2.0 REVIEW OF LITERATURE

In this chapter, the literature pertinent to the present study has been reviewed.

2.1 Background

The first known case of candidal infection as _oral thrush_ was described in a book —Epidemicsl by Hippocrates in fourth century (Chander, 1996). Oral Thrush is a type of fungal infection (mycosis) caused by _Candida_ species on mucous membrane of mouth (Walsh and Dixon 1996). Candidiasis is also technically known as candidosis, moniliasis, and oidiomycosis (James et al., 2006). _Candida albicans_ is the leading pathogen in oral infection but other _Candida_ species are also associated with this condition. The invasive _Candida_ infections are increasing in aging people (Ten Cate et al., 2009).

The incidence of candidiasis depends upon the immune status of individuals. It is higher in immunocompromised hosts; concurrent infections with other organisms like HIV have been reported. Besides causing infections in humans, _Candida_ species cause infections in animals also (Smith, 1967, Foley and Schlafer 1987). The majority of such cases found in pigs where invasion of the epithelium in the oesophageal area was demonstrated. Also, _Candida_ species are responsible for abortion in cattle, crop mycosis in broiler chickens (Wyatt and Hamilton 1975).

The ability of _C. albicans_ to show alteration in cell morphology increases its virulence in opportunistic infections (Hogan et al., 2004). It possesses certain virulence factors such as adherence to endothelial and epithelial cells, pseudohyphae formation, phenotypic switching, production of proteinases and modification of antigen make up etc. _Candida albicans_ and _Candida tropicalis_ are known to produce aspartyl proteinase which also contribute to their pathogenicity (Kuleta et al., 2009). Several antifungal drugs such as polyenes, azoles, allylamines and echinocandins are used in treatment of fungal infections. Among azoles, first generation trizoles such as fluconazole is commonly used in treatment of superficial and invasive fungal infections (Chen et al., 2007). The resistance to antifungal agents have been reported in recent years and continues to grow and evolve consequently complicating the management of the patients. Various mechanisms may are involved in the resistance development process (Pfaller et al., 2012). However not a single mechanism is responsible for development of drug resistance in _Candida_ species.

_Candida_ species are leading pathogens in blood stream infections, about 27% of blood stream infections are caused by _C. albicans_ (Harriott et al., 2009). Also, _Candida_ species have been reported as the fourth most common cause of hospital acquired bloodstream infections in United States and Scotland (Chander 1996).
In India, *Candida* species are major pathogens among fungal infections (Rani *et al.*, 2002), with *Candida albicans* being the most predominant species. However, other *Candida* species such as *C. glabrata, C. parapsilosis and C. tropicalis* have been implicated in candidasis (Chakrabrati *et al.*, 1992). *Candida* has been emerged as 7th most common pathogen associated with the nosocomial infections (Chander, 1996). In Taiwan, *Candida tropicalis* is the main organism isolated from various hospitals (Rani *et al.*, 2002). Recently, *Candida dubliniensis* has been described as one of non *Candida albicans* species associated with oral candidiasis in HIV infected patients (Morgan *et al.*, 1998).

### 2.2 Clinical manifestations of Candidiasis

*Candida* species are associated with oropharyngeal Candidiasis in HIV and AIDS patients (Morgan *et al.*, 1998). The other forms of the candidiasis are; superficial candidiasis, deep candidiasis, and disseminated candidiasis in neonates, vulvovaginal candidiasis in post pubertal women and chronic disseminated candidiasis in patient with severe neutropenia (Kwon-Chung *et al.*, 1992).

Nosocomial infections are the main threats in present time, therefore, proper management of such infections is required (Ruhnke *et al.*, 2000, Odds *et al.*, 2007) and *Candida* species are considered as the main fungal pathogens affecting individuals with reduced immunity mainly in HIV infected and AIDS patients (Morgan *et al.*, 1998, Mishra *et al.*, 2007). In such patients, mainly the oropharyngeal Candidiasis is the most common condition. Chakrabarti and co-workers (1992), studied the incidence of candidaemia in hospital in India over a ten years period. *Candida* species were frequently isolated during the study from blood culture, in which 50% strains were *Candida albicans*, followed by *C. guilliermondii* (17%), *C. tropicalis* (15%) and *C. parapsilosis* (8%). The increased level of candidaemia was reported in patients with varying symptoms. Rani and co-workers (2002) reported high prevalence of *Candida* species in the blood of neonates in the northern region of India as they found 31.71% blood samples culture positive. In the hospital studies, these workers observed that *Candida tropicalis* was predominant organism in neonates in 92% cases followed by *Candida albicans* and *Candida kefyr* which constituted only 4% of total cases.

