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Antifungal Susceptibility Profile of *Candida albicans* Causing Vulvovaginal Candidiasis Among Pregnant Women

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Abstract

Candida infection is a common disorder that can cause vulvovaginal candidiasis (VVC) among adult women. *Candida albicans* is the main etiologic agent of VVC. This study was to determine the antifungal susceptibility of *C. albicans* isolated from pregnant women in Hospital Tengku Ampuan Afzan (HTAA), Kuantan, Malaysia. A hundred and twenty-five isolates of yeast were cultured on Sabouraud dextrose agar (SDA) from a high vaginal swab (HVS). These yeast isolates were identified by the odor and morphology of the yeast colony, and were further identified by germ tube test and subsequently sub-cultured on CHROMagar *Candida* and cornmeal agar. The antifungal susceptibility test of *C. albicans* was carried out on Fluconazole and 5-Fluorocytosine using the Epsilometer test (E-test) method. A total of 30 *C. albicans* isolates were susceptible to 90% of Fluconazole and 100% of 5-Fluorocytosine. As for minimum inhibitory concentration (MIC) determined by the E-test method, Fluconazole showed that 13.3% of *C. albicans* was sensitive in the range of 0.023-0.19 µg/ml and 76.7% in a range of 0.25-2.0 µg/ml, while 56.7% was sensitive to 5-Fluorocytosine in range of 0.003-0.094 µg/ml and 43.3% in a range of 0.125-4.0 µg/ml. The resistance rate of Fluconazole was only 10%, which is in the range of 8-32 µg/ml. In this study, most of *C. albicans* was sensitive towards both antifungals which shows the effectiveness among VVC infection. In conclusion, the risk of multidrug resistance among C. albicans in the high vaginal swab (HVS) sample remains low, and the antibiotics used to treat VVC remain effective.

Keyword: Antifungal susceptibility, *Candida albicans*, vulvovaginal candidiasis

INTRODUCTION

Candida infection is a common disorder that mostly can cause vulvovaginal candidiasis among adult women. It is a normal commensal organism in the vagina and colonized on human skin. This opportunistic fungal infection usually infected the female lower genital tract, the vulva and vagina. Vulvovaginal candidiasis (VVC) is an opportunistic yeast infection usually infected female lower genital tract, the vulva and vagina. Usually, patients infected with VVC coming with a complaint of abnormal vaginal discharge. About 75% of woman may experience at least one episode of VVC during lifetime. In pregnancy, VVC may lead to systemic infection in neonates such as underweight birth and premature baby. The infection occurs among pregnant women mostly because of hormonal changes and gestational diabetes causing yeast in the vagina to be out of balance and overgrowth. *Candida albicans* is the main etiologic agent of VVC. It is a normal commensal organism in the vagina and colonized on human skin. Because of certain factors, overgrowth of *C. albicans* may occur and lead to VVC infection. The recent treatment of antifungal in Malaysia is Miconazole, Ketoconazole, Clotrimazole, Nystatin, Econazole, Fluconazole, Terbinafine, Selenium sulphide and Amorolfine (Masri et al., 2015). However, there is an increasing number of cases of *Candida* infection that are resistant to antifungal commonly because of long treatments with those drugs which lead to the development of multidrug-resistant towards *C. albicans*. Eventually, alternative antifungal should be identified which susceptible to *C. albicans*.

C. albicans is a Gram-positive oval organism with buds and dark purple. It has a unique characteristic that is capable to grow in various morphological forms. The four main morphology is yeast-like, hyphae, pseudohyphae and chlamydospore form (Bottcher et al, 2016). The pathogenicity of *C. albicans* depends on two main factors, that is, virulence factors of this pathogen and human's immune status. Adhesion to the host cell, secretion of hydrolytic enzymes, dimorphic phenotype (yeast to filamentous form or hyphae), thigmotropism, phenotypic switching, modulating the host's immune system, and formation of biofilm on biotic (host cell) and abiotic (example: catheter) surfaces are all factors that influence *C. albicans* pathogenicity. The virulence factor also includes fitness characteristics linked to quick adaptation to changes in ambient pH, metabolic flexibility, and stress response (Mayer et al., 2013).

