REVIEW OF THE LITERATURES
2. Review of the literatures.

2.1 Fungal infections.

Fungal infections may be divided into two broad categories: (i) nosocomial and (ii) community acquired. Virtually all nosocomial fungal infections may be considered opportunistic mycoses; because fungi those are ordinarily nonpathogenic, harmless saprobes may cause life-threatening infection in seriously ill and/or immunocompromised patients. In contrast, community-acquired fungal infections encompass not only opportunistic mycoses but also the endemic mycoses, in which susceptibility to the infection is acquired by living in geographical area constituting the natural habitat of a pathogenic fungus.

Falkow, (1997) recognized two basic types of pathogenic microorganisms (i) primary fungal pathogens that commonly cause diseases among at least a portion of otherwise healthy, normal individuals, and (ii) opportunistic fungal pathogens (figure I) that cause disease only in individuals who are compromised in their innate and/or acquired immune defenses. Primary pathogen depends on its ability to replicate and be transmitted in a particular host population, whereas an opportunistic pathogen does not depend on such transmission. Fungi that cause human diseases cannot be easily distinguished as primary or opportunistic, Figure II presents some examples of different fungal diseases. Fungal agents of disease (mycoses) that are endemic to specific regions of the world are known to be able to initiate infection in normal, apparently immunocompetent individuals.

Over the past two decades, the incidence of both fungal infections (primary & opportunistic) has increased dramatically.
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Figure I: The common Opportunistic fungal infections.
Figure II: Examples of fungal infections:
1. Pencilliosis (*Penicillium marneffei*).
2. Daeryocystitis (*C. albicans & Aspergillus niger*).
3. Cellulitis (*T. beigelii*).
4. Onychomycosis (*T. rubrum*).
5. Corneal ulcer (*Fusarium*).
6. Sporotrichosis (*Sporothrix schenckii*).
7. Cutaneous candidisis (*C. albicans*).
8. Cutaneous *Cryptococcus* infection.
9. Histoplasmosis (*Histoplasma capsulatum*).
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The morbidity and mortality associated with these infections are substantial, and it is as a result that fungal diseases have emerged as important public health problems. Various risk factors associated with the development of fungal infections have been enumerated in figure III.

**Figure III: Risk factors predisposing to development of fungal infections.**
2.2 Fungal infections in the patients with Human Immunodeficiency Virus Infection.

Fungi frequently cause disease in patients with human immunodeficiency virus (HIV) infections. The spectrum of illness ranges from asymptomatic mucosal candidiasis to overwhelming disseminated infection and life-threatening meningitis (Tumbarello et al, 1999; Tylenda et al, 1989; Bertout et al, 1999). The importance of fungal diseases among patients with HIV infection was recognized in the early days of the acquired immunodeficiency syndrome (AIDS) epidemics. Fungal infections were reported in many of the earlier patients described with a new acquired cellular immunodeficiency in 1981 (Gottlieb et al, 1981; Masur et al, 1981). Soon after, the centers for disease control and prevention (CDC) proposed a case definition of fungal opportunistic infections associated with AIDS, these are summarized in table I.

**Table I: Fungal infections included in the 1993 AIDS surveillance case definition.**

<table>
<thead>
<tr>
<th>Fungal infections</th>
<th>Affected area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candidiasis</td>
<td>Bronchi, Trachea, or Lung</td>
</tr>
<tr>
<td>Candidiasis</td>
<td>Esophageal</td>
</tr>
<tr>
<td>Coccidioidomycosis</td>
<td>Disseminated or extra pulmonary</td>
</tr>
<tr>
<td>Cryptococcosis</td>
<td>Extrapulmonary</td>
</tr>
<tr>
<td>Histoplasmosis</td>
<td>Disseminated or extra pulmonary</td>
</tr>
</tbody>
</table>

Modified from Anaissie et al, 2003
Review of the literatures

Despite advances in diagnosis and treatment, the epidemiological status of the human immunodeficiency virus (HIV) infection is far from under control in most of the developing countries.

2.3 Candida species.

_Candida_ species are classified as yeasts with a predominantly unicellular mode of development. Figure IV shows the taxonomic classification of _Candida_ species and its phylogenetic relationship to other yeast species. The growing problems of mucosal and systemic candidiasis reflects the enormous increase in the pool of patients at risk and the increased opportunity that exists for _Candida_ species to invade tissues normally resistant to invasion (Odds, 1998). _Candida_ species are true opportunistic pathogens that exploit advances mechanisms to gain access to the circulation and deep tissues. The increased prevalence of local and systemic diseases caused by _Candida_ species has resulted in numerous new clinical syndromes, the expression of which primarily is dependent upon the immune status of the host.

_Candida_ species produce a wide spectrum of diseases, ranging from superficial mucocutaneous disease to invasive illnesses, such as hepatosplenic candidiasis, _Candida_ peritonitis, and systemic candidiasis (Prasad _et al_, 2002; Anaissie _et al_, 2003). Lately, _Candida_ species has been reported to be the sixth most commonly isolated pathogen and the fourth most prevalent bloodstream pathogen isolated.
<table>
<thead>
<tr>
<th>KINGDOM</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHYLUM</td>
<td>Ascomycota</td>
</tr>
<tr>
<td>SUBPHYLUM</td>
<td>Ascomycotina</td>
</tr>
<tr>
<td>CLASS</td>
<td>Ascomycetes</td>
</tr>
<tr>
<td>ORDER</td>
<td>Saccharomycetales</td>
</tr>
<tr>
<td>FAMILY</td>
<td>Saccharomycetaceae</td>
</tr>
<tr>
<td>GENUS</td>
<td>Candida</td>
</tr>
</tbody>
</table>

**Figure IV:** Taxonomic classification of *Candida* and its phylogenetic relation to their yeast species.
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Management of serious and life threatening invasive candidiasis remains severely hampered by delay in diagnosis and the lack of reliable diagnosis methods that allow detection of both fungemia and tissue invasion by *Candida* species (White *et al.*, 2005; Anaissie *et al.*, 2003; Khan *et al.*, 1998; Odds 1988). On daily basis, virtually all physicians are confronted with a positive *Candida* isolate obtained from one or more of various anatomical sites. *Candida* infections may involve any anatomical structure.

More than 100 species of *Candida* exist in nature; only few species are recognized as causing disease in humans. Seven species in the genus of *Candida* are known opportunistic human pathogens (Table II)

*C. albicans* is the cause of and accounts for approximately more than 50–60 % of candidiasis in humans. *C. glabrata* recently has become important because of its increasing incidence worldwide (Nedret *et al.*, 2002), and it is intrinsically less susceptible to azoles and amphotericin B. Some of the *Candida* species, *C. lusitaniae* and *C. guilliermondii* are important because of their resistance to fluconazole. Another important *Candida* is *C. krusei*, it is of clinical significance because of its intrinsic resistance to azoles and it's lower susceptibility to all other antifungals, including amphotericin B (Mukherjee *et al.*, 2005).
Table II Species commonly causing Candidiasis and its frequency

<table>
<thead>
<tr>
<th>Medically significant Candida species</th>
<th>% Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans (the most common species identified)</td>
<td>50-60%</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>15-20%</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>10-20%</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>6-12%</td>
</tr>
<tr>
<td>Candida guilliermondi</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>Candida lusitaniae</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>1-3%</td>
</tr>
</tbody>
</table>

