Chapter 1

Review of Literature
Chapter - 1

1. Review of the literatures

1.1 Fungal infections

Fungal infections may be divided into broad categories: (i) nosocomial and (ii) community acquired. 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.

Diacovich and Gorvel, (2010) 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 1.1) 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 dose not depends on such transmission. Fungi that cause human diseases cannot be easily distinguished as primary or opportunistic, (Figure 1.2) 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. Various risk factors associated with the development of fungal infections have been enumerated in (Figure 1.3).

1.2 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. 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 first patients described with a new acquired cellular immunodeficiency in 1981 (Gottlieb et al, 1981; Masur et al, 1981).
Fig 1.1  Examples of fungal infections

1. Pencilliosis (pencillium marnefei)
2. Dacryocysitis (C. albicans and 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. Histoplosmosis (Histoplasma capsulatum)
Fig 1.2  The common opportunistic fungal infections.
Fig 1.3  Risk factors predisposing to development of fungal infections.
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 1.1).

### 1.3 Candida species

*Candida* species are classified as yeasts with a predominantly unicellular mode of development. (Figure 1.4) 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 (Chunchanur, 2009). *Candida* species are true opportunistic pathogens that exploit advances mechanism 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 (Kofla & Ruhnke, 2011). Lately, *Candida* species has been reported to be the sixth most commonly isolated pathogen and the fourth most prevalent bloodstream pathogen isolated. Seven species in the genus of *Candida* are known opportunistic human pathogens (Table1.2)

### 1.4 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, (Figure1.5) shows the different clinical of oropharyngeal candidiasis (Lattif *et al*, 2004). 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 (Ship *et al*, 2007). (Figure1.6) 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 (Goswami *et al*, 2006). 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).

### 1.4.1 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 (Enwuru *et al*, 2007) the chronic asymptomatic phase and overt AIDS (Liu X *et al*, 2001; Fidel,). (Figure 1.7) shows the hypothetical defect in host defense against oropharyngeal candidiasis in HIV infection.

### 1.4.2 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 (De Luca *et al*, 2008) in HIV infected patients.

### 1.5 Vulvovaginal candidiasis

Vulvovaginal candidiasis is a significant problem for women of child bearing age. Approximately 75% of women experiences at least one episode of vulvovaginal candidiasis during their lifetime (Hainer & Gibson, 2011). The risk factors for vulvovaginal candidiasis are summarized in (Figure 1.7). 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 (Falagas *et al*, 2006). Several
Table 1.1  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
Fig 1.4  taxonomic classification of candida and its phylogenetic relationship to other yeast species.
Table 1.2  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>
Fig 1.5 different clinical pictures of oropharyngeal candidiasis in HIV/AIDS patients.
Adapted from de Repentigny et al, 2004.

Fig1.6 Hypothetical effect in host defence against oropharyngeal candidiasis in HIV infection.
Fig 1.7  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.
exogenous factors including antibiotics or oral contraceptive usage, pregnancy, hormone replacement therapy (HRT) and uncontrolled diabetes mellitus predispose women to vulvovaginal candidiasis (Hainer & Gibson, 2011). In contrast, premenarchal and post menopausal women who are not receiving HRT suffer from vulvovaginal candidiasis (Hainer & Gibson, 2011) 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 (Figure 1.7).

### 1.5.1 Pathogenesis of vulvovaginal candidiasis

Between 85% and 90% of yeast isolated from the vagina are *C. albicans* strains. (Figure 1.7) shows the Risk factors in pathogenesis of vulvovaginal candidiasis. 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* (Marrazzo, 2010).

*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 (Donders *et al*., 2008).

### 1.5.2 Host factors

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 (Amouri *et al*., 2010).

Colonization does not appear to increase with progress decline in CD4+-T-cell count (de Repentigny, *et al*, 2004).

### 1.6 Global burden of diabetes

Diabetes mellitus is a heterogeneous metabolic disorder and consider being a group of varying etiology and pathogenesis. Diabetes mellitus is now taking its place as one of the main threat to human health in the 21st century (Martinez-Ramirez *et al*., 2006). The top 10 countries of
the world in terms of the people with diabetes for 1995 and projected changes in their positions in 2011 are shown in (Table1.4).