Bassetti and co-workers (2006), conducted a study on Candidaemia, of the total 182 cases examined, 40% were due to *C. albicans*, 23% due to *C. parapsilosis*, 15% *C. glabrata* and 9% due to *C. tropicalis* in Geneva. This study shows the high involvement of *Candida albicans* and other non *Candida albicans* species with *Candidaemia* in hospital environment.
Yang and co-workers 2008, studied patient of candidasis from nine hospitals in Taiwan and determined their susceptibility to fluconazole. It was concluded from the study that the increase in the nosocomial Candida associated infections were due to emergence of drug resistance to fluconazole and other antifungals drugs. Fluconazole resistance was observed during the study in 35 isolates out of 88 patients treated. The percent distribution of resistance to fluconazole in total resistant isolates was 46.5% C. tropicalis, C. albicans 36.8% and C. glabrata 30.8% respectively. Another clinical condition, endocarditis has been reported due to increased use of prosthetic intravascular devices although it was considered as a rare disease earlier. This condition is seen during different stages such as (i) during the intensive care unit stay and (ii) transitory candidaemia after surgery occurs which leads to colonization of the prosthetic valve sites and biofilm formation. Also, alterations of the mental status of the individuals lead to candidaemia-associated sepsis syndrome. Candida is frequently isolated in nosocomial nonpostneurosurgery spondylodiscitis (Venditti et al., 2009). However, it is infrequently associated with osteomyelitis.

2.3 Morphology of Candida species

As mentioned earlier in the chapter 1.0 on introduction, there are 163 known species of Candida and about 20 of them are associated with various types of infections in human. Different Candida species possess different phenotypic traits on the basis of which they can be differentiated from each other, when grown on different selective and differential media. On Sabroaud’s Dextrose agar (SDA), Candida albicans produces white to cream colored, smooth and soft colonies, whereas Candida tropicalis, C. glabrata, and C. guilliermondii produce white to cream colored, C. parapsilosis cream to yellowish colonies.

On Hicrome Candida differential agar, Candida albicans produces apple green colonies, Candida tropicalis dull blue to purple colonies that diffuse into the surrounding agar with pale pink edge. C. guilliermondii produce small pink to purple colonies while C. glabrata form white, large glossy, pale pink to violet colonies and C. parapsilosis white to pale pink colonies.

Candida albicans is capable of forming a wide range of polarized and expanded cellular morphology ranging from pseudohyphae to true nonconstricted hyphae. Filamentous form of this fungus consists of uninucleated compartments partitioned by septa (Gow, 1997).
The cell wall is essential part of organisms in every aspect of the biology and pathogenicity. Recent studies on cell wall have revealed that it is a very useful organelle. The major components of Candida cell wall are glucan and chitin which are associated with structural rigidity. In addition to these, mannan is the most abundant component. Glucan polymers constitute cell wall of C. albicans and are less active. The protein portion of cell wall including mannoprotein and nonmannoproteins, consist of 40 or more moieties. The role of these cell wall proteins is very important in adhesion to host tissues (Lajean et al., 1998). The serological determinants in this organism have been determined by agglutination reactions against the antisera raised against cell surface components. Two main group of antigens; cell surface and cytoplasmic antigens have been observed. The cell wall is not antigenically stable and shows variable response under in vitro and in vivo growth conditions. The lysed cells also contain significant antigenic components. The glycoprotein extracts of cell wall of this organism are lethal, pyrogenic and behave much like bacterial endotoxins (Kwon-Chung et al., 1992).

