Introduction

Vulvovaginal candidiasis (VVC) is a disease caused by the abnormal growth of yeast-like fungi on the mucosa of the female genital tract ***(Souza et al., 2009).*** Although *Candida species* occur as normal vaginal flora, opportunistic conditions such as diabetes, pregnancy and other immune depressants in the host enable them to proliferate and cause infection ***(Pam et al., 2012).***

There are approximately 200 *Candida species,* among which are *Candida albicans, glabrata, tropicalis, stellatoidea, parapsilosis, catemilata, ciferri, guilliermondii, haemulonii, kefyr and krusei.* *Candida albicans* is the most common species ***(Pam et al., 2012).***

The most frequent cause of VVC is *Candida albicans*. Non-*Candida albicans* species of *Candida*, predominantly *Candida glabrata*, are responsible for the remainder of cases ***(Ge et al., 2010).*** It is estimated that 75% of women experience at least one episode of vulvovaginal candidiasis throughout their life and 40-50% of them have at least one recurrence ***(González et al., 2011).***

Most patients with symptomatic VVC may be readily diagnosed on the basis of microscopic examination of vaginal secretions. Culture is a more sensitive method of diagnosis than vaginal smears, especially in a suspected patient with a negative result for microscopy ***(Khosravi et al., 2011).***

Antifungal agents that are used for treatment of VVC include imidazole antifungals (e.g., butoconazole, clotrimazole, and miconazole), triazole antifungals (eg, fluconazole, terconazole), and polyene antifungals (e.g., nystatin) ***(Abdelmonem et al., 2012).*** The azoles, particularly fluconazole, remain among the most common antifungal drugs used for prophylaxis and treatment ***(Pietrella et al., 2011).*** It is recommended in various guidelines as the first drug of choice because it is less toxic and can be taken as a single oral dose ***(Pam et al., 2012).***

Inappropriate management of VVC may result in the progression to complicated vaginitis, which often causes long-term physical and mental discomfort, significant economic burden from treatment, and considerable negative effect on sexual relations ***(Ge et al., 2010).***

Over the next decade, antifungal resistance may become an increasingly crucial determinant of the outcome of antifungal treatment ***(Agha et al., 2010).*** Thus, an understanding of the mechanisms underlying the resistance to fluconazole in *Candida albicans* is not only essential for the development of new antifungals, but also important in the selection of the appropriate antifungals for the patients at the earliest time ***(Li-juan et al., 2010).***

Several mechanisms of resistance to azoles have been described in *Candida albicans*. These include increased expression of the drug efflux pump genes such as MDR1, CDR1 and CDR2 ***(Wang et al., 2009).*** Also, fluconazole resistance in *Candida species* has been associated with over expression of ERG11 gene which encodes for lanosterol 14α-demethylase which results in production of a large amount of lanosterol 14α-demethylase and this favours continuous synthesis of ergosterol and maintenance of the integrity of the cell wall which enables *Candida* to resist fluconazole**.** This type of resistance has been associated with fluconazole widespread use as prophylactic, empiric and definitive therapy of candidal and other fungal infections ***(Pam et al., 2012).***

Fluconazole resistance is diagnosed by standardized antifungal testing by either broth microdilution or disc diffusion assays. Antifungal susceptibility testing requires 48–72 h following identification, which often comes too late to influence a timely decision on patient management. Molecular techniques provide a faster and more accurate assessment of resistance than classical methodologies. Nucleic acid-based diagnostics are the fastest growing component of many clinical laboratories (***Perlin, 2009).***

Aim of the Work

The aim of this work was to:

1. Determine the in vitro antifungal susceptibility profile of different *Candida* species especially fluconazole in cases of vulvovaginal candidiasis
2. Investigate fluconazole resistant and susceptible dose dependent (SDD) isolates for the expression of ERG11 gene.
3. Guide empirical antifungal treatment for the suspected cases of candidal vulvovaginitis.