1.6.1 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 (Goswami et al, 2006).

Several studies have examined possible mechanisms whereby hyperglycemia inhibits neutrophil function (Plosker et al, 2004). In a study the evaluated neutrophil killing of *C. albicans* in the presence of increased concentration of glucose. Results showed that 50mmol/l (900 mg/dl) of glucose decreased oxidative killing of *C. albicans* by neutrophils (Siebenhofer et al, 2004).

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* (Siebenhofer et al, 2004).

1.6.2 Studies on vulvovaginal candidiasis from India

Vulvovaginal candidiasis is not a reportable disease and because there are no active surveillance studies in India. There are very few published studies on vulvovaginal candidiasis (Table 1.5) 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 by different factors: recall bias, accuracy of diagnosis, patient selection, referral bias and confounding affects of widespread local antifungal.

1.7 Neonatal candidiasis

1.7.1 Overview

Invasive candidiasis in neonates is a serious and relatively common cause of late onset sepsis associated with a high mortality. A review of the first 26 cases reported in the English literature up to 1984 reported a mortality rate of 54% (Clerihew et al, 2007). More recent data from the
Table 1.3  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
Table 1.4  Reports of vulvovaginal candidiasis from Indian patients over the year (1961-2011).

<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 Diabetic</td>
<td>2000</td>
<td>78</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>2009</td>
<td>1050</td>
<td>20.47</td>
<td>46.9</td>
</tr>
<tr>
<td>Present study</td>
<td>2010-11</td>
<td>80</td>
<td>30</td>
<td>39.5</td>
</tr>
</tbody>
</table>
NICHD Neonatal Research Network centers reported a death rate of 28.1% in the neonates with fungemia (Stoll & Hansen, 2003).

Premature neonates and particularly low birth weight infants require invasive diagnostic and aggressive therapeutic interventions, many of which increase the risk factors for developing *Candida* infections. In addition the immaturity of the immune system especially among preterm neonates, which mainly involves T-cells and neutrophils, further predisposes this population to infections (Stoll *et al*, 2002). Indeed, the anti-*Candida* activity of lung macrophages in neonates has been shown to be reduced (Stoll & Hansen, 2003).

Neonatal candidiasis can be subdivided into two categories: (i) Catheter-related candidemia (ii) Disseminated or invasive candidiasis

Catheter-related candidemia refers to infants with central vascular catheters in place and candidemia that resolves rapidly after catheter removal and initiation of therapy. Disseminated or invasive candidiasis refers to persistent candidemia if a catheter was in place and removed, and/or the isolation of *Candida* from other normally sterile body sites. However, as in adults, there are no clinical criteria or diagnostic tests that allow a differentiation of these two groups at presentation (Butler *et al*, 1990). Up to 75% of cases of neonatal candidiasis present with infection of two or more organs. Unifocal osteomyelitis, meningitis, and renal candidiasis are the most common presentations, otherwise infection of any combination of blood, kidneys, meninges, heart, eye, (Fanaroff *et al*, 1998).

A distinctive form of cutaneous candidiasis that may become invasive and that is known as Congenital Candidiasis is discussed separately below.

### 1.7.2 Epidemiology

The incidence of invasive candidiasis in neonatal care units ranges between 1.6 and 4.5% (Sarkar *et al*, 2006). More recent data from the NICHD Neonatal Research Network centers on late-onset sepsis in very low birth weight neonates, has reported that fungal pathogens explain 9% of all bloodstream infections (Bliss *et al*, 2008). Neonates may acquire *Candida* by vertical or nosocomial transmission (Tiraboschi *et al*, 2007). In vertical transmission, acquisition may occur either during gestation or at the time of delivery. In both cases an ascending route from the mother's vagina is
involved. Vaginal candidiasis occurs frequently among pregnant women, especially in the last trimester. Rates as high as 56% have been reported (Ariff et al, 2011). Candida carriage rates among neonates vary between 30 to 60% (Baş et al, 2011). Interestingly as gestational age falls, the rates of candidal colonization appear to rise (Roilides, 2011). Acquiring Candida does not always translate into systemic infection, but previous colonization is a required step before the step of occurrence of invasive candidiasis.