The antigenic structure of Candida has been studied in detail to understand pathogenesis of candidal infections and their diagnosis. Two major serogroups A or B of Candida albicans strains have been reported, of which serotype A is epidemiologically important. The distributions of these serotypes groups can vary depending on the geographic origin of the isolates. Recently, the occurrence of serotype B has been shown on the increase in candidasis (Stephane et al., 1996).

2.3.1 Germ tube formation

The change of C. albicans from commensal form to the pathogenic is strongly related with its ability to reflect dimorphic nature and conversion from the yeast to the hyphal (germ tube) form (Vardar-Unlu et al., 1998). Germ tube formation in Candida albicans is partially controlled by product released by cells during the yeast phase of this fungus on incubation at 37°C in tissue culture medium or fetal calf serum. This germ tube regulatory substance is stable under lyophilization and heating upto 70°C, but inactivated at pH of 4.0 and 9.5. Production of this factor does not appear to be a universal characteristic of yeasts as this factor could not be found from either Cryptococcus laurentii or Candida parapsilosis (Hazen et al., 1979).
2.3.2 Chlamydospore formation

The chlamydospore production is a distinctive feature of the fungus *Candida albicans*. However, it does not occur in model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. This feature has been used for a long time for the characterization of *Candida albicans*. Chlamydospores are presumably responsible for the survival of this fungus under harsh environmental conditions, but this assumption needs proof. Although the Chlamydospores do not contribute significantly in the pathogenesis of *Candida* infections as limited studies have been carried out to demonstrate their function. The advances of molecular techniques and the recent discovery of mating in *C. albicans* have stimulated the studies to understand the structure and function of these spores (Staib et al., 2007). Little information is available about the pathways that control their development, their formation is genetically controlled. A total of six genes; *ISW2*, *MDS3*, *RIM13*, *RIM101*, *SCH9* and *SUV3* – are needed for effective chlamydospore formation. Mutations in *ISW2*, *SCH9* and *SUV3* genes may completely abolish chlamydospore formation. However, their formation is delayed if mutations occur in other three genes; *RIM13*, *RIM101* and *MDS3* genes (Nobile et al., 2006).

2.3.3 Pseudohyphae and hypha formation

An important feature of *Candida* biology is its ability to grow as yeast, pseudohyphal and hyphal forms. The altering morphology is linked to the capacity of this organism to cause disease. The hyphal form of this fungus has a significant role in causing disease by penetrating epithelial cells, consequently damaging the tissue (Pappas et al., 2011). Pseudohyphal form has been regarded as a morphological growth form that exhibits characteristics distinct from budding yeast phase and hyphal form of this fungus. The pseudohyphae exhibit unipolar cell divisions (Veronica et al., 2009). Transcription factors Efg1p and Cph1p control transcription required for hyphal growth of *C. albicans* (Ian et al., 2010).

2.4 Genome of *Candida albicans* and other *Candida* species

The size of *Candida albicans* is about 15.6 Mb. Nucleotide sequencing of the whole genome carried out at Stanford University was assembled into 412 contigs. Sequence analysis are complicated due to presence of repeated sequence and polymorphism between homologous chromosomes. Hiroji and co-workers (2005) assigned 16 of contigs, ranging in length from 7309 to 267,590 bp to chromosome 7.
The nucleotide sequence of 16 regions was determined. Sequence analysis was carried out and 404 genes have been predicted, 11 of which included at least one intron. The *Candida* Genome Database (CGD) is now playing an important role in maintaining up-to-date version of the genome sequence (Martha *et al.*, 2007).

Fitzpatrick and co-workers (2010) developed the *Candida* Gene Order Browser (CGOB) which is an online tool that helps to compose different *Candida* species. This browser contains all available genome sequences of *Candida albicans* (SC5314 and WO-1), *Candida dubliniensis*, *Candida tropicalis*, *Candida parapsilosis*, *Lodderomyces elongisporus*, *Debaryomyces hansenii*, *Pichia stipitis*, *Candida guilliermondii* and *Candida lusitaniae*.