Candida Species

A.Historical aspect

Candida albicans is normally a harmless commensal of host epithelial tissues. Actually, about 60% of the healthy population is estimated to carry *Candida* species ***(Williams et al., 2013).*** However, within a few decades, *Candida* species have progressed from infrequent pathogens that were largely considered nuisance contaminants to important and common human pathogens causing a wide spectrum of superficial and deep disease ***(Vazquez and Sobel, 2011).***

Candida albicans is known since 400 BC when the renowned Greek physician, Hippocrates, identified a microbial infection and he named it as “thrush,” which is caused by this pathogen. However, it was not studied like any other model organism till late twentieth century ***(Kabir et al., 2012).***

The first binomial suggested by Zopf in 1890 was *Monilia albicans*. This name gained wide acceptance over a long period and is still wrongly used. Berkhout in 1923, after recognizing the differences between *Monilia* species isolated from rotting plants and fruits and the yeasts isolated from medical cases, established the genus *Candida* to accommodate the latter. Berkhout proposed the name *Candida* from the Latin toga *Candida*, which referred to the white robe worn by Candidates for the Roman senate. *Albicans* also comes from the Latin albicare which means "to whiten" ***(Parihar et al., 2011).***

B.Classification of *Candida*

Taxonomic classification:

Candida is a genus of yeast that consists of 150-200 species. They are imperfect unicellular dimorphic fungi which multiply mainly by budding similar cells from their surface and form hyphae and/or pseudohyphae. They were earlier assigned to the family deuteromycetes, indicating a lack of sexual reproduction. However, several pathogenic and non pathogenic *Candida* species have been identified to have a sexual stage ***(Meurman et al., 2007).***

The genus *Candida* consists of a heterogeneous group of yeast species that usually exists in two forms: yeast (simple cells) and hyphae (mycelium and pseudo mycelium). The genus *Candida* belongs to mitosporic fungi (fungi with asexual reproduction, or by mitosis rather than meiosis). They do not exhibit sexual reproduction but some species have a teleomorphic form (sexual reproduction) ***(Bialkova and Subik 2006).*** Taxonomically, *Candida* belongs to the phylum *Ascomycota*, subphylum *Saccharomycotina*, class *Saccharomycetes,* order *Saccharomycetales*, family *Saccharomycetaceae*, and genus *Candida* ***(Perez-Nadales et al., 2014)*** (Table 1)**.**

Table (1): Taxonomic classification of *Candida*

Phylum	*Ascomycota*
Subphylum	*Saccharomycetes*
Class	*Saccharomycetes*
Order	*Saccharomycetales*
Family	*Saccharomycetaceae*
Genus	*i.* *Candida* *ii.* Other genus: *Endomyces (Geotrichum sp.)* *Kluyveromyces(Candida pseudotropicalis)* *Geotrichum*
Species of *Candida* genus	*Candida albicans* *Non Candida albicans*

(Quoted from Tille, 2014)

Of the genus *Candida* only few species can cause disease in humans. Although more than 17 different species of *Candida* have been reported as pathogens, more than 90% of invasive infections are attributed to five species, *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei*. Less commonly reported are infections due to *Candida kefyr, Candida guilliermondii, Candida lusitaniae*, *Candida stellatoidea*, and *Candida dubliniensis* ***(Vazquez and Sobel, 2011)*** (Table 2)**.**

Table (2): Disease characteristics of different *Candida* species

Candida species	Characteristics/disease states
Candida albicans	Remains the major fungal pathogen of humans and the most common cause of mucosal and systemic fungal infection It is closely related to several other yeasts, including *Candida africana* and *Candida dubliniensis*; however, they are easily discriminated using molecular techniques.
common non-albicans Candida species	
Candida glabrata	(Synonym: *Torulopsis glabrata*) has become important because of its increasing incidence worldwide and decreased susceptibility to antifungals. *Candida glabrata* is the second most common cause of candidemia following *Candida albicans*.
Candida parapsilosis	Is the third most common cause of candidemia, especially in patients with intravenous catheters. This species produces slime as a virulence factor enabling it to adhere to environmental surfaces and skin of hospital personnel.
Candida tropicalis	Is the third or fourth most commonly recovered *Candida* species from blood cultures. Leukemia and prolonged neutropenia are major risk factors for *Candida* tropicalis candidemia.
Candida krusei	Is the fifth most common bloodstream isolate. *Candida* krusei is of clinical significance because of its intrinsic resistance to fluconazole and reduced susceptibility to most other antifungal drugs.
Candida kefyr	Is a rare species that occasionally causes disease in immunocompromised host.
Candida guilliermondii	Is a rare species. Candidemia and invasive disease in the neutropenic host due to *Candida* guillermondii, are reported occasionally. Of clinical importance is the decreased sensitivity of this species to fluconazole and relatively high minimum inhibitory concentrations (MIC) to echinocandins.
Candida lusitaniae	Is uncommon, but of clinical importance because of intrinsic or secondary resistance acquisition to amphotericin B. It is typically found in patients with hematological malignancies and those in intensive care units.
Candida stellatoidea	Is a heterogeneous species with at least two confirmed subtypes, I and II. Type I differs from *Candida albicans* in several genetic characteristics and is not considered a mutant of *Candida albicans*. Type II is a sucrose-negative mutant of *Candida albicans* serotype A. *Candida stellatoidea* produces germ tubes in vitro.