Candidal nosocomial acquisition is being recognized as a very important form of transmission in the neonatal ICU (NICU) (Kristóf et al, 2010). Indeed, important outbreaks of candidemia in NICU have been identified (Roilides, 2011). Such colonization is actually a general phenomenon: hand carriage rates among health workers in seven NICU across the USA have been found to be of around 30% (Roilides, 2011).

1.7.3 Neonatal Candidiasis and Candida species

Initial reports on neonatal candidiasis consistently found Candida albicans to be responsible for the majority of cases (Dutta & Palazzi, 2011). However, similar to change of the epidemiologic patterns that has been observed in adults, a changing spectrum of species is being noted among neonates. This change is characterized by a progressive decrease in the rate of isolation of Candida albicans and an emergence of non-albicans species (Dimopoulos et al, 2008).

Unlike the situation with adults however, it is Candida parapsilosis that is becoming the most prevalent species. In some centers it has replaced C. albicans as the most frequent species (Lin et al, 2005).

1.7.4 Clinical manifestations

The classic clinical picture of systemic candidiasis in neonates is indistinguishable from bacterial sepsis (Badran et al, 2008).

All of the following signs may be seen: Temperature instability, hypotension, respiratory deterioration and apnea, abdominal distension, guaiac positive stools, carbohydrate intolerance. Among these, respiratory dysfunction and apnea were the most common presenting signs in large series, being present in about 70% of cases (Badran et al, 2008).
significant proportion of neonates will present simultaneously with localized signs of candidal infection at one or more other sites:

### 1.8 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 (Bouza & Muñoz, 2008). 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 1.8). They can be classified in to five groups on the basis of their molecular mechanism of action; their structure and mode of actions have been described in figure 1.9 and table 1.6 respectively.

#### 1.8.1 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.
Fig 1.8 Structures of antifungals used in Candidiasis treatment
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Adapted from Anaissie et al, 2003

Fig 1.9 Site and mechanism of action of different classes of antifungals on a typical fungal cell.
## Table 1.5  Antifungals and their mode of action.

<table>
<thead>
<tr>
<th>Antifungal</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>Polysporin</td>
<td>Amphotericin B</td>
<td>0.3–1.5 mg/kg/day</td>
<td>Interaction with ergosterol, elevation of fungal membrane permeability, cell death</td>
<td>Primary resistance may be seen in <em>Pseudoallescheria boydii</em>, <em>Geotrichum candidum</em> spp. <em>Aspergillus</em> spp., <em>Rhodotorula</em> spp., and <em>Candida</em> spp.</td>
</tr>
<tr>
<td>Liposomal</td>
<td>Amphotericin B</td>
<td>0.25–4.0 mg/kg/day</td>
<td>Similar to amphotericin B</td>
<td>Similar to liposomal formulations of amphotericin B</td>
</tr>
<tr>
<td>Fluconazole</td>
<td></td>
<td>100 mg/ day orally divided every 6 h</td>
<td>Similar to itraconazole but more selective inhibition of fungal sterol synthesis</td>
<td>Active against Candida and Cryptococcus spp. Primary antifungal resistance common among mold species, particularly Aspergillus spp. Rapid emergence of resistance in <em>Candida</em> and <em>Cryptococcus</em> restricts use of GRTs in monotherapy</td>
</tr>
<tr>
<td>Itraconazole</td>
<td></td>
<td>200 mg orally once a day</td>
<td>Inhibition of cytochrome P450 14alpha-demethylase, accumulation of sterols leading to perturbation of fungal cell membrane</td>
<td>Poor oral absorption and drug interactions are common reasons for clinical resistance. Interference with human steroid biosynthesis seen</td>
</tr>
<tr>
<td>Vansolan</td>
<td></td>
<td>200 mg/ intravenously every 24 h</td>
<td>Similar to itraconazole, but less selective inhibition of fungal sterol synthesis</td>
<td>Active against C. albicans and <em>Cryptococcus</em> spp. No activity against invasive molds. Primary resistance also common with less invasive <em>Candida</em> spp. and less invasive <em>C. albicans</em> in patients with immunosuppression</td>
</tr>
<tr>
<td>Posaconazole</td>
<td></td>
<td>200 mg/ intravenously every 24 h</td>
<td>Improved activity over fluconazole against invasive molds. As with fluconazole, drug interactions and poor absorption are common causes of clinical resistance. Marked interpatient variability in plasma concentrations secondary to variation in CYP3A genotypes affecting drug metabolism. Resistance may be seen with Posaconazole</td>
<td>More active than itraconazole against invasive molds, including <em>Aspergillus</em> and <em>Fusarium</em> spp. Not active against <em>Zygomycetes</em>. Cross-resistance with fluconazole? Multiple independent variations in serum concentrations secondary to variation in P450 genotypes affecting drug metabolism.</td>
</tr>
<tr>
<td>Echinocandins</td>
<td></td>
<td>70 mg/ intravenously day 1, then 50 mg daily every 24 h</td>
<td>Inhibition of cell wall glycosyl synthetase leading to lytic death in fungal cell</td>
<td>Rapidly fungicidal against <em>Candida</em> spp. Including endophytic resistant species. Excretion essentially limited to <em>Fusarium</em> spp. No activity against <em>Aspergillus</em> spp. Unlikely to be used as monotherapy for any antifungal agent</td>
</tr>
<tr>
<td>Anfotericin B</td>
<td></td>
<td>350 mg orally every day</td>
<td>Inhibition of respiratory enzymes in ergosteryl and ergosterol synthesis</td>
<td>Poor intrinsic activity against common yeast and mold species. Used as monotherapy, particularly in combination with azoles in the treatment of early-stage endocarditis and infections</td>
</tr>
</tbody>
</table>