2.3.1 Disease spectrum.
Diseases caused by Candida species cover a diverse range of pathologic effects and are associated with a plethora of underlying host factors that predispose persons to infections with these organisms. Table III summarize the recognized forms of Candida infection. The forms of disease most commonly caused by Candida involve the oral cavity (thrush), the female genitalia, the skin and nails. Candida infections of deep tissues are almost always the result of hematogenous spread of Candida organism from an endogenous or, less often, an exogenous site.
<table>
<thead>
<tr>
<th>Type of disease</th>
<th>Major predisposing/Risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oropharyngeal infection</td>
<td>HIV infection, Age extremes, Denture wearers, Antibiotic use, Radiotherapy for head and neck cancer, Inhaled and systemic corticosteroids, Cytotoxic chemotherapy, Hematologic malignancies</td>
</tr>
<tr>
<td>Esophagitis</td>
<td>Systemic corticosteroids, AIDS, Cancer, Stem cell or solid organ transplantation</td>
</tr>
<tr>
<td>Lower gastrointestinal infection</td>
<td>Cancer, Surgery</td>
</tr>
<tr>
<td>Vulvovaginal infection</td>
<td>Oral contraceptive, Pregnancy, Diabetes mellitus, Antibiotic use</td>
</tr>
<tr>
<td>Infection of the skin and nails</td>
<td>Local moisture and occlusion, Immersion of hands in water, Peripheral vascular disease</td>
</tr>
<tr>
<td>Coetaneous congenital candidiasis</td>
<td>Intrauterine foreign body, Prematurity</td>
</tr>
<tr>
<td>Chronic mucocutaneous candidiasis</td>
<td>T-lymphocyte defects</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>Indwelling urinary catheter, Urinary obstruction, Urinary tract procedures, Diabetes mellitus</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>Aspiration</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>Major surgery, Previous bacterial endocarditis or valvular disease, Intravenous drug abuse, Long-term central venous catheter</td>
</tr>
<tr>
<td>Pericarditis</td>
<td>Thoracic surgery, Immunosuppression</td>
</tr>
<tr>
<td>Central nervous system (CNS) infection</td>
<td>CNS surgery, Ventriculo-peritoneal shunt, Ocular surgery</td>
</tr>
<tr>
<td>Ocular infection</td>
<td>Trauma, surgery</td>
</tr>
<tr>
<td>Bone and joint infection</td>
<td>Trauma, Intra-articular injections, Diabetic foot</td>
</tr>
<tr>
<td>Abdominal infection</td>
<td>Recurrent perforation, Repeat abdominal surgery, Anastomical leaks, Pancreatitis, continuous ambulatory peritoneal dialysis</td>
</tr>
<tr>
<td>Hematogenous infection</td>
<td>Solid-organ transplantation, Colonization, prolonged antibiotic use, abdominal surgery, Intensive care support, Total parenteral nutrition, Hemodialysis, Immunosuppression, Stem cell or liver transplantation</td>
</tr>
</tbody>
</table>
2.4 Oropharyngeal candidiasis in HIV/AIDS patients.

Pseudomembranous and erythematous variants of oropharyngeal candidiasis represent the most common clinical presentations of mucosal candidiasis associated with HIV-infection, figure V shows the different clinical of oropharyngeal candidiasis (Lattif et al, 2004). The pseudomembranous and erythematous variants of oropharyngeal candidiasis represent the most common clinical presentations of mucosal candidiasis associated with HIV-infection. Further clinical variants include angular cheilitis, exfoliative cheilitis (Reichart et al, 1997) and Candida-associated palatal papillary hyperplasia (Reichart et al, 2000). Utilizing established clinical diagnostic criteria is facilitated the recognition of these specific forms of oral candidiasis in HIV-infected patients (Greenspan et al, 2000). Symptoms may include burning pain, altered taste sensation, and difficulty swallowing liquids and solids (Fichtenbaum et al, 2000).

The pseudomembranous form can be easily diagnosed by demonstrating the presence of candidal yeast and pseudohypha on wet mounts or a stained smear of material obtained by swabbing the lesions and is confirmed by isolation of Candida species on culture. Because procurement of oral tissue samples is restricted for ethical reasons (Reichart et al, 2000) only a limited number of studies have been conducted to determine the histopathologic and ultrastructural features of oropharyngeal candidiasis in HIV infection (Eversole et al, 1997; Romagnoli et al, 1997). In erythematous candidiasis, Candida hyphae are few while blastoconidia may be found on an atrophic epithelial surface. In contrast, hyphae are numerous and extend into the spinous cell layer in pseudomembranous candidiasis, accompanied by parakeratosis, acanthosis, and spongiosis of the infected superficial epithelium (Reichart et al, 2000). Figure V presents some of the clinical pictures of oropharyngeal candidiasis in HIV/AIDS patients.
In addition, to the marked contrast in penetration of the epithelium by *C. albicans* in pseudomembranous and erythematous candidiasis, these two forms of oropharyngeal candidiasis are distinguished by the nature and intensity of the mucosal inflammatory cell response (Reichart et al, 1995; Romagnoli et al, 1997). The erythematous form in both HIV-infected and uninfected patients is characterized by abundant neutrophilic microabcesses in the parakeratin layer of the epithelium, while microabcesses are rarely found in pseudomembranous candidiasis, even underneath foci of extensive hyphal colonization of the parakeratin layer. Indeed, some HIV-infected patients with pseudomembranous candidiasis have almost no epithelial inflammatory response (Eversole et al, 1997; Reichart et al, 1995).
Figure V: Different clinical pictures of oropharyngeal candidiasis in HIV/AIDS patients.
2.4.1 Oropharyngeal candidiasis and the CD4+ T-cell count of the HIV/AIDS patients.

Oropharyngeal candidiasis can occur at any time during the course of HIV infection, including primary HIV infection (Pena et al, 1991) the chronic asymptomatic phase and overt AIDS (Abgrall et al, 2001; Chiou et al, 2000; Schuman et al, 1998). Figure VI shows the hypothetical defect in host defense against oropharyngeal candidiasis in HIV infection. During the chronic asymptomatic phase, both erythematous and pseudomembranous candidiasis is predictive of progressive immunodeficiency and onset of AIDS, independently of the CD4+-T-cell count (Dodd et al, 1991; Nielsen et al, 1994).

Oral burdens of C. albicans are augmented in HIV-infected patients even prior to the first episode of oropharyngeal candidiasis (Tylenda et al, 1989; Wertz et al, 1991) and the intensity of carriage increases significantly in the progression from asymptomatic Candida carrier to an episode of oropharyngeal candidiasis (Wertz et al, 1991). CD4+-T-cell counts measure the number and percentage CD4+T-cells. CD4+-T-cells, also known as helper T-cells, are a specific type of white blood cell that helps identify and attack bacteria, fungi, and germs. Helper T-cells also regulate the production of antibodies and cytokines. The HIV virus targets CD4+-T-cells and attacks them. HIV binds to the surface of CD4+-T-cells and uses it to reproduce itself, destroying the CD4+-T-cell in the process. The destruction of CD4+-T-cells weakens the immune system. As HIV progresses, the body attempts to produce new lymphocytes to replace those destroyed. Despite the effort, CD4+-T-cells levels gradually decline over the course of HIV infection. Because of this process, CD4+-T-cell counts are useful in determining the strength of the immune system. A healthy, HIV-negative adult CD4+-T-Cell count is between 800 and 1200 cells. CD4+-T-cell counts below 500 demonstrate moderate immune
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system damage; a count below 200 or a percentage less than 14 percent suggests severe immune system damage.

Figure VI presents the hypothesis of the defect in host defense against Oropharyngeal candidiasis in HIV infection.
Figure VI: Hypothetical defect in host defense against oropharyngeal candidiasis in HIV infection.
2.4.2 Impact of Antiretroviral Therapy.

The introduction in 1996 of Highly Active Anti Retroviral Therapy (HAART) including protease inhibitors dramatically reduced the prevalence of oropharyngeal candidiasis (Arribas et al, 2000; Cauda et al, 1999) in HIV infected patients. Over a period of 12 months after starting antiretroviral treatment including a protease inhibitor, significant decreases were found in the prevalence of oropharyngeal candidiasis (from 30-56% to 1-9%) (Arribas et al 2000, Martins et al, 1998) the number of relapses of oropharyngeal candidiasis, the rate of asymptomatic oral carriage of *C. albicans* (Martins et al, 1998), and oral candidal burdens (Arribas et al, 2000). An equally striking decrease in the incidence of *Candida* esophagitis ranging from 29 to 42% occurred during the period from 1996 to 1998 compared with the first half of the decade (pre-HAART) (Detels et al, 2001; Ives et al, 2001; Kaplan et al, 2000). A comparable decline in the incidence of esophageal candidiasis has been observed in HIV-infected children since the introduction of HAART (Kaplan et al 2000).

2.5 Vulvovaginal candidiasis.

Vulvovaginal candidiasis is a significant problem for women of childbearing age. Approximately 75% of women experiences at least one episode of vulvovaginal candidiasis during their lifetime (Sobel, 1990; Sobel et al, 1998). In majority of cases, the yeast is present in the absence of clinical symptoms. Carrol et al, (1973) found *C. albicans* in the vagina of 50 out of 303 pregnant women but only one it was isolated from healthy vagina. Hurley (1975) recognized vaginal thrush as the common of all infectious diseases during pregnancy and put the incidence at 15–16% in her patients. The incidences of vulvovaginal candidiasis have been increasing markedly during pregnancy with abrupt decline in the immediate post partium period (Odds and Abbot, 1980).
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The risk factors for vulvovaginal candidiasis are summarized in figure VII. Acute episodes of vulvovaginal candidiasis often occur during pregnancy and during the luteal phase of the menstrual cycle, when levels of progesterone and estrogen are elevated (Witkin, 1991). Several exogenous factors including antibiotics or oral contraceptive usage, pregnancy, hormone replacement therapy (HRT) and uncontrolled diabetes mellitus predispose women to vulvovaginal candidiasis (Sobel, 1990; Sobel et al, 1998). In contrast, premenarchal and postmenopausal women who are not receiving HRT suffer from vulvovaginal candidiasis (Sobel, 1998). Sobel (1990 and 1992) pointed out that there also exists a subset of women (5–10%) who experience recurrent vulvovaginal candidiasis, defined as 3 to 4 episodes per annum in the absence of any recognized predisposing factors, including menstrual cycle pattern. The relevant factor may be estrogen-mediated deposition of glycogen in the vaginal epithelium. Women using oral contraceptive, the glucose content of the vagina is 50–80% higher than in women not on the pill and that vaginal pH may rise as much as 6.5 (Sobel, 1992; Sobel, 1998). Strict criteria for the diagnosis of vulvovaginal candidiasis are uncertain.
Figure VII: Risk factors in pathogenesis of vulvovaginal candidiasis. HIV, Human immunodeficiency virus, HRT, hormone replacement therapy; O. C., oral contraceptive; IUD, intrauterine contraceptive device; VVC, vulvovaginal candidiasis.
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The list of possible clinical symptoms of the vulvovaginal candidiasis includes pruritus, vaginal discharge, genital burning sensation, dyspareunia and dysuria and the most common sign of the infection are abnormal discharge, vaginitis and vulvitis.