Vulvovaginal candidiasis is quite common among women, and some of them experience repeated occurrences of this infection, which is known as Recurrent Vulvovaginal Candidiasis (RVVC) (Mayer et al., 2013). This infection is a serious illness that can cause morbidity in pregnancy, including abortion, candida chorioamnionitis, premature birth, emotional stress, and immune system suppression in pregnant women (Kabir&Ahmad, 2013). VVC is caused by three main factors: high levels of progesterone and estrogen, uncontrolled hyperglycemia, and contraceptive use (Yadav and Prakash, 2016).

In comparison to bloodstream infections, there are few investigations on vaginal Candida infection in Malaysia. Fluconazole is a typical VVC therapy that is effective against the most common *Candida* species (Masri et al., 2015). The antifungals are divided into numerous categories, including Azoles, Polyenes, Pyrimidine Analogs, Allylamine, and Echinocandins. The Clinical and Laboratory Standard Institute (CLSI), which provides lower minimum inhibitory concentration (MIC) breakpoints for antifungal medications, is the most recent standard in antifungal susceptibility interpretation (Dovnik et al, 2015).

Physician frequently prescribes Clotrimazole for vulvovaginal candidiasis treatment since it is effective and less harmful. According to earlier research, all *Candida* species are responsive to the polyene antifungals Nystatin and Amphotericin B. *C. albicans* is highly sensitive to the azole group, with 78% and 79% of *C. albicans* being sensitive to Fluconazole and Clotrimazole, respectively (Dovnik et al., 2015), while another study found that Fluconazole and Clotrimazole are susceptible to 100% and 83% of *C. albicans*, respectively.

The sensitivity of *C. albicans* to Caspofungin (98.2%), Voriconazole (94%), Amphotericin B (93%), Ketoconazole (90.6%), and Fluconazole (89.5%) was also discovered (Kalairasan et al, 2017). Nevertheless, according to Al-mamari et al. (2014), *C. albicans* was resistant to Fluconazole and Clotrimazole in 76% and 55% of cases, respectively. The use of broad-spectrum antibiotics, diabetics, AIDS patients, and the growth of drug-resistant fungus are all common causes of antifungal medication resistance (Zarei-Mahmoudabadi et al, 2016).

The objective of the study was to determine the antifungal susceptibility of *C. albicans* that cause VVC isolated from pregnant women. Due to the rise in fungal infections and antifungal treatment resistance, researchers are looking into antifungal susceptibility profiles in emerging countries. The outcome of the HVS culture shows either *C. albicans* or *Candida* spp. by germ tube test if the patients have vaginal candidiasis. Because distinct isolates of *C. albicans* from various samples may have distinct antifungal patterns, specific identification and antifungal profiles are necessary to identify *C. albicans* isolated from HVS. Furthermore, we can determine which drugs are resistant to avoid treatment failure and present patients with alternative medications.

METHODOLOGY

This study was carried out at Microbiology Unit, Department of Pathology, Hospital Tengku Ampuan Afzan (HTAA). A total of 125 isolates of *C. albicans* are required in this study to determine the antifungal profile among *C. albicans.* The sample was cultured aseptically on blood agar, Mac Conkey agar, sabouraud dextrose agar (SDA) and incubated for 24 hours at 36-37°C. After incubation, yeast colonies were determined by the odor which like bread yeast smell and the morphology of yeast which was whitish, creamy and dense on SDA.

The preliminary test used in identification was the germ tube test. Germ tube was discovered to contribute as a virulent factor in the pathogenesis of *C. albicans.* The purpose of the germ tube is to check the production of the germ tube of *Candida*. One of the single yeast colonies was taken and mixed into sterile tryptone water in the sterile test tube. After that, 1 ml of human serum was added into the mixture and incubated for three hours at 37°C. Next, by using a sterile pipette, the mixture in the test tube was pipetted and put into the Kova slide. Kova slide was placed at the microscope under 40x to check the formation of the germ tube.

A single colony of each 125 of yeast from the samples that yield the germ tube was taken to be sub-cultured on CHROMagar *Candida*, followed by incubation at 37°C for 24 to 48 hours. Then, the isolates were inoculated on cornmeal agar with Tween 80. The yeast on the media was streaked and stabbed after 48 hours of incubation, then covered with a sterile coverslip and incubated at room temperature (25°C) for another 48 hours. After incubation, the colony on cornmeal agar was observed by using a microscope (40x lens).