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*Note: The table provides an overview of various antifungal agents and their mechanisms of action, along with their clinical applications and potential resistance profiles.*
Table 1.6 Antifungals and date of release 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>
(Barrett-Bee & Dixon, 1995). These synthetic compounds are divided into three different subgroups: (i) azole antifungal agents, (ii) allylamines, and (iii) morpholine derivatives.

### 1.8.2 Azoles

The primary mode of action of these agents has been demonstrated to be the inhibition of sterol biosynthesis (Oldfield, 1999) through the selective inhibition of P450 14α-demethylase (P450_{14αdm}) (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 P45014αDM.

### 1.8.3 Antifungal drug resistance

There are basically two clinical types of resistance: (i) innate and (ii) acquired. The emerging of yeast isolates resistant to azole antifungal agents, especially fluconazole is matter of concern (Balkis et al, 2002). The mechanisms of antifungal drug resistance included number of factors as explained in the following sections. (Figure 1.10) presents the mechanisms by which a *C. albicans* cell might develop resistance.

#### 1.8.3.1 Clinical factors

Over the last several decades, aggressive anticancer chemotherapy has resulted in a population of patients that are increasingly exposed toazole drugs for antifungal treatment and prophylaxis. (Jaworski et al, 2011). Figure 1.11 summarizes the clinical factors involved in the development of resistance. The factors, which contribute to the resistance, are summarized below.

- Change to a more resistant species of *Candida* e.g replacement by *C. krusei* or *C. glabrata*
- 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 and Cellular mechanisms of drug resistance
Fig 1.10  Mechanisms by which a C. albicans cell might develop resistance

1. The entry of drug is prevented at the cell wall/ cell membrane level
2. The drug is pumped out by efflux pumps which are over-expressed in presences 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
Fig 1.11 Factors which may contribute to clinical resistance
1.8.3.2 Molecular mechanisms of development of drug resistance

Several different mechanisms may be responsible for the development of drug resistance in \textit{C. albicans}. (Kontoyiannis \& Lewis, 2002) demonstrated that many fluconazole-resistant, clinical \textit{C. albicans} isolates displayed strongly increased mRNA levels of \textit{CDR1} or \textit{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. **Alterations in the sterol biosynthesis pathway**

   Inhibition of 14\(\alpha\)-DM by fluconazole not only results in ergosterol depletion but also in the accumulation of the methylated sterol 14\(\alpha\)-methylergosta-8, 24 (28)-dien-3\(\beta\), 6\(\alpha\)-diol, which inhibits cell growth (Balkis \textit{et al}, 2002).