DNA typing techniques capable of fingerprinting *Candida* isolates reveal long-term vaginal colonization with the same strain of *Candida* over months and years. Several genetic, biological, and behavioral factors are associated with increased rate of asymptomatic vaginal colonization with *Candida* (figure VII), including recent antibiotic use, pregnancy, use high estrogen-content oral contraceptive, and uncontrolled diabetes mellitus. Other contraceptive measures, including the intrauterine device, diaphragm, vaginal sponge, and spermicidal nonoxynol-9, may also act as risk factors for *Candida* colonization. Some evidence exists that sexual intercourse frequency may influence the incidence of vulvovaginal candidiasis (Foxman, 1990). The rarity of *Candida* isolation in premenarchial girls and lower prevalence of *Candida* vaginitis after menopause emphasize the hormonal dependence of the infection. *Candida* vaginitis virtually occurs only in elderly women in the presence of uncontrolled diabetes mellitus or associated with the use of exogenous estrogen replacement therapy (Soble, 2003).

2.5.1 Pathogenesis of vulvovaginal candidiasis.

Between 65% and 90% of yeast isolated from the vagina is *C. albicans* strains (Ribeiro *et al*, 2001; Sobel. 1990; Tietz *et al*, 1995). The reminders are due to other species, the most common of which are *C. glabrata* and *C. tropicalis*. Non-*C. albicans* *Candida* species are capable of inducing vaginitis and are often more resistant to conventional therapy. Figure VII shows the Risk factors in pathogenesis of vulvovaginal candidiasis. There is some evidence of an increase in yeast vaginitis due to non-*C. albicans*
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Candida species, especially *C. glabrata* (Cauwenbergh, 1989). Risk factors for *C. glabrata* include diabetes, old age and previous use and abuse of over-the-counter antifungal agents may be selective for relatively resistant *C. glabrata* (Ferris, 1996).

For *Candida* organism to colonize the vaginal mucosa they must first adhere to the vaginal epithelial cells. *C. albicans* adheres in significantly higher number to vaginal epithelial cells than do *C. tropicalis* and *C. krusei*. Germination of *Candida* enhances colonization and facilitates tissue invasion. Factors that enhance or facilitate germination (e.g., estrogen therapy and pregnancy) tend to precipitate symptomatic vaginitis, whereas measures that inhibit germination (e.g. bacterial flora) may prevent acute vaginitis in women who are asymptomatic carriers of yeast. Other virulence factors include proteolytic enzymes, mycotoxins, phospholipase elaboration, and iron use. *Candida* vaginitis is seen predominantly in women of childbearing age, and only in the minority of cases can a precipitating factor be identified to explain the transformation from asymptomatic carriage to symptomatic vaginitis (Sobel, 2004; Xu & Sobel, 2004).

2.5.2 Host factors.

During pregnancy the clinical attack rate is maximally increased in the third trimester, but symptomatic recurrences are more common throughout pregnancy. It is generally thought that the high levels of reproductive hormones raise the glycogen content in the vaginal environment and provide an excellent carbon source for *Candida* species growth and germination. The likely but more complex mechanism is that estrogens enhance vaginal epithelial cell avidity for *Candida* adherence, and a yeast cytosol receptor or binding system for female reproductive hormones has been documented. These hormones also enhance
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Mycelium formation by the yeast cells. Low estrogen oral contraceptives have not been found to cause an increase in Candida vaginitis. Vaginal colonization with Candida is more frequent in diabetic women, and uncontrolled diabetes predisposes to symptomatic vaginitis. Glucose tolerance tests have been recommended for women with recurrent vulvovaginal candidiasis; however, the yield is low, and testing is not justified in otherwise healthy premenopausal women (Bohannon, 1998; de Leon et al, 2002).

Genetic predisposes to vaginal colonization with Candida has been recently shown by Chaim et al, (1997) who showed that women prone to vulvovaginal candidiasis are significantly more likely to be genetic and phenotypic nonsecretors of blood group antigens. The latter serve as buccal and vaginal epithelial cell membrane receptors. Similarly, controlled cohort studies reveal that vaginal colonization with Candida, although high in human immunodeficiency virus (HIV) negative high-risk behavioral women, is significantly higher in HIV-positive women. Colonization does not appear to increase with progress decline in CD4+ T-cell count (Schuman et al, 1998).

2.6 Global burden of diabetes

Diabetes mellitus is a heterogeneous metabolic disorder and is considered being a group of varying etiology and pathogenesis. Hyperglycemia is the major cause of increased susceptibility of diabetic patients to vulvovaginal candidiasis, increased glucose levels in genital tissue enhances yeast adhesions and growth. Diabetes mellitus is now taking its place as one of the main threat to human health in the 21st century (Zimmet, 2000). Prevalence of diabetes in adults worldwide was estimated to be 4% in 1995 and was expected to rise to 5.4% by the year 2025, an increase of 35%. The prevalence of diabetes is higher in industrialized countries than in the developing countries.
The number of adults with diabetes in the world will rise from 135 millions in the year 1995 to (150 million in 2000; 220 million in 2010) 300 million in the year 2025, an increase of 122% (King et al, 1998). The majority of this increase will occur in developing countries. There will be an increase of 42% i.e. from 51 to 72 millions in the developed countries and an increase three fold i.e. from 84 to 228 millions in developing countries. The highest increase in the prevalence between 1995 and 2025 will be for China (68%) and India (59%). Thus, by the year 2025 more than 75% of the diabetic people will be residing in the developing countries as compared to 62% in 1995. India, China and USA are the countries with the largest number of people with diabetes at present and will remain so in year 2025 (king et al, 1998). The top 10 countries of the world in terms of the people with diabetes for 1995 and projected changes in their positions in 2025 are shown in table IV.
Table IV: Changes in the positions of top ten courtiers for estimated number of adults (in million) with diabetes in 1995 and 2025.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Countries</th>
<th>Year 1995</th>
<th>Courtiers</th>
<th>Year 2025</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>India</td>
<td>19.4</td>
<td>India</td>
<td>57.2</td>
</tr>
<tr>
<td>2</td>
<td>China</td>
<td>16.0</td>
<td>China</td>
<td>37.6</td>
</tr>
<tr>
<td>3</td>
<td>USA</td>
<td>13.9</td>
<td>USA</td>
<td>21.9</td>
</tr>
<tr>
<td>4</td>
<td>Russia</td>
<td>8.9</td>
<td>Pakistan</td>
<td>14.5</td>
</tr>
<tr>
<td>5</td>
<td>Japan</td>
<td>6.3</td>
<td>Indonesia</td>
<td>12.4</td>
</tr>
<tr>
<td>6</td>
<td>Brazil</td>
<td>4.9</td>
<td>Russia</td>
<td>12.2</td>
</tr>
<tr>
<td>7</td>
<td>Indonesia</td>
<td>4.5</td>
<td>Mexico</td>
<td>11.7</td>
</tr>
<tr>
<td>8</td>
<td>Pakistan</td>
<td>4.3</td>
<td>Brazil</td>
<td>11.6</td>
</tr>
<tr>
<td>9</td>
<td>Mexico</td>
<td>3.8</td>
<td>Egypt</td>
<td>8.8</td>
</tr>
<tr>
<td>10</td>
<td>Ukraine</td>
<td>3.6</td>
<td>Japan</td>
<td>8.5</td>
</tr>
<tr>
<td>All other Nation</td>
<td>-----</td>
<td>49.7</td>
<td>-----</td>
<td>103.6</td>
</tr>
<tr>
<td>Total</td>
<td>-----</td>
<td>135.3</td>
<td>-----</td>
<td>300.0</td>
</tr>
</tbody>
</table>

* Modified from King et al 1998
2.6.1 Vulvovaginal candidiasis in diabetic patients

Diabetes is a proven predisposing factor for vulvovaginal candidiasis, along with pregnancy use of broad-spectrum antibiotics, high estrogen dose oral contraceptives, obesity, and drug addiction (de Leon et al, 2002). Symptomatic vulvovaginal candidiasis infections have been shown to be more prevalent in patients with diabetes than in the general population (Peer et al, 1993; Goswami et al, 2000) and are usually attributed to yeast infection by women themselves. In other surveys, occurrence of vulvovaginal candidiasis among diabetic women ranged from 7 to > 50% (Peer, 1993; Rowe, 1990).