Candida dubliniensis	Identified initially from the oral cavity of HIV-positive patients. This species rarely is associated with candidemia or invasive disease. *Candida dubliniensis* is identified by germ tube and chlamydospore production, by an inability to grow at 45 °C, and by using commercially available yeast identification kits.
Candida rugosa	Although a rare cause of candidemia, is important because of frequent nystatin and azole-resistance and association with catheters and parenteral nutrition; it is susceptible to amphotericin B and echinocandins.
Less common non-albicans Candida species	
Candida catenulate	A natural contaminant of dairy products, especially camembert cheeses; causes cutaneous, and mucosal infection; candidemia (one case report in a patient with gastric carcinoma who frequently ate cheese)
Candida ciferii	Cutaneous, onychomycosis
Candida famata	Candidemia, endophthalmitis, peritonitis due to CAPD (continuous ambulatory peritoneal dialysis)
Candida haemulonii	Candidemia, cutaneous
Candida inconspicua	Candidemia in neutropenics,oropharyngeal, esophageal, and vaginal candidiasis in diabetics and HIV-positive patients
Candida lipolytica	Low virulence, catheter-related candidemia, colonizes the stool, sputum, and mouth, although not associated with mucosal infections
Candida norvegensis	Recovered from the gastrointestinal (GI) and respiratory tract, rarely causes candidemia and peritonitis
Candida pelliculosa	The most frequent of the uncommon causes of invasive candidiasis; cases of invasive candidiasis including: candidemia, endocarditis, intraabdominal abscess, pyelonephritis, cerebral ventriculitis
Candida pulcherrima	Recovered from skin and nails of normal host
Candida rugosa	Associated with nosocomial candidemia and invasive candidiasis in burn patients, neutropenics, and catheterassociated infections
Candida utilis	Utilized primarily in industry; a rare cause with only two cases of invasive candidiasis
Candida viswanathii	Recovered from respiratory tract and rare cause of meningitis
Candida zeylanoides	Low virulence, associated with catheter-related infections and septic arthritis
Candida africana	In 2001, *Candida Africana* was described as a new species or variant of *Candida albicans* in human isolates. It was recovered from vaginal, perianal, glans penis, and skin samples.

(Vazquez and Sobel, 2011)

C.Epidemiological aspects:

1. Pathogenesis of *Candida* infection

a) Natural Habitat:

Members of the genus *Candida* are ubiquitous. They are frequently recovered from the hospital environment, including respirators, and medical personnel ***(Puebla, 2012).*** While most *Candida* species are found in the environment, approximately a dozen or so are associated with colonization and infection of humans. *Candida* is a part of the normal flora in healthy individuals, and is usually confined to the mucosal surfaces of the oral cavity, gastrointestinal and urogenital tracts, and vagina, with newborns being colonized soon after birth ***(Moran et al., 2012; Tsai et al., 2013).***

b) Different Ways for the Pathogenesis:

Depending on the underlying host defects, the microorganism may cause a wide variety of infections ranging from mucosal infections to life threatening disseminated candidiasis. Therefore, development of candidiasis depends on a delicate balance between the fungi and the host’s immune status which determines the commensal or parasitic relationship ***(Mishra et al., 2007).***

i. Infectious forms:

Yeast, pseudo-and true hyphae ***(Murphy, 2000).***

ii. Source of infection and mode of transmission:

Candida species can be present in clinical specimens as a result of environmental contamination, colonization or actual disease processes. *Candida albicans* infections or colonization are mostly endogenously acquired while *Candida parapsilosis* infection has environmental origin and *Candida tropicalis* infection can be either endogenous or environmental ***(Betty et al., 2007).***

Humans are first exposed to fungus *Candida albicans* when passing through the vaginal canal during birth. In this course *Candida* colonizes the buccal cavity, and upper and lower parts of the gastrointestinal tract of the newborn. *Candida* rarely cause disease in immunocompetent hosts though often exposed to infectious spores. Disease results when *Candida* accidentally penetrates host barriers or when immunologic defects or other debilitating conditions exist that favor *Candida* entry and growth ***(Khan et al., 2010).***