Jackson and co-workers (2009) sequenced the genome of *C. dubliniensis* and compared it with the known *C. albicans* genome sequence. The genome sequences of these two organisms are similar and conserved throughout, 168 species-specific genes were identified, which includes some known gene encoding hyphal-specific virulence factors. Among 115 pseudogenes confirmed in *C. dubliniensis* are orthologs belonging to other filamentous growth regulator (FGR) genes. Their studies suggest that the recent evolutionary history of *C. albicans* and *C. dubliniensis* varies significantly. While gene families corresponding to pathogenesis are more elaborated in *C. albicans* (Fig 2.1) as compared to *C. dubliniensis*, the later has lost genomic capacity and important pathogenic functions. This might explain why *C. albicans* is more pathogenic than *C. dubliniensis* (Jackson *et al.*, 2009).

**Fig 2.1** Graphical representation of protein coding genes in *Candida albicans*SC5314
(Adapted from *Candida* genome database)
2.5 Epidemiology of candidiasis

The incidence of oral, vaginal and oesophageal candidosis occur mostly in patients with HIV infection. The occurrence of oesophageal candidosis varies between subgroups of the HIVinfected individuals. Most of candidal infections in AIDS patients are caused by the *C. albicans*. Also, some surveys reveals an increased prevalence of *C. albicans* serotype B strains in AIDS patients (Odds *et al.*, 1990). As discussed earlier in section 2.3 under morphology, two serogroups have been identified on the basis of antigenic make up.

Invasive candidiasis is a condition of major medical and clinical importance. Its occurrence increased over the past 50 years, requiring standards of medical care. The global disease load of invasive candidal infections is difficult to quantify because of geographical differences. The involvement of non-albicans *Candida* spp. to invasive infection is increasing. Such infections continue to represent a major problem with increasing mortality and morbidity in the group of hospital patients (Hobson *et al.*, 2003).

There has been a change in the incidence and epidemiology of invasive fungal infections during past three decades (Giri *et al.*, 2012). This may be attributed to; increase in AIDS epidemic, increased number of population receiving immunosuppressive therapy and frequent use of antibiotics. The main risk factors for candidal infections include use of broad-spectrum antibiotics, cancer chemotherapy, colonization of sites by organism and increased use of indwelling vascular catheters like central venous catheters.

Eggimann *et al.*, 2003 observed that a significant proportion of patients became colonised with *Candida* sp. during their hospital stay. However, only few subsequently developed severe infection. Clinical outcomes of severe infection manifest very early but not specific until late in the course of the disease, thus making diagnosis difficult. Nosocomial invasive candidiasis occurs in 1-8% of individuals admitted in hospitals.

The rate of blood-stream candidal infections has increased by almost 500% in hospitalized patients since 1980s (Pfaller *et al.*, 1995). This increase is due to increased mortality and a long stay in the hospital. In the US, *Candida* spp. remains the fourth most common bloodstream agent leading to 8% of all nosocomial blood-stream infections. One-third of candidal blood-stream infections are caused by non *albicans Candida* species. The most of such infections arise from an endogenous colonization.
Nosocomial transmission or 'crossinfection' and the development of resistance to antifungal agents present new and significant problems. Recent studies suggest that in the intensive care unit setting, *Candida* may be isolated from the hands 15-54% of healthcare workers. The strains of *Candida* left on the hands may be shared among infected patients. Molecular typing and epidemiological investigations suggest that cross-infection of *Candida* species is an important aspect of candidal blood-stream infection.

Phillips and co-workers (1997), conducted retrospective study of hospital-acquired urinary tract infections (UTI) occurring in neonates admitted to a neonatal intensive care unit between 1989-1995. In this study, *Candida* spp. were associated with 25 of 60 (42%) UTI. The candidemia was found in 13 of 25 (52%) candidal UTI cases which was more often than bacteremia. Renal fungus balls were present in 35% of infants with candidal UTI.