Several studies have examined possible mechanisms whereby hyperglycemia inhibits neutrophil function (Fidel 2002). In a study the evaluated neutrophil killing of *C. albicans* in the presence of increased concentration of glucose was slower. Results showed that 50mmol/l (900 mg/dl) of glucose decreased oxidative killing of *C. albicans* by neutrophils (Willis et al, 2000; Fidel 2002).

This mechanism was further assessed by observing the oxidative phase of killing using Lucigenin-enhanced chemiluminescence, which showed that the increases in glucose concentration led to reduced chemiluminescence output, suggesting the existence of aldose reductase activity in neutrophils.

Differences in virulence of various strains of *Candida* species may also affect phagocytosis in patients with diabetes (Fidel & Sobel 1996). In a study addressing these subjects, leukocytes from normal subjects revealed no differences in their ability to engulf virulent and avirulent strains of *C. albicans* (Fidel 2002).
2.6.2 Studies on vulvovaginal candidiasis from India

Vulvovaginal candidiasis is not a reportable disease because there are no active surveillance studies in India. There are very few published studies on vulvovaginal candidiasis (table V) and this study is one of the few studies on vulvovaginal candidiasis in diabetic patients not only from India but worldwide. The paucity of research work on vulvovaginal candidiasis may be hampered by several different factors: recall bias, accuracy of diagnosis, patient selection, referral bias and confounding affects of widespread local antifungal.
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Table V: Reports of vulvovaginal candidiasis from Indian patients over the year (1961-2005)

<table>
<thead>
<tr>
<th>First Author</th>
<th>Year of study</th>
<th>No. of patients studied</th>
<th>Yeast %</th>
<th>C. albicans %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dutt-Choudhuri</td>
<td>1961</td>
<td>800</td>
<td>25</td>
<td>12.8</td>
</tr>
<tr>
<td>Daftary</td>
<td>1963</td>
<td>100</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>Desai</td>
<td>1966</td>
<td>183</td>
<td>31.1</td>
<td>14.2</td>
</tr>
<tr>
<td>Raut</td>
<td>1971</td>
<td>544</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>Daftary</td>
<td>1980</td>
<td>500</td>
<td>32.8</td>
<td>-</td>
</tr>
<tr>
<td>Goswami</td>
<td>2000</td>
<td>88</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>Goswami</td>
<td>2000</td>
<td>78 Diabetic</td>
<td>46</td>
<td>26</td>
</tr>
<tr>
<td>Nandan</td>
<td>2001</td>
<td>193</td>
<td>-</td>
<td>16.5</td>
</tr>
<tr>
<td>Present study</td>
<td>2001</td>
<td>75</td>
<td>25</td>
<td>42</td>
</tr>
<tr>
<td>Present study</td>
<td>2005</td>
<td>100 Diabetic</td>
<td>45</td>
<td>46.6</td>
</tr>
</tbody>
</table>
2.7 Treatment of candidiasis

The therapeutic options for treating fungal infections, often caused by the emerging new pathogens whose incidence has increased due to the AIDS pandemic and use of immunosuppressive drugs in transplant and cancer patients, are limited by relative low number and structural variety of antifungals (Kolaczkowski & Goffau, 1997). High morbidity and mortality persist for systemic fungal infections due to pathogenic yeast and mold. Antifungal agents belonging to different chemical classes are available with different Site of action (Figure VIII). They can be classified into five groups on the basis of their molecular mechanism of action; their structure and mode of actions have been shown in figure IX and table VI respectively.

![Structures of antifungals used in Candidiasis treatment](image-url)
Figure IX: Site and mechanism of action of different classes of antifungals on a typical fungal cell.

Adapted from Anaissie et al, 2003.
Table VI  Antifungals and their mode of action

<table>
<thead>
<tr>
<th>Antifungals</th>
<th>Trade name(s)</th>
<th>Usual adult dose</th>
<th>Mechanism(s) of action</th>
<th>Spectrum/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyenes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Fungon</td>
<td>0.3-1.0 mg/kg/day</td>
<td>Interaction with ergosterol, interference of fungal membrane, increased membrane permeability to antifungal agents, cell death</td>
<td>Primary resistance may be seen in Pseudallescheriaboydii, Sceloporus spp., Fusarium spp., Aspergillus terreus, and A. flavus. Both primary and secondary resistance has been reported for rare yeasts (Trichosporon beigeli, Candida lindemayeri, and C. guilliermondii)</td>
</tr>
<tr>
<td>Liposomal formulations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambisome</td>
<td>Abelcet</td>
<td>0.25-4.00 mg/kg/day</td>
<td>Similar to amphotericin B</td>
<td>Similar to lipid formulations</td>
</tr>
<tr>
<td>Amphotericin</td>
<td>Nystatin*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluconopyrimidines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluconazole</td>
<td>Azole</td>
<td>200-400 mg orally once a day</td>
<td>Inhibition of cytochrome P450 14α-demethylase, accumulation of lanosterol leading to perturbation of fungal cell membrane, fungastasis</td>
<td>Poor oral absorption and drug interactions are common reasons for clinical resistance. Interference with human sterol biosynthesis seen</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>Sporanox</td>
<td>200 mg intravenously every 12 h for the first 2 days, then 200 mg intravenously once a day or orally twice a day</td>
<td>Similar to fluconazole</td>
<td>Improved activity over fluconazole against invasive moulds. As with ketoconazole, drug interactions and poor absorption (capsules) are common causes of clinical resistance. Marked interpatient variability in serum concentrations leading to resistance in P450 genotype affecting drug metabolism. Cross-resistance may be seen with fluconazole</td>
</tr>
<tr>
<td>Voriconazole*</td>
<td></td>
<td></td>
<td></td>
<td>Like itraconazole, more active than fluconazole against invasive moulds including Aspergillus and Fusarium spp. No activity against Zygomycetes. Cross-resistance with fluconazole? Marked interpatient variability in serum concentrations leading to resistance in P450 genotype affecting drug metabolism</td>
</tr>
<tr>
<td>Posaconazole*</td>
<td></td>
<td></td>
<td></td>
<td>More active than itraconazole against invasive moulds, including Aspergillus and Fusarium spp. No activity against Zygomycetes. Oral absorption and drug interactions potential sources of clinical resistance. Currently, no intravenous formulation</td>
</tr>
<tr>
<td>Echinocandines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspofungis</td>
<td>Canados</td>
<td>70 mg intravenously every day, then 50 mg every 24 h</td>
<td>Inhibition of cell wall glucan synthesis, leading to susceptibility of fungal cell to osmotic lysis</td>
<td>Rapidly fungicidal against Candida spp. including azole-resistant species. Spectrum essentially limited to Candida and Aspergillus spp. Less active against C. parapsilosis and C. guilliermondii</td>
</tr>
<tr>
<td>Micafungin* (FK463)</td>
<td></td>
<td></td>
<td></td>
<td>Same as caspofungin</td>
</tr>
<tr>
<td>Allylamines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terbinafine</td>
<td>Lamisil</td>
<td>250 mg orally every day</td>
<td>Inhibition of squinate epoxidase, resulting in ergosterol dehydroxylation and accumulation of toxic sterols, fungistasis</td>
<td>Poor intrinsic activity against common yeast and moulds. Precludes use as monotherapy. Exhibits activity in combination with azoles in the treatment of azole-resistant oral candidiasis and aspergillosis</td>
</tr>
</tbody>
</table>


The treatment of deeply invasive fungal infections has lagged behind bacterial infections, as there are substantially fewer antifungals than antibacterial drugs (Ghannoum & Rice, 1999) table VII shows the main antifungal, which are in use now with the year given when they were discovered. At the same time, an emergence of fungal species, which develop resistance to the most commonly used antifungals has compounded this problem (Prasad et al, 2000; Prasad et al, 1995; Franz et al, 1998a; Franz et al, 1998b).