Antifungal susceptibility test (AST) was carried out after the yeast was confirmed to be *C. albicans*. This test was employed to determine the antifungal profile among *C. albicans* by

testing Fluconazole and 5-Flurocytosine using the Epsilometer test (E-Test). Rosewell Park Memorial Institute (RPMI)-glucose agar medium was used to perform this test.

This research was registered under Ethical Committee approval from Faculty Engineering and Life Sciences (ECFBHS) UNISEL (Ref. No.: J160108E) and Medical Research and Ethics Committee (MREC) from Ministry of Health Malaysia (Research ID: 29587).

RESULTS AND DISCUSSION

A total of 125 *C. albicans* were isolated and identified in this study. Unfortunately, only 30 from 125 yeast samples proceeded with antifungal susceptibility test and only two types of the antifungal drug were applied because of budget limitation. This cross-sectional study involved HVS samples were taken from pregnant women from the gynae ward in Hospital Tengku Ampuan Afzan (HTAA), various health clinics and other district hospitals. The majority of HVS samples taken were Malay women, followed by Chinese, Indian and Orang Asli with 74.4%, 12%, 8.8% and 4.8% respectively (Table 1).

Table 1 The frequency and percentage of races infected with VVC

Race	Frequency (n)	Percentage (%)
Malay	93	74.4
Chinese	15	12.0
Indian	11	8.8
Orang Asli	6	4.8

There was no evidence that races are linked to vulvovaginal candidiasis risk factors. The high number of Malay patients could be related to Malays' predilection for having their antenatal check-up at HTAA. Meanwhile, the lowest result for Orang Asli may be related to social factors such as lack of knowledge, remoteness, and financial concerns.

C. albicans and *Candida dubliniensis* were observed to produce germ tubes after three hours of incubation. From the yeast cell, a short hyphal extension was generated with an absence of constriction at the point of origin. The extension of the germ tube is three to four times in length of the yeast cell without any nucleus. There are several types of *Candida sp.* detected in other studies and identified by using CHROMagar *Candida* that yield different colony colors. A total of 125 yeast that positive in germ tube test was isolated on CHROMagar *Candida* shows light green colony colors indicating to *C. albicans.* A newly discovered *Candida* species, *C. dubliniensis* that also generated in the germ tube, can be distinguished by this selective agar (Figure 1). On the contrary, all isolates of *C. albicans* cultured on cornmeal agar showed the presence of chlamydospores which can be described as large, spherical, thick-walled, and refractile cells that are usually 8±1 μm in diameter in a single form.

Figure 1 Light green colony of *C. albicans* on CHROMagar *Candida*

In the germ tube test, it was suggested to take the single colony of yeast to avoid other bacteria mix up together. The employment of human serum in the germ tube test might be hazardous as the possibility of the serum be infected with HIV or hepatitis virus is high. Extra precautions were taken, such as using fresh or frozen serum and allowing enough time for incubation to avoid the formation of pseudohyphae by *Candia tropicalis*, which could be misinterpreted for germ tubes. However, the germ tube test is still the gold standard in a procedure that is used in most *C. albicans* studies (Deorukhkar et al., 2012, Badiee & Alborzi, 2011, Aslam et al., 2008). Consistent with our results, all 125 isolates of yeast were positive in germ tube test, in which indicated the presence of *C. albicans* and this test was considered as presumptive identification test.

A total of 125 isolates of yeast that produce germ tube was successfully identified as *C. albicans* by using CHROMagar *Candida* and cornmeal agar. This finding demonstrates that *C. albicans* that produce germ tubes grow their colonies on CHROMagar Candida in a light green colour. The chlamydospore was observed in good shape, cornmeal agar was incubated at 25°C which the most appropriate temperature for chlamydospore formation. Incubation at 30°C to 37°C can inhibit the generation of chlamydospore. Under the microscope, singles of chlamydospore can be spotted during the observation. Unlike yeast cells, chlamydospores cannot resist heat, starvation, or dryness. Cornmeal agar encourages the growth of chlamydospores by providing a rich source of complex carbon and feeding.