Yap and co-workers (2009) studied episodes of candidaemia in intensive care unit at Hong Kong during the 9 years of the study period. They observed 128 patients with episodes of candidaemia in which 72 had albicans candidaemia and 56 non-albicans candidaemia. They also observed increase in the incidence of *C. tropicalis*. Fluconazole and amphotericin B were used for treatment of such infections but only 89 (70%) of the patients received appropriate anti-fungal treatment. Their results reveal a high prevalence of candidaemia in the intensive care unit (ICU), appearing during early part of the stay. Candidaemia cases in these units may be associated with high morbidity and mortality. An increase in the proportion of device-associated candidal infections has also been observed by Kojic *et al.*, (2004). Production of biofilms on synthetic materials by *Candida* spp, help in adherence of this fungus on devices. Treatment of device-associated candidal infections can be more challenging to medical personnel. Prompt removal of the infected device is required to initiate treatment of such infections which have a great medical and economic impact.

### 2.6 Pathogenesis of candidial infections

*Candida* species cause vaginitis, oropharyngeal infection, thrush, candidemia, urogenital tract infections and serious systemic infections in immunocompromised patients. Infections of skin and superficial mucosal sites result as a consequence of battle between fungal virulence and host defence
Host express epidermal proliferation and T-cell immune responses to prevent fungal invasion, besides, inflammatory responses and other nonspecific inhibitors also play significant role. *Candida albicans* express mainly three types of surface adhesion molecules to colonize epithelial surfaces. The enzyme aspartyl proteinase facilitates initial invasion of cells. Deeper invasion of keratinized epithelia is enhanced by hypha formation. Proteinase and phospholipase enzyme production are regarded as virulence factors possessed by the *Candida* species responsible for catheter related candidemia in intensive care unit (ICU) patients having indwelling devices. Vinitha and co-workers (2008), found 74.56% of the isolates recovered from blood while 44.73% produced phospholipase enzymes.

Pathogenesis of *Candida* species requires expression of various virulence factors at each step of the process. Rapid alteration of the phenotype in *C. albicans* may be a significant factor in high pathogenicity of this species (Odds *et al.*, 1994). Most of the clinical manifestations in Candidal infections are due to the biofilm formation by this organism. The cells of this fungus have dramatic biofilm forming ability. Biofilm formation may be a key factor for long term survival of *Candida* species (Chander, 1996).

Waleed and co-workers (2007) demonstrated that the genome of *Candida albicans* contains many short sequence repeats (SSRs) in genome. These are stable upon transition of colonization to infection in immuno-compromised patients. In non-neutropenic patients this transition may coincide with variation in several of the short sequence repeats (SSRs).

### 2.7 Virulence factors of *Candida* species

*Candida* species have some virulence factors that contribute to pathogenesis. Production of secreted aspartyl proteases and phospholipases, other host recognition receptors (adhesins) and morphological dimorphism are major virulence factors. Beside these factors, phenotypic switching is induced by changes in antigen structure, colony morphology contribute to virulence. Tissue tropism is another factor which also contribute to virulence which results in cell flexibility and may lead to adaptation of the organism to the host environment (Calderone and Fonzi 2001).

*Candida* species are known to produce certain extracellular hydrolytic enzymes. Secreted aspartyl proteinases (Saps), which directly contribute to *C. albicans* pathogenicity by degrading host cell proteins (Mardegan *et al.*, 2006).

During recent years, non-*Candida albicans* Candida (NCAC) species such as *Candida glabrata*, *Candida tropicalis* and *Candida parapsilosis* have been reported to produce biofilms and adhesion
to the tissues which contribute to their virulence (Silva et al., 2010). The drug resistant strains of Candida tend to be more pathogenic due to various biochemical and physiological changes occurring during the development of drug resistance. These changes may be due to alteration in composition of cell wall polysaccharides, increased and extensive hypha formation, increase in adherence to cells and increased biofilm formation by the drug resistant species (Naillis et al., 2010). Also, the expression of some proteins is related to the morphological growth form of the fungus and may also play a significant role in fungal morphogenesis (Lajean et al., 1998).

Production of hemolysin is another virulence factor of Candida species. Candida albicans shows hemolytic activity on glucose-enriched blood agar. This activity is present in the cells and secreted into the culture medium during the growth. Candida species produce a hemolytic factor which acquires iron from host erythrocytes resulting in hemolysis (Manns et al., 1994).