**Table VII: Antifungals and year in which released for treatment of fungal infections.**

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassiumlodide</td>
<td>1903</td>
</tr>
<tr>
<td>Nystatin</td>
<td>1951</td>
</tr>
<tr>
<td>Amphotericin</td>
<td>1956</td>
</tr>
<tr>
<td>Flucytosine</td>
<td>1964</td>
</tr>
<tr>
<td>Miconazole</td>
<td>1978</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>1981</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>1990</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>1992</td>
</tr>
<tr>
<td>Lipid associated amphotericin</td>
<td>1990s</td>
</tr>
</tbody>
</table>
2.7.1 Ergosterol biosynthesis inhibitors

Among the most important groups of antifungals are compounds that interfere with the biosynthesis of ergosterol, the principal sterol in pathogenic fungi. All ergosterol biosynthetic inhibitors presently used in medicine inhibit enzymes in the post-squalene segments of the fungal sterol biosynthetic pathway. This segment of the pathway consists of enzymes such as squalene epoxidase, sterol 14α-demethylase, Δ^{14} reductase and Δ^{8}→Δ^{7} isomerase (Barrett-Bee & Dixon 1995). These synthetic compounds are divided into three different subgroups: (i) azole antifungal agents, (ii) allylamines, and (iii) morpholine derivatives.

2.7.2 Azoles

The primary mode of action of these agents has been demonstrated to be the inhibition of sterol biosynthesis (Asai et al. 1999) through the selective inhibition of P450 14α-demethylase (P450_{14adm}) (encoded by ERG11) which occurs following the stoichiometric interaction of the N-3 (imidazole) or the N-4 (triazoles) substituents of the azole ring with the haem ring of the P450{14aDM}. This monooxygenase will, in the absence of azole, catalyze the oxidative removal of the C-14 methyl group from the sterol molecule (lanosterol or eburicol). Such demethylation proceeds via three sequential hydroxylation reactions (Aoyama et al., 1989) and has been demonstrated to be essential for the generation of a sterol molecule able to orientate correctly within the phospholipid bilayer and thus fulfill the regulatory role of the sterols in the maintenance of membrane fluidity and stability. While the triazoles such as fluconazole appear to target 14α-DM almost exclusively, the imidazoles such as ketoconazole have a
broader spectrum of action and also act on various monooxygenases. In most fungal cells, ergosterol is the sterol best suited to maintain membrane integrity and activity (Nes et al, 1978). A complete block of ergosterol synthesis is known to block the cell growth and affects the physical state of the membrane. Many fungal species are sensitive to azoles whereas Mucor species, C. krusei and Aspergillus fumigatus are intrinsically resistant to fluconazole and ketoconazole but sensitive to itraconazole and voriconazole with the exception of Mucor species (Marichal et al, 1995).

2.7.3 Antifungal drug resistance.
There are basically two clinical types of resistance: (i) innate and (ii) acquired. Some fungi are inherently resistant to a particular antifungal agent, whereas others have developed resistance over time, sometimes during therapy. The emerging of yeast isolates resistant to azole antifungal agents, especially fluconazole is a matter of concern. The mechanisms of antifungal drug resistance included number of factors (Prasad et al, 2002).

Figure X presents the mechanisms by which a C. albicans cell might develop resistance.
Figure X: Mechanisms by which a *C. albicans* cell might develop resistance.

1. The entry of the drug is prevented at the cell wall/cell membrane level.
2. The drug is pumped out by efflux pumps which are over-expressed in presence of drugs.
3. The target enzyme is overproduced, so that the drug does not inhibit the biochemical reaction completely.
4. The drug target is altered so that the drug cannot bind to the target.
5. The cell has a by-pass pathway that compensates for the loss-of-function inhibition due to the drug activity.
6. The drug is sequestered in an organelle thereby preventing it to reach its target site.
7. Chromosomal rearrangements which lead to a loss of the chromosomes which harbor efflux pump encoding genes.
8. Some fungal enzymes that convert an inactive drug to its active form are inhibited.
2.7.3.1 Clinical factors

Over the last several decades, aggressive anticancer chemotherapy has resulted in a population of patients that are increasingly exposed to azole drugs for antifungal treatment and prophylaxis. An increased rate of patient mortality due to candidiasis has been reported in patients suffering from cancer, HIV/AIDS or undergoing organ transplantation (Khan & Gyanchandani, 1998; Nguyen et al, 1996). Figure XI summarizes the clinical factors involved in the development of resistance. The role of azoles in treating Candida infections in the above patients has been expanding. Recently, advances in clinical microbiology have enabled us to detect and define azole.

Table VIII shows the factors, which contribute to the resistance.

Table VIII: Lists of those factors, which contribute to the resistance.

- Change to a more resistant species of Candida e.g. replacement by C. krusei or C. glabrata.
- Change to a more resistant strain of C. albicans
- Genetic alterations that render a strain resistant. Drug pressure leads to the generation of resistant cells with specific random mutations
- Transient gene expression by which a cell can alter its phenotype to become resistant in the presence of drug
- Cellular mechanisms of drug resistance.
Figure XI: Factors that may contribute to clinical resistance.
2.7.3.2 Molecular mechanisms of development of drug resistance.

Several different mechanisms may be responsible for the development of drug resistance in *C. albicans*. These include alterations in the sterol biosynthesis pathway, overexpression of the *ERG11* gene encoding the drug target enzyme 14α-DM, mutations in the *ERG11* gene which result in reduced affinity of 14α-DM to fluconazole, and reduced intracellular drug accumulation, which is correlated with the overexpression of membrane transport proteins. In contrast, inactivation of the drug, a frequent cause of resistance to antibiotics in bacteria, has not been described as a resistance mechanism in *C. albicans*. The following sections summarize the genetic evidence that is available for the involvement of the various mechanisms in fluconazole resistance. Sanglard *et al*, (1995) demonstrated that many fluconazole-resistant, clinical *C. albicans* isolates displayed strongly increased mRNA levels of *CDR1* or *MDR1* in comparison with matched susceptible isolates and accumulated less intracellular fluconazole.

The molecular mechanisms of development of drug resistance may include the following:

1. **Alteration in the sterol biosynthesis pathway.**

Inhibition of 14α-DM by fluconazole not only results in ergosterol depletion but also in the accumulation of the methylated sterol 14α-methylergosta-8, 24 (28)-dien-3β, 6α-diol, which inhibits cell growth (Kelly *et al*, 1997).
2. Mutations in the *Erg11* gene encoding the drug target enzyme 14-alpha-DM

A frequent cause of drug resistance is mutations in the target structure that reduce its binding to the drug without preventing function.

3. Overexpression of the *ERG 11*

In the presence of fluconazole, *C. albicans* up-regulates the *ERG11* gene, presumably as a feedback mechanism to make up for ergosterol depletion (Albertson *et al*, 1996; Franz *et al*, 1998a). Franz *et al*, (1998a) reported that even in the absence of fluconazole some fluconazole-resistant isolates express *ERG11* mRNA at higher levels than matched susceptible isolates in the presence of the drug.

4. Chromosomal alterations

An alteration in chromosomal copy number in response to selection pressure, a regulatory principle of gene expression in lower fungi has also been recently discovered in *C. albicans*. Perepnikhatka *et al*, (1999) have shown that the exposure of *C. albicans* cells to fluconazole resulted in the non-disjunction of two specific chromosomes in drug resistant mutants. Drug exposure for different time periods led to the gain of one copy of chromosome 3 and in the loss of a homologue of chromosome 4. While at least two genes *CDR1* and *CDR2* are localized on chromosome 3 none of the genes associated with drug resistance are situated on chromosome 4.
5. Drug import

Defects in drug import are a common mechanism of drug resistance. Many hydrophilic drugs, for example the anticancer antimetabolite methotrexate, cannot easily diffuse through the plasma membrane and have to use specific transporters for this purpose.

6. Overexpression of genes encoding drug efflux pumps

An important mechanism of fluconazole resistance is reduced intracellular accumulation of the drug. In recent years, it became evident that fluconazole is actively transported out of the cells in an energy-dependent manner and that an enhanced drug efflux is caused by the over expression of genes encoding membrane transport proteins. The CDR gene family in C. albicans comprises many more genes, but apart from CDR1 and CDR2 no evidence for the involvement of other members of the gene family in fluconazole resistance has been obtained so far (Balan et al, 1997; Franz et al, 1998b). Recently, a gene that is homologous to MDR1, FLU1 has been isolated by its ability to confer fluconazole resistance on hyper susceptible S. cerevisiae transformants (Calabrese et al, 2000).
2.8 Combination antifungal therapy

High morbidity and mortality persist for systemic fungal infections due to pathogenic yeast (Gudlaugsson et al., 2003; Ribaud et al., 1999). The availability of new antifungal agents belonging to different chemical classes (i) polyenes, (ii) pyrimidines (iii) azoles and (iv) echinocandins as therapeutic option for fungal infections with novel mechanism of action. However, treatment is often complicated by high toxicity, low tolerability, or narrow spectrum of activity. These difficulties have driven recent efforts to determine the efficacy of combination therapy in the treatment and management of infections. The most common rationales behind the studies focused on combination therapy are based on (i) mechanism of action, combining agents with complementary targets within the fungal cells (polyenes plus azoles or echinocandins, antifungals plus immune factors), (ii) spectrum of action (combining agents potent against different organisms) and (iii) stability and pharmacokinetic/pharmacodynamic characteristics (Mukherjee et al., 2005).