2. **Mutations in the \textit{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. **Over expression of the \textit{ERG11} gene**

   In the presence of fluconazole, \textit{C. albicans} upregulates the \textit{ERG11} gene, presumably as a feedback mechanism to make up for ergosterol depletion (White \textit{et al}, 2002). (Vanden Bossche \textit{et al}, 1998) reported that even in the absence of fluconazole some fluconazole-resistant isolates express \textit{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 \textit{C. albicans}. (White \textit{et al}, 1998) have shown that the exposure of \textit{C. albicans} cells to fluconazole resulted in the non-disjunction of two specific chromosomes in drug resistant mutants.

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.

1.9 Combination antifungal therapy

High morbidity and mortality persist for systemic fungal infections due to pathogenic yeast (White et al, 1998). 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. (Table1.10) shows the efficacy of drug combination against Candida species. 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 (White et al, 1998).

1.10 Antifungal susceptibility testing

The method described in M27-A2 of the NCCLS is intended for testing yeasts that cause infections. 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 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.

1.11 Molecular typing of fungal strains

DNA fingerprinting techniques are likely to answer the following questions:

- To understand the dynamic of an infection.
- To verify the relationship between commensalisms and infection.
- Identify the origin of an infection.
- Monitor the emergence of drug-resistant strains.
- Determine if specific strains are more likely to cause disease.
Recognize if more than one strain is causing infection and their relative proportions.

Identify if relapse of infection is due to the same or different organisms.

The molecular methods for epidemiologic typing used are as summarized in (Table 1.9).

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 3) 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 measurers of relatedness among moderately-related strains. Dendrone is a computer-assisted which was developed for analyzing and comparing DNA fingerprinting patterns. DNA fingerprinting of the infectious fungi has becomes 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 (Lattif, 2000). DNA fingerprinting is required to understand: (a) the dynamics of an infectious organism in human population, (b) the complex relationship between commensalisms and infection, (c) identify the origin of an infection and (d) monitor the emergence of drug resistant strains.

The most common methods to DNA fingerprint the infectious fungi are:

(1) **Multilocus enzyme electrophoresis.**
(2) Restriction fragment length polymorphism without hybridization.
(3) Restriction fragment length polymorphism with hybridization probes.
(4) Random amplified polymorphic DNA and related PCR-Based fingerprinting methods.
(5) Electrophoretic karyotyping.

1.11.1 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 shortcomings of one method may be overcome by the other one, but at the same time it may have its own shortcomings. Still the desirable characteristics of an ideal method are: identifying the same strain in independent sets of isolates, identify micro evolutionary changes in a strain, cluster moderately related strains, identify unrelated isolates and should be resistant to homoplasy (i.e. convergence of karyotyping patterns without a common ancestor).

The methods currently used for DNA fingerprint of infectious fungi are:

1.11.1.1 Restriction fragment length polymorphism (RFLP).

RFLP without probe hybridization has been applied to a variety of infectious fungi including *C. albicans* (Shin, 2005). RFLP is one of the earliest molecular techniques developed to compare inters-train relationships between microbial strains, including a wide variety of fungal species, was restriction enzyme analysis. This 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.
Table 1.7  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 (Restriction fragment length polymorphism)</td>
<td>Candida species, Aspergillus species, Cryptococcus neoformans, Trichsporon begelii, Histoplasma capsulatum</td>
</tr>
<tr>
<td>Restriction endonulease analysis genomic analysis of genomic DNA</td>
<td>Candida species, Aspergillus species, Malassezia species, H. capsulatum</td>
</tr>
<tr>
<td>Pulsed field del electrophoresis Electrophoretic karyotypic restriction Endonuclease digestion with rare cutters</td>
<td>Candida species, C. neoformans</td>
</tr>
<tr>
<td>Polymerase Chain reaction fingerprinting</td>
<td>Candida species, Aspergillus species, C. neoformans, H. capsulatum, Pneumocystis carinii</td>
</tr>
<tr>
<td>Protein-based methods Immunoblot fingerprinting</td>
<td>Candida species, Aspergillus species</td>
</tr>
<tr>
<td>Polycrylamide gel electrophoresis of Cellular proteins</td>
<td>Candida species</td>
</tr>
<tr>
<td>Multilocus enzyme electrophoresis</td>
<td>Candida species, C. neoformans</td>
</tr>
</tbody>
</table>
1.11.1.2 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 a repetitive elements. 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 (Lattif, 2011). The DNA fingerprinting pattern is amenable to computer-assisted analysis.