Most of the fungal pathogens of human can grow in more than one morphological form. Some of fungal pathogens produce filamentous hyphae at site of the infection. These include Candida albicans, C. dubliniensis, Malassezia spp. These opportunistic pathogens produce hyphae for invasion of the biotic or abiotic substrate which they adhere to. The hyphae also play some other important role such as translocation between host environments, colony consolidation and nutrient uptake (Brand, 2012).

2.7.1 Biofilm formation by Candida species

Candida species are most prevalent in oral cavity specially Candida albicans. These have high mortality rate in invasive infections. A number of virulence factors correspond to survival of these organisms such as attachment to surfaces and formation of biofilm (Ten Cate et al., 2009). Candida albicans causes infections often due to its ability to form biofilms (Naillis et al., 2010). Candida dubliniensis is another species, an opportunistic pathogen which has also been recently implicated in oropharyngeal candidiasis in human HIV. Most of clinical manifestations of candidiasis are associated with biofilm formation by these organisms. C. dubliniensis is known to form biofilms on the surfaces of biomaterials (polystyrene and acrylic) within 24 to 48 hrs. Biofilm formation by C. dubliniensis may help this species to maintain itself as a normal commensal and pathogenic to humans (Ramage et al., 2001). After adherence, growth, proliferation, the biofilms of C. dubliniensis at maturation are composed of a dense network of yeast cells and hyphal elements (Thein et al., 2007).

2.7.1.1 Possible Mechanisms of biofilm formation

There is synergistic degradation of certain substrates when bacteria form biofilms on fungal surfaces. More complex synergistic associations occur for the nutrient acquisition between bacteria and fungi.
The molecular mechanisms of these interactions are not well understood (Hogan et al., 2010). A wide arsenal of glycoproteins has been located at the outer surface of cell wall and secretion of signaling molecule in Candida species play an important role in biofilm formation. Other factors such as cell wall biosynthetic enzymes, cross linking enzymes and cell wall proteins are also involved in biofilm formation (Ten Cate et al., 2009). Candida albicans start the biofilm formation through three distinct developmental growth phases. These phases can transform adherent blastospores to a definitive cellular communities enclosed in a polysaccharide matrix. Biofilm formation by C. albicans have a highly heterogeneous architecture when examined under microscope, which is composed of cellular and noncellular elements (Chandra et al., 2001). Other factors that modulate the biofilm forming ability of C. albicans and non-albicans Candida species include; hydrodynamic conditions and ambient oxygen gradients for growth (Thein et al., 2007).

2.8 Antifungal agents
Four classes of antifungal drugs, polyenes, azoles, allylamines and echinocandins which are used in treatment of fungal infections. Among azoles, first generation trizoles such as fluconazole is used in treatment of superficial and invasive fungal infections (Chen et al., 2007). The current use of the available antifungal agents has been increased by 30% since the year 2000. Besides this increase in the availability of antifungal agents, only 15 agents got approval for clinical use. The therapeutic choices have been also increased; however, there is a great need of detailed knowledge of every antifungal agent for several reasons; differences in the mode of action of antifungal agent, bioavailability, chemical composition, interactions with other drugs and associated side effects (Thompson et al., 2009). The mechanism of action of these drugs and detail account of various types of antifungal agents used for treatment of Candidiasis is presented in Fig 2.2 and 2.3 respectively.

2.8.1 Polyenes
Amphotericin B (AMB) and nystatin are the most frequently used agents among polyenes. The application of Nystatin is limited to topical use because of certain side effects such as nausea, vomiting and diarrhea etc when given in higher doses (Ellis et al., 2002). The binding of this drug to cell membrane disrupts its permeability by forming oligo- dendromes forming pores with efflux of potassium and other intracellular molecules leading to fungal cell death (Ben-Ami et al., 2008). Amphotericin B is a broad spectrum polyene drug which was discovered in 1957. This drug has affinity for ergosterol and cholesterol. On application, it causes pores in the fungal cell wall resulting in the leakage of essential molecules. This drug has activity against most of fungal pathogens both
yeast like and mycelial type. Conventional amphotericin B and liposomal formulations of amphotericin B (Fungisome™) are used in the treatment of various fungal infections. In addition to their use in antifungal therapy, various forms of amphotericin B are also used in treating non fungal diseases such as Chagas disease, cutaneous leishmaniasis and leprosy.