Fluconazole and 5-Fluorocytosine were found to be susceptible to *C. albicans* (90% and 100% respectively). Only 10% of Fluconazole was resistant against the *C. albicans*. The quality control range was based on the Clinical and Laboratory Standard Institute (CLSI) M27-S4 as reference. The isolation of *C. albicans* on CHROMagar *Candida* and cornmeal agar should take from a fresh colony from SDA, not from repeated sub-culturing. This is because some *Candida* strains may be lost the ability to produce chlamydospore and may not produce a clear-cut color of *C. albicans*. Mostly, at low concentrations of Fluconazole and 5-Fluorocytosine E-test, 0.0023-0.19 µg/ml and 0.003-0.094 µg/ml respectively, able to inhibit the growth of *C. albicans*. Fluconazole is one of the first-line drugs in the treatment of fungal. The results are consistent with a study by El Feky et al., (2015) that shows a high prevalence of susceptibility of *C. albicans* to this Fluconazole which is 89.5%. A similar finding was also reported by Gandhi et al., 2015 which is 78%. However, there is not much latest study on 5-Fluorocytosine as treatment in VVC. 5-Fluorocytosine is under second-line drug treatment. A study of VVC reveals that the azole group is less effective against non-albicans *Candida* and uses 5-Fuorocytosine as another alternative in treatment (Whaley et

al., 2017). 5-Fluorocytosine is rarely used as a single treatment, it is usually used in combination with other antifungals, such as Amphotericin B and Fluconazole for better effect (Dovnik et al., 2015).

The antifungal susceptibility test was determined by E-test showed 56.7% was susceptible for 5-Fluorocytosine in range concentration is 0.003 – 0.094 µg/ml and 43.3% in range concentration of 0.125 – 4.0 µg/ml. Likewise, fluconazole showed that 13.3% of *C. albicans* was susceptible in range concentration of 0.023 – 0.19 µg/ml and 76.7% in range concentration of 0.25 – 2.0 µg/ml. The resistance rate of Fluconazole contributes 10% with a concentration of 8 µg/ml and 12 µg/ml. The E-test method shows specifically the minimum inhibitory concentration (MIC) of *C. albicans* which more precise compared to the disk method.

The MIC of an antifungal by E-test which is made of a plastic strip with a predetermined gradient is utilized. Antifungal MICs were calculated using the scale where the growth inhibition curve edge crossed the strip. It indicates that the scale was read at the point when growth was inhibited. Scale reading was problematic in a test of azole MICs due to trailing, which is the slow but continuous growth of *Candida* sp. even at high drug concentrations (Badiee and Alborzi, 2011). Hence, the scale was determined at the concentration where the colony size was decreased. It is preferable to read the Fluconazole MIC after 24 hours. MIC of this determined at the point of complete (100%) or almost complete (95%) inhibition and usually, a zone of inhibition is seen.

This antifungal pattern demonstrates that the majority of pregnant women infected with VVC are treated successfully. Patients with recurrent vulvovaginal candidiasis (RVVC) and long-term medication therapy may develop resistance to the treatment. Previous research from a separate region five years ago shown a small increase in Fluconazole resistance (Mohamadi et al., 2015).

Although it is only slightly increased, it still becomes our concern to overcome this problem. The physician usually prescribes topical drug, clotrimazole, the same group as fluconazole which is azole group as the treatment because it less toxic and suitable treatment plan for pregnant women (Masri et al., 2015). At the laboratory in HTAA, samples from sterile sites such as blood and cerebral spinal fluid (CSF) will proceed with further test in the mycology laboratory. However, the lack of antifungal tests performed for VVC infection could lead to an increase in medication resistance, endangering pregnant women and their babies.

CONCLUSION

Vulvovaginal candidiasis (VVC), often known as vaginal thrush, is a condition that affects predominantly women in their reproductive years. *C. albicans* is the most common cause of VVC, however other species such as *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* are also involved. This study shows the high prevalence of antifungal sensitivity of *C. albicans* in VVC infection among pregnant women at Hospital Tunku Ampuan Afzan, Kuantan. As the first-line antifungal treatment for yeast infection, fluconazole is the best option. 5-Fluorocytosine is more likely to be utilized as a treatment for non-albicans *Candida* infection. Fluconazole and 5-fluorocytosine, at their lowest concentration levels, are able to limit the development of *Candida albicans*, and the risk of developing MDR is relatively low.

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