Candidemia in neonates is usually endogenously acquired through host gastrointestinal tract translocation (which is defined as the passage of viable microbes from the gastrointestinal tract through its mucosal lining to extraintestinal sites, such as the mesenteric lymph node (MLN), spleen, liver, kidneys, and blood), given the immaturity of the neonatal gut wall integrity and immunity. However, healthcare-related exogenous transmission from fomites such as biofilms within implantable devices and indwelling catheters, contaminated total parenteral nutrition, and contact with the colonized hands and fingernail beds of healthcare workers (HCW) has been reported sporadically and in the outbreak setting in hospitals as evidenced by molecular epidemiologic studies ***(Panackal, 2013).***

The major source of vaginal yeasts is the gastrointestinal tract, through a process called endogenous transference. The yeasts are carried through self-inoculation to the vagina, in which they adapt and develop, and may cause immediate disturbances or make up a reservoir for future infections ***(Carrara et al., 2009).***

iii. Virulence Factors

The identification of virulence-associated factors in *Candida* species is complicated by the fact that they are opportunistic pathogens that usually exist in harmony with the human host as part of the commensal flora and only cause infection when host's immunity is deficient ***(Moran et al., 2012).*** Strikingly, *Candida albicans* is able to adapt to a wide range of environmental changes such as pH, nutrient shift (low nutrient or low glucose) and temperature, and can infect virtually every human organ ***(Linde et al., 2010).***

To establish an infection successfully, *Candida albicans* must adapt to different niches at various anatomic sites of the host and express infection-associated genes. The products of infection associated genes contribute to *Candida albicans* pathogenicity, and function as virulence factors. Researchers have reported several virulence factors for *Candida albicans*, including morphological transition, adhesins, secreted hydrolytic enzymes, and phenotypic switching ***(Karkowska-Kuleta et al., 2009).***

Other important virulence factors are genes as (HWP1, ECE1, HYR1 and ALS3) involved in the interaction with cells of the immune system as well as genes involved in nutrient acquisition, stress response, and interaction with other host cells ***(Linde et al., 2010).***

1- Dimorphic transition:

Candida albicans is able to undergo reversible morphological changes between yeast, pseudohyphal, and hyphal forms of growth in response to environmental cues and can successfully infect many different anatomical sites of the human host ***(Lu et al., 2011).*** All forms are present in tissue specimens. Yeast cells may be disseminated more effectively, whereas hyphae are thought to promote invasion of epithelial and endothelial tissue and help evade macrophage engulfment ***(Grubb et al., 2009; Vazquez and Sobel, 2011).***

2- Phenotypic switching:

Phenotypic switching involves reversible and heritable switching between alternative cellular phenotypes. In this transition, white cells are round and give rise to smooth, domed colonies, whereas opaque cells are bean shaped and give rise to flatter, translucent colonies. It affects *Candida albicans* pathogenicity, mating efficiency, and biofilm formation White and opaque cells have also been shown to differ in their interaction with immune cells. White cells secrete a chemoattractant for leukocytes, whereas opaque cells do not. White-opaque switching may be an adaptive mechanism to help *Candida albicans* cells escape the attention of the host immune system ***(Alby and Bennett, 2009).***

3- Secretion of enzymes:

Virulence is enhanced by proteolytic enzymes, toxins, and phospholipase elaborated by yeast. These proteolytic enzymes, with broad substrate specificity, destroy free and cell-bound proteins that impair fungal colonisation and invasion ***(Sobel, 2007).*** These enzymes facilitate adherence to host tissue, rupture of cell membranes, invasion of mucosal surfaces and blood vessels, and evasion of the host's immune response ***(Fisher et al., 2011).***

4- Adherence and* Biofilm formation*:

The pathogenesis of *Candida* infection involves a cascade of events including adhesion, colonization, and invasion. Once attached, the yeast can replicate and colonize the host. When host defenses are impaired, such as in the neonate, colonizing yeast can invade from endogenous sites ***(Stechenberg, 2008).***

In addition, *Candida albicans* can adhere to abiotic substrates, such as medical devices, and form biofilms. Biofilm is a dense colonial source of cells, which are continually released into the immediate environment, providing a reservoir for persistent sources of infection. They are much more resistant to antimicrobial agents and host immune factors in comparison to planktonic cells ***(Abd Elsattar et al., 2011).***

Some *Candida albicans* molecules are related to adherence and known as adhesins. The agglutinin-like sequence (Als) protein family is the most recognized of these adhesions ***(Tsai et al., 2013).***

5- Evasion of the immune system:

In addition to destruction of immunoglobulins, cells of *Candida albicans* can bind platelets via fibrinogen ligands in the bloodstream, resulting in the yeast being surrounded by a cluster of platelets which may have the effect of camouflaging them from the immune system during dissemination ***(Fisher et al., 2011).***