2.8.2 Azoles
The derivatives of azoles, are the most frequently used drugs for treating fungal infections. These compounds contain a five member azole ring attached by carbon nitrogen bonds to other aromatic rings. They are fungistatic in nature and inhibit sterol 14-α-demethylase enzyme of ergosterol pathway. However, they differ in binding pattern to this enzyme, which is responsible for the varied antifungal activity. Azoles are usually considered safe for clinical use. The knowledge of drug-drug interactions and adverse effects like elevations in the level of liver transaminase, skin rashes and other visual disturbances associated with the use of these antifungal drugs is necessary while precombining them (Chen and Sorrell 2007). Azoles inhibit the cytochrome P-450 dependent 14-α demethylase enzyme (Fig-2.3) which plays an important role in the synthesis of other molecules like cholesterol, retinoic acid and glucocorticoid etc.

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**Fig 2.2** Mechanisms of action of different classes of antifungal agents.

Amongst the azole class of antifungal agents, fluconazole is commonly used in treatment of candidal infections. This drug is soluble in water, administered orally, have a long half life and penetrates readily in CSF. It is effective against wide range of fungi like *Candida* and *Dermatophytes* species and is given at the dose rate of 150 mg/day for 2-3 months for treating patients with pityriasis and onycomycosis. The frequent use of fluconazole therapy may lead to development of resistance in *Candida* species against this drug.

Itraconazole, another azole is a lipolytic compound which binds to plasma protein albumin and degrades it into several metabolites which are excreted in bile and urine. Voriconazole and Posaconazole primarily inhibit isoenzymes 3A4 and 2C9 and have small effect on 2C19 thus, facilitating drug–drug interactions because of shared metabolic pathway (Fig-2.3). While treating candidal infections with these drugs combined with: HMG-CoA reductase inhibitors viz; benzodiazepines, phenytoin, carbamazepine, cyclosporine, tacrolimus, sirolimus, methylprednisolone, proper precautions need to be followed since these drugs inhibit cholesterol pathway and some protease inhibitors such as ritonavir, indinavir and saquinavir (Kramer et al., 1990 and Jeng et al., 2001).
2.8.3 Allylamine and Benzylamines
These are the newly developed synthetic agents and have activity against a number of pathogenic fungi. These drugs inhibit squalene epoxidase, another key enzyme of ergosterol pathway. However, their action is selective due to their interaction with other pathways. All allylamine and benzylamines act initially on ergosterol pathway, hence, possess fungicidal activity. The main agents belonging to this group of antifungals are; Naftifine, Terbinafine and Butenafine (Chander, 1996).

2.9 Antifungal drug resistance and role of ERG 11 gene
Besides improvement of antifungal therapies in past 30 years, antifungal resistance is still a major problem confronting clinicians (Vandeputte et al., 2012). Antifungal drug resistance continues to grow and evolve consequently complicating management of the patients (Pfaller et al., 2012). Four major possible mechanisms of antifungal resistance to azoles have been described in Candida spp. These include; decreased intracellular drug concentration by activation of efflux systems or reduction of drug penetration, modification of the target site, upregulation of the target enzyme and development of bypass pathways. However, there is no single predominant mechanism associated with development of antifungal drug resistance. The resistance to fluconosine may result from defects in its metabolism through enzymatic mutations and resistance to amphotericin B may be mediated by increased catalase activity or defects in ergosterol biosynthetic pathway (Peman et al., 2009).

Ruhnke and co-workers (1994), observed resistance to fluconazole in Candida species isolated from HIV symptomatic patient having oropharyngeal Candidiasis. These workers have shown that there is regular increase in MIC to fluconazole in Candida albicans. Several other workers have also reported the emergence of fluconazole resistance in Candida species. White and co-workers (2002) suggested that mutations in certain genes of Candida lead to emergence of antifungal drug resistance. Pfaffer and co-workers (2003) tested 7,837 isolates of Candida against fluconazole and found 351 isolates resistant to this drug. The failure of chemotherapy has been reported due to prolonged use of antifungal agents over time