Figure XII shows the schematic representation of depletion and enhancement theories of interactions between polyenes and azoles, as an example for combination antifungal agents.
Review of the literatures

Figure XII: Schematic representation of depletion and enhancement theories of interactions between polyenes and azoles.
2.9 Antifungal susceptibility testing

Invasive fungal infections have increased over the past two decades causing formidable morbidity and mortality among immunocompromised hosts, especially patients with HIV/AIDS. The frequency of nosocomial fungal infections has increased also amongst cancer, organ transplant, burn and surgical patients. Due to the life threatening nature of these infections and reports of drug resistance, susceptibility testing of yeast pathogens has become very important. National Committee Clinical Laboratory Standard (NCCLS, 2002) document 27-A2 gives the reference method for broth dilution antifungal susceptibility testing of yeast. The method described in M27-A2 of the NCCLS is intended for testing yeasts that cause infections. These yeasts encompass Candida species and Cryptococcus neoformans. M27-A2 is a reference standard being developed through a consensus process to facilitate the agreement among laboratories in measuring the susceptibility of yeasts to antifungal agents. The document has focused on developing for available antifungal agents (NCCLS 1997, 2002), and reference minimal inhibitory concentration (MIC) ranges for microdilution testing of the antifungal. MIC is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.
2.10 DNA Fingerprinting of fungal strains.

Interest in assessing the genetic relatedness of isolates of the same species has grown rapidly as we have delved deeper into the epidemiology of a variety of fungal diseases (Lockhart et al, 1997; Pizzo et al, 2002; Soll, 2000; Thanos et al, 1996; Williams et al, 1995). Also to understand an infectious disease fully and to unravel its epidemiology, there is an absolute requirement to be able to identify individual strains of species responsible for the infection. Thus, given the increased importance of fungal diseases during the past two decades, and with a view to improving the treatment and prognosis of these infections, a variety of strains typing techniques have been developed to improve understanding to how and why fungi cause disease.

Effective DNA fingerprinting techniques are likely to answer the following questions:

1. To understand the dynamics of an infection.
2. To verify the relationship between commensalisms and infection.
3. Identify the origin of an infection.
4. Monitor the emergence of drug-resistant strains.
5. Determine if specific strains are more likely to cause disease.
6. Recognize if more than one strain is causing infection and their relative proportions.
7. Identify if relapse of infection is due to the same or different organisms.

The molecular methods for epidemiological typing used are as summarized in table IX.
**Table IX: Molecular methods for epidemiologic fungal strains typing.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Fungal pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Southern Hybridization analysis</td>
<td>Candida species, Aspergillus species</td>
</tr>
<tr>
<td>(Restriction fragment length Polymorphism)</td>
<td>Cryptococcus neoformans,</td>
</tr>
<tr>
<td></td>
<td>Trichsporon begelii, Histoplasma capsula</td>
</tr>
<tr>
<td>Restriction endonuclease analysis</td>
<td>Candida species, Aspergillus species</td>
</tr>
<tr>
<td>genomic analysis of genomic DNA</td>
<td>Malassezia species, H. capsulatum</td>
</tr>
<tr>
<td>Pulsed field del electrophoresis</td>
<td>Candida species, C. neoformans</td>
</tr>
<tr>
<td>Electrophoretic karyotypic restriction</td>
<td></td>
</tr>
<tr>
<td>Endonuclease digestion with rare cutte</td>
<td></td>
</tr>
<tr>
<td>Polymerase</td>
<td>Candida species, Aspergillus species</td>
</tr>
<tr>
<td>Chain reaction fingerprinting</td>
<td>C. neoformans, H. capsulatum,</td>
</tr>
<tr>
<td></td>
<td>Pneumocystis carinii</td>
</tr>
<tr>
<td>Protein-based methods</td>
<td>Candida species, Aspergillus species</td>
</tr>
<tr>
<td>Immunoblot fingerprinting</td>
<td></td>
</tr>
<tr>
<td>Polycrylamide gel electrophoresis of</td>
<td>Candida species</td>
</tr>
<tr>
<td>Cellular proteins</td>
<td></td>
</tr>
<tr>
<td>Multilocus enzyme electrophoresis</td>
<td>Candida species, C. neoformans</td>
</tr>
</tbody>
</table>
DNA fingerprinting has become the major method for assessing the genetic relatedness of microorganisms in epidemiological studies. DNA fingerprinting methods in many cases involve comparisons of patterns which are assumed to reflect genetic relatedness and which are generated by some form of electrophoresis. Patterns which have been used include: 1) electrophoretically separated digestion fragments of whole cell DNA stained with ethidium bromide (RFLP) electrophoretically separated digestion fragments of whole cell DNA hybridized with a range of DNA probes including ribosomal sequences, mitochondrial sequences, moderately repetitive sequence dispersed throughout genome, sub-telomeric sequences and complex probes amplified primer extension products (e.g., RAPD) and 4) electrophoretically separated chromosome (e.g., TAFE, OFAGE, CHEF). Before using a particular DNA fingerprinting system the user must be sure that it effectively distinguishes between genetically unrelated strains, is capable of identifying the same strain in separate samples, and provides measures of relatedness among moderately-related strains. DENDRONE is a computer-assisted program, which was developed for analyzing and comparing DNA fingerprinting patterns.

DNA fingerprinting of the infectious fungi has become an important subdiscipline of medical mycology. As DNA fingerprinting is more frequently applied to a variety of epidemiological problems, it becomes increasingly evident that there are criteria, which can be used to assess the resolution of a particular fingerprinting method, and strategies have evolved to verify the efficacy of a fingerprinting method (Pujol et al., 1997; Soll, 2000).
DNA fingerprinting is carried out to understand:
(1) The dynamics of an infectious organism in human population.
(2) The complex relationship between commensalisms and infection.
(3) Identify the origin of an infection.
(4) Monitor the emergence of drug resistant strains.

2.10.1 Features of an ideal DNA fingerprinting method.
The efficacy of one typing method over the other is an issue of debate as no method is foolproof. The other one may overcome the shortcomings of one method, but at the same time it may have its own shortcomings. An ideal DNA fingerprinting method should be able to:
1. Identify same strain in independent sets of isolates.
2. Identify micro evolutionary changes in a strain.
3. Cluster moderately related strains.
4. Identify unrelated isolates.
5. Should be resistant to homoplasy (i.e. convergence of karyotyping patterns without a common ancestor)
The shortcoming of fungal fingerprinting as compared to bacteria is that in bacteria even RFLP is a sufficient method due to small size of the genome. However, the fungal genome is large, so simple method like RFLP is of no use. Another problem encountered in fungal DNA fingerprinting is that it is diploid, wherein both the alleles may be different. Thus to design an efficient method for fungal DNA fingerprinting that correctly identifies different strains in terms of being unrelated, moderately related or closely related is a very difficult assignment. Table IX summarized the molecular methods used for epidemiological typing of fungal pathogens. A
good DNA fingerprinting method should provide quantitative data that reflect genetic distance. The methods currently used for DNA fingerprint of infectious fungi are:

2.10.1.1 Restriction Fragment Length Polymorphism (RFLP).

RFLP without probe hybridization has been applied to a variety of infectious fungi including *C. albicans* (Magee *et al*, 1992; Spitzer & Spitzer 1992; Bart-Delabesse *et al*, 1993; Clemons *et al*, 1997).

RFLP is one of the earliest molecular techniques developed to compare inter-strain relationships between microbial strains, including a wide variety of fungal species, and restriction enzyme analysis. RFLP method relies on the analysis of strain specific banding patterns generated by electrophoresis of DNA fragments resulting from the digestion of genomic DNA with specific restriction endonucleases. These strain specific patterns result from polymorphisms in the recognition sites of individual restriction enzymes. Though easy to perform, this technique generates very complex fingerprint patterns, which are very difficult to interpret objectively and are also not amenable to computer-assisted analysis.

2.10.1.2 Restriction fragment Length Polymorphisms with Specific-Species DNA Fingerprinting Probes.

To improve the sensitivity of RFLP, the DNA fingerprint patterns generated by using restriction enzymes, can be probed in Southern blot hybridization test with labeled species-specific DNA
probes that contain repetitive elements. The results are less complex hybridization banding patterns, which are readily amenable for further analysis. DNA fingerprinting using species-specific probe is time consuming and expensive and involves complex labeling and hybridization procedures that are tedious to apply to a large numbers of strains. However, this method give highly resolved as well as selectively visualizing a limited number of fragments that provide a more highly resolved fingerprint pattern for analysis (Soll, 2000). The DNA fingerprinting pattern is amenable to computer-assisted analysis. Details of some DNA probe for various fungal pathogens are listed in the table X.
### Review of the literatures

**Table X: Available species specific probes for various fungal pathogens**

<table>
<thead>
<tr>
<th>Species</th>
<th>Fingerprinting probe</th>
<th>Size of the probe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Candida species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. <em>Candida albicans</em></td>
<td>27A</td>
<td>6.7 kb</td>
</tr>
<tr>
<td></td>
<td>Ca3</td>
<td>11.0 kb</td>
</tr>
<tr>
<td></td>
<td>CARE2</td>
<td>1.06 kb</td>
</tr>
<tr>
<td>2. <em>Candida dubliniensis</em></td>
<td>Cd1</td>
<td>15.5 kb</td>
</tr>
<tr>
<td></td>
<td>Cd24</td>
<td>10.0 kb</td>
</tr>
<tr>
<td></td>
<td>Cd25</td>
<td>16.0 kb</td>
</tr>
<tr>
<td>3. <em>Candida glabrata</em></td>
<td>Cg6</td>
<td>14.8 kb</td>
</tr>
<tr>
<td></td>
<td>Cg12</td>
<td>9.0 kb</td>
</tr>
<tr>
<td>4. <em>Candida tropicalis</em></td>
<td>Ct3</td>
<td>18.0 kb</td>
</tr>
<tr>
<td></td>
<td>Ct14</td>
<td>20.0 kb</td>
</tr>
<tr>
<td>5. <em>Candida parapsilosis</em></td>
<td>Cp3</td>
<td>15.0 kb</td>
</tr>
<tr>
<td><strong>Other fungal species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. <em>Cryptococcus neoformans</em></td>
<td>CNRE-1</td>
<td>-</td>
</tr>
<tr>
<td>7. <em>Aspergillus flavus</em></td>
<td>PAF28</td>
<td>-</td>
</tr>
<tr>
<td>8. <em>Aspergillus fumigatus</em></td>
<td>λ 3.9 (Af 3.9)</td>
<td>-</td>
</tr>
<tr>
<td>9. <em>Histoplasma capsulatum</em></td>
<td>Yps-3</td>
<td>-</td>
</tr>
</tbody>
</table>
2.10.1.3 Microsatellite typing by using oligonucleotide probes

A variation of the previous methods is used of radio-labeled oligonucleotides, rather than species-specific sequences, as DNA fingerprinting probes. The target sequences on the basis of which these primers are designed are homologous to microsatellite sequences. Microsatellite are 1-6 bp tandem repeats, scattered randomly through the genome of the eukaryotic organisms including fungi. These sequences generally consist of mono, di, tri and tetra nucleotide motifs in multiple tandems repeat. One advantage of this method is that the same-labeled probe can be used on a wide range of species. Though useful, this method has been applied in a limited number of studies on medically important fungal species (Soll, 2000)

2.10.1.4 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE determines the relationship between fungal strains by comparing the molecular karyotype profile of individual strains. By the PFGE technique the DNA from individual yeast chromosome can be isolated. The DNA is gently isolated from cells to avoid breakage and subjected to electrophoresis through agarose gel under special conditions that permit separation of molecules as large as 2000 kb (Singer & Berg, 1991). Conventional electrophoretic techniques do not separate duplex DNAs that are much longer than 20 kb. In PFGE the DNA is exposed to fields in alternating orientations, then even very large molecules can be separated according to size (Singer & Berg 1991). This allow very large DNA molecules and fragments to reorient and migrate through the pores of agarose gel at varying rates, and following staining of DNA fragments results in a specific karyotypic banding
A number of variations of PFGE have been developed and applied to the analysis of fungal species with varying success including homogenous electric field (CHEF) electrophoresis, orthogonal-field-alteration gel electrophoresis (OFAGE) and field inversion gel electrophoresis (FIGE). Although PFGE have been demonstrated to be very effective at discriminating between separate strains of a wide range of medically important fungi. PFGE is time consuming; this is one of the factors that have limited the use of PFGE (Olive & Bean, 1999).

2.10.1.5 Random Amplification of Polymorphic DNA (RAPD):
RAPD analysis depends on the use of oligonucleotides primers of arbitrarily chosen sequences (either singly or pairs) in low stringency PCR amplification reactions. Fingerprints or profiles of amplimers are generated by electrophoresis of the amplified products on agarose gels. Minor sequences differences between strains can results in increased or decreased annealing of primers, resulting in the presence or absence of specific amplimers and therefore differences in fingerprint pattern. This method is called Randomly Amplified Polymorphic DNA (RAPD), arbitrarily primed PCR (Ap-PCR) (Welsch & McClelland, 1990; Welsch & McClelland 1991; Williams et al, 1990) differentially amplified fragments (DAFs), and amplified fragment length polymorphisms (AFLPs) (Caetano-Anolles, 1993; Vos et al, 1995).

Although each technique has unique aspects, all rely on a principle of genome-wide screening in an arbitrary, or non-targeted, fashion, and use non-locus specific primers. The goal is to find a maximal number of useful polymorphism that can be applied to the construction of a map, or if recombination inbred
or similar genetic stocks are being used, finding closely linked markers to a gene of interest. Once the markers are found, they can develop into sequence-confirmed amplified fragments (Paran & Michelmore, 1993; Thomson et al, 1997). That allows specific primers to be synthesized that can specify the analysis for a particular locus. The RAPD method of DNA fingerprinting has been used for most of the infectious fungi and has been successfully applied for example to *C. albicans* (Robert et al, 1995; Steffan et al, 1997), *C. dubliniensis* (Coleman et al, 1997), *C. parapsilosis* (Lott et al, 1993), *C. tropicalis* (Lin & Lehmann, 1995) and for *C. glabrata* (Becker et al, 2000). The problem of RAPD is that, every methodological aspect of PCR can affect reproducibility. Artifactual variation can occur as a result of small differences in the primer to template concentration ratio, the temperatures during the amplification reaction, and the concentration of magnesium in the reaction mixtures (Ellsworth et al, 1997; Soll, 2000).

### 2.10.1.6 Multilocus enzyme Electrophoresis (MLEE).

This method identifies allelic polymorphisms that lead to altered electrophoresis mobility of proteins in polyacrylamide gels. MLEE has been successfully used in population studies of fungi including *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *Cryptococcus neoformans* (Arnavielhe et al, 1997; Boerlin et al, 1996; Bertout et al, 1999).

MLEE technique is unable to detect silent mutations that do not lead to changes in the Electrophoretic mobility of proteins. MLEE has been used to assess genetic diversity, gene flow and
population or genotypic structures besides being useful for
typing of systemic and epidemiological studies of fungi.

2.11 The computer assisted DENDRON.

DENDRON is a computer-assisted system (Figure XIII), which was
developed for analyzing and comparing DNA fingerprinting
patterns. In the DENDRON system, an autoradiogram or
fluorogram of a fingerprinting gel containing a number of lanes,
each with visible complex banding pattern, is scanned in to the
DENDRON database with a compatible scanner. The digitized
image can then be viewed on the computer monitor with the clarity
and detail of the original image. The gel can be scanned directly
from an autoradiogram or fluorogram, in which case the scanner
uses direct light or it can be scanned from a case DENDRON
computes similarity coefficients between every possible pair of
strains in a gel, and for all possible pairs between gels.

New strains can be compared with strains analyzed previously as
long as the same standards were contained in the gel for
normalization. The similarity coefficients are presented in a matrix,
and can be used to generate a dendrogram. One of the major
functions of a dendrogram is to demonstrate clustering for groups
of genetically similar strains, and to separate unrelated groups of
strains. When a gel pattern is of acceptable quality, either in its
original form or after processing, the DENDRON program identified
lanes, scans the lanes for pixel density, identifies bands and
categorizes them for intensity. Once the positions and intensities of
the bands in the pattern of each lane have been logged in the
database of DENDRON, a similarity coefficient ($S_{AB}$) is computed for
every pair of strains, generating a matrix of values.
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2.12 Virulence Factors of Candida species

The ability of Candida species to colonize, penetrate, and damage host tissues depends on imbalances between Candida virulence attributes and specific defects in host immune defenses. C. albicans possesses a multiplicity of properties, including adhesions, dimorphism, phenotypic switching, molecular mimicry of mammalian integrins, and secretion of hydrolytic enzymes, each with a low propensity for enhancing fungal infection and none necessarily dominant, and all, even in combination, unable to fully overcome intact host defenses (Odds, 1988). Hydrolytic enzymes are probable virulence factors in pathogenic Candida species (Cutler, 1991; Hube, 1996; White et al, 1995). Among these, C. albicans Saps, under the control of multigene family (SAPI to SAP10) expressing distinct isoenzymes that are regulated differentially at the mRNA level in vitro (Monod et al, 1998) are implicated in the breakdown of several host substrates. Evidence has been presented that phospholipase B, expressed by at least
two genes (*PLB1* and *PLB2*) (Leidich *et al*, 1998) also contributes to the pathogenesis of candidiasis by the degradation of host tissues (Ghannoum, 2000).

2.12.1 **The glyoxylate cycle is required for the fungal virulence.**

*C. albicans*, a normal component of mammalian gastrointestinal flora, is responsible for the most fungal infections in immunocompromised patients. *Candida* is normally phagocytosed by macrophages and neutrophils, which secrete cytokines and induce hyphal development in this fungus (Calderone & Fonzi, 2001). Neutropenic patients, deficient in these immune cells, are particularly susceptible to systemic candidiasis.

The majority of the cases of candidiasis in humans are caused by *C. albicans*. *C. albicans* is an opportunistic pathogen and causes infections in different anatomical locations [Odds, 1988; Prasad *et al*, 2002]. The frequency of this infection has risen drastically in the recent decades due to the increasing number of the immunocompromised patients [Fidel, 2002a, b; Fidel *et al*, 2003]. The *C. albicans* infections resistant to antifungal agents have been increasingly documented in immunocompromised and/or critically ill patients. This organism expresses several virulence factors that contribute to the pathogenesis. These include: (I) phenotypic switching of genes [Mitchell, 1998], (II) transition between blastospores and hyphal forms [van Burik & Magee, 2001], (III) adherence capacity [Kennedy *et al* 1992], (IV) hydrolytic enzymes *PLB1* and (V) secreted aspartyl proteinases *SAPI-10* [Naglik *et al*, 2003].
Recently it has been found that *C. albicans* harbors enzymes involved in the glyoxylate cycle, which helps its survival inside the macrophages during infection, especially the two key specific enzymes namely, isocitrate lyase and malate synthase [Lorenz & Fink, 2001]. Earlier studies have shown that glyoxylate cycle has a role in the virulence of many pathogens, like *Mycobacterium tuberculosis* [McKinney et al, 2000] and *Cryptococcus neoformans* [Rude et al, 2002]. Recently the glyoxylate cycle was found to be required for virulence of the plant pathogenic fungus *Magnaporthe grisea* [Wang et al, 2003].

Genes coding for the glyoxylate cycle have now been shown to be required for virulence in both bacteria and fungi that can survive inside a macrophage. Inhibition of the glyoxylate cycle should block nutrient availability and prevent survival of these pathogens inside the macrophage. Acetyl-coenzyme A can only be assimilated through glyoxylate cycle, which bypasses the catabolic steps of the tricarboxylic acid cycle (figure XIV) in which the two carbon atoms are lost as CO₂ in mammalian systems, and the cycle is absent in mammalian system. Thus the glyoxylate cycle is the only route for the synthesis of glucose in this environment. Compounds that inhibit nutrient availability have been developed into effective herbicides, for example glyphosate and imidazolinones, because their targets are enzymes exclusively produced by plants and not animals. Glyoxylate cycle enzymes are therefore the prime targets for antibacterial and antifungal agents.
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Figure XIV: The glyoxylate cycle and its interrelationship with other pathways.
2.13 Identification and diagnosis of the yeasts

In a clinical mycology laboratory, yeasts are always identified by a combination of morphological and biochemical criteria. Majority of yeast isolates found in the laboratory are Candida species, Cryptococcus species, Rhodotorula, Trichosporon, and Hasenula.

2.113.1 Criteria for Identification & diagnosis of yeasts:

The principal morphological criteria include:

(i) Appearance and colour of colonies (pigment production).
(ii) Size and shape of cells.
(iii) Presence of a capsule.
(iv) Production of hyphae and/ or pseudohyphae.
(v) Ability to produce germ tubes.
(vi) Ability to produce chlamydoconidia,

Criteria (v) and (vi) form the basis of rapid tests for the identification of C. albicans.

The principal biochemical criteria include:

(i) Assimilation of carbohydrates;
(ii) Fermentation of sugars.

There are other more specific tests for the identification of particular genera or species. Some of the traditional methods are cumbersome and time consuming to perform. Consequently, a number of rapid tests and commercial identification systems have been developed and introduced into microbiology laboratories over the past decade.
2.13.2 Direct identification of *Candida* species using differential media.

The incorporation of fluorogenic or chromogenic substrates directly into the growth agar media to reveal species-specific enzyme activity allows for easier discrimination of *Candida* colonies in mixed yeast populations than does Sabouraud-dextrose agar. This allows rapid identification of *Candida* species on the primary isolation medium. These media may avoid or diminish the need for subculture and further biochemical tests, and considerably simplify the identification procedure. One such most commonly used media nowadays is the chromogenic agar (CHROM agar) media. This media definitively identifies three medically important *Candida* species namely, *C. albicans*, *C. tropicalis*, and *C. parapsilosis* (Figure XV).
Figure XV: Differential of *Candida* species by isolation on CHROMagar Candida. The green colonies are *C. albicans*; the blue-gray colonies are *C. tropicalis*, and large pale rough colony is *C. krusei*. The pink colonies are yeast species. Only *C. albicans*, *C. krusei* and *C. tropicalis* can be dependably recognized on this medium; other species have colonies ranging from a very pale to a dark pink.
2.13.2 PCR-based identification of pathogenic *Candida* species.

The frequency of invasive fungal infections has risen dramatically in recent years. Early and accurate diagnosis of these infections is important for several reasons (i) including timely institution of antifungal therapy and (ii) to decrease the unnecessary use of toxic antifungal agents. In addition, the availability of accurate and timely diagnoses could reduce the use of empirical antifungal therapy, thereby reducing antifungal selection pressure and the emergence of antifungal resistance.

Standard approaches to the laboratory diagnosis of fungal infection included (i) direct microscopic visualization for the presence of organism (ii) histopathologic demonstration of fungi within tissue section in case of invasive fungal infections and (iii) cultivation of the causative fungus and its subsequent identification. However, these approaches are not sufficiently sensitive and/or specific diagnosis to diagnose invasive fungal infections (Guiver *et al*, 2001).

That is why over the past several years, the development and application of molecular diagnostic techniques has initiated a revolution in the diagnostic and monitoring of infectious diseases. Nucleic acid techniques, such as plasmid profiling, various methods for generating restriction fragment length polymorphisms, and the polymerase chain reaction (PCR), are making increasing inroads in to clinical laboratories (Kanbe *et al*, 1999; Lehmann *et al*, 1992; Liu *et al*, 1996; Mannarelli & Kurtzman, 1998; Melo *et al*, 1998)
2.14 Identification of *Candida* species by Randomly Amplified Polymorphic DNA.

There has been a significant increase in the number of reports of systemic and mucosal infections caused by *Candida* species with the increase in the number of immunocompromised patients (Fujita *et al.*, 1995; Lattif *et al.*, 2004). *C. albicans* is the most frequently isolated causative agents of candidiasis in humans. However, in the recent years it has been shown that Non-*C. albicans* *Candida* species have been isolated with increasing incidence from cases of candidiasis (Girmenia *et al.*, 1996; Perea *et al.*, 2002).

The differentiation of *Candida* species has routinely been performed on the basis of morphological features and biochemical reactions. Nonetheless, the phenotypic variations of some *Candida* species have led to much uncertainty in differentiation *Candida* species (Einsele *et al.*, 1997; Sandhu *et al.*, 1995). Conventional means of identification of pure cultures of *Candida* species include lengthy and time-consuming morphological, fermentation and assimilation tests that can take several days to identify the isolates in a culture (Fujita & Hashimoto, 1992; Hui *et al.*, 2000) and clinical yeast isolates are sometimes misidentified when automated biochemical systems are used (Dooley *et al.*, 1994). In immunocompromised patients the clinical appearance of the *Candida* infection is often very complex and identification of the organism up to species level is difficult (Dodd *et al.*, 1991; Dooley *et al.*, 1994). Early diagnosis and management of the candidiasis is essential for these patients.

A number of PCR methods for the differentiation of some *Candida* species have been reported (Burgener-Kairuz *et al.*, 1994; Einsele
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et al, 1997; Elie et al, 1998; Haynes & Westerneng, 1996; Jordan, 1994; Meyer et al, 2001; Shin et al, 1997; Shin et al, 1999). Also, many PCR-based methods that use several unique or multicopy molecular targets for the highly sensitive detection of C. albicans in culture or biological samples have been developed. The ideal method for the rapid and accurate identification of microorganism, particularly in a clinical laboratory, would have minimum sample preparation, would analyze samples directly and would be rapid, automated, accurate and relatively inexpensive (Einsele et al, 1997; Sandhu et al, 1995).

Rapid identification of Candida species has become more important because of an increase in infections caused by species other than C. albicans, including species innately resistant to azole antifungal drugs, the most used drugs for treating patients with candidiasis.