ, Silva NC, Dias ALT (2019) *Candida* spp and phagocytosis: Multiples evasion mechanisms. Antonie van Leeuwenhoek, doi: 10.1007/s10482-019-01271-x
35. Pappas P G, Rex JH, Sobel JD, Filler SG, Dismukes WE, Walsh TJ, et al (2004) Guidelines for treatment of candidiasis. Clinical Infectious Diseases 38(2): 161-189.
36. Rajilić-Stojanović M, de Vos WM (2014) The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiology Reviews 38(5): 996-1047.
37. Revie NM, Iyer KR, Robbins N, Cowen LE (2018) Antifungal drug resistance: evolution, mechanisms and impact. Current Opinion in Microbiology 45: 70-76.
38. Sardari A, Zarrinfar H, Mohammadi R (2019) Detection of *ERG11* point mutations in Iranian fluconazole-resistant *Candida albicans* isolates. Current Medical Mycology 5(1): 7.

39. Sobel JD (2002) Pathogenesis of recurrent vulvovaginal candidiasis. *Current Infectious Diseases Reports* 4(6): 514-519.
40. Sobel JD (2016) Recurrent vulvovaginal candidiasis. *American Journal of Obstetrics and Gynecology* 214(1): 15-21.
41. Tsitsopoulou A, Posso R, Vale L, Bebb S, Johnson E, White PL (2018) Determination of the prevalence of triazole resistance in environmental *Aspergillus fumigatus* strains isolated in South Wales, UK. *Frontiers in Microbiology* 9: 1395, doi: 10.3389/fmicb.2018.01395.
42. Venkateswarlu K, Denning DW, Manning NJ, Kelly SL (1996) Reduced accumulation of drug in *Candida krusei* accounts for itraconazole resistance. *Antimicrobial Agents and Chemotherapy* 40 (11): 2443-2446.
43. Viegas S, Assuncao R, Martin C, Nunes C, Osteresch B, Twarużek M (2019) Occupational exposure to mycotoxins in swine production: Environmental and Biological Approaches. *Toxins* 11(2): 78, doi: 10.3390/toxins11020078.
44. Vierira de Melo AP, Zuza-Alves DL, da Silva-Rocha WP, Canário de Souza LBF, Francisco EC, Salles de Azevedo Melo A, et al (2019) Virulence factors of *Candida* spp obtained from blood cultures of patients with candidemia attended at tertiary hospitals in Northeast Brazil. *Journal de Mycologie Médicale* 29(2): 132-139.
45. Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD (2017) Azole antifungal resistance in *Candida albicans* and emerging non-*albicans* *Candida* species. *Frontiers in Microbiology* 7: 2173, doi: 10.3389/fmicb.2016.02173.
46. Wiederhold NP (2017) Antifungal resistance: Current trends and future strategies to combat. *Infection and Drug Resistance* 10: 249-259.
47. Xiang M-J, Liu J-Y, Ni P-H, Wang S, Shi C, Wei B, et al (2013) *Erg11* mutations associated with azole resistance in clinical isolates of *Candida albicans*. *FEMS Yeast Research* 13 (4): 386-393.

Figures

Figure 1

Budding yeast cells and pseudohyphae in HVS wet mount preparation

Figure 2

Budding yeast cells with pseudohyphae in HVS Gram stain

Figure 3

Colour scheme for the differentiation of Candida species

Figure 4

Hemolytic activity by *Candida* sp. on sheep blood Sabouraud dextrose agar (SDA)

Figure 5

Phospholipase enzyme production by *Candida* sp. on egg yolk agar plate

Figure 6

Biofilm formation by *Candida* sp. on polystyrene tube wells

Figure 7

Antifungal Drug Susceptibility test on RPMI 1640 Agar

Figure 8

Agarose gel electrophoresis of PCR products of ERG11 genes amplified from the genomic DNA of *Candida* species.