1.11.1.3 Microsatellite typing by using oligonucleotide probes

A variation of the previous methods is use of radiolabeled 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 microstellite sequences. Micorsatellite are 1-6 bp tandem repeats, scattered randomly through the genome of the eukaryotic organisms including fungi.

1.11.1.4 Random amplification of polymorphic DNA (RAPD)

RAPD analysis depend 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 variously; randomly amplified polymorphic DNA (RAPD), arbitrarily primed PCR (Ap-PCR) (Lattif, 2011), differentially amplified fragments (DAFs), and amplified fragment length polymorphisms (AFLPs) (Lattif, 2011). 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 developed into sequence-confirmed amplified fragments (Lattif, 2011), which allow 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* (Lattif, 2011), *C. dubliniensis* (Lattif, 2011), *C. parapsilosis* (Lattif, 2011), *C. tropicalis* (Lattif, 2011) and for *C. glabrata* (Lattif, 2011). 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 (Lattif, 2011).

1.11.1.5 Multilocus enzyme electrophoresis (MLEE)

This method identifies allelic polymorphisms that lead to altered electrophoresis mobility of proteins in polyarylamide gels. MLEE has been successfully used in population studies of fungi including *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *Cryptococcus neoformans* (Boriollo *et al*, 2010).

1.12 The computer assisted DENDRON

DENDRON is a computer assisted system (Figure 1.13) which was developed for analyzing and comparing DNA fingerprinting patterns. In the DENDRONE system, an autoradiogram or fluorogram of a fingerprinting gel containing a number of lanes, each with visible complex banding pattern, is scanned into the DENDRONE 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 dendrograms 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.

### 1.13 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. Hydrolytic enzymes are probable virulence factors in pathogenic Candida species (Lattif, 2011). Evidence has been presented that phospholipase B, expressed by at least two genes (PLB1 and PLB2) (Lattif, 2011) also contributes to the pathogenesis of candidiasis by the degradation of host tissues (Lattif, 2011).

#### 1.13.1 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.

The frequency of Candida infection has risen drastically in the recent decades due to the increasing number of the immunocompromised patients (Ramírez et al, 2009). Recently the glyoxylate cycle was found to be required for virulence of the plant pathogenic fungus Magnaporthe grisea (Ramírez et al, 2009). Acetyl-coenzyme A can only be assimilated through glyoxylate cycle, which bypasses the catabolic steps of the tricarboxylic acid cycle (Figure 1.14) in which the two carbon atoms are lost as CO2 in mammalian systems. Thus the glyoxylate cycle is the only route for the synthesis of glucose in this environment.

### 1.14 Identification and diagnosis of the yeasts

In a clinical mycology laboratory, yeasts are always identified by a combination of morphological and biochemical criteria.

#### 1.14.1 Criteria for Identification and diagnosis of yeasts
Fig 1.12  The DENDRON system.
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;

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) Assimilation of nitrates;
(iii) Fermentation of sugars.

(iv) 1.14.2. Direct identification of Candida species using differential media

(v) 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 (Figure 1.14).

(vi) 1.15. PCR-based identification of pathogenic *Candida* species

(vii) 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)

(viii) 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.

1.15.1 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 (Lattif, 2011) 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 (Lattif, 2011).

Fig 1.13  the metabolic pathway of glyoxilate cycle
Fig 1.14 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.

Adopted and modified from Anaissie *et al*, 2003
1.16 Objective:

In view of the present background we initiated our study with the following objectives

1. To look the incidence and distribution of *Candida albicans* and non-albicans species as etiological agent of vaginitis in pregnant and non-pregnant women in North India.

2. To look the prevalence of Candida species and potential risk factors for vulvovaginal candidiasis in North India.

3. To look the Molecular Epidemiology of Invasive Candidiasis and antifungal susceptibilities of Candida spp. to antifungal agents in Neonates: A NICU experience from Northern India.

4. To understand the molecular epidemiology of the occurrence of Candida in full term neonates and their mothers: a concordance study.

5. To understand the mode of transmission of Candida albicans in extremely low birth weight (ELBW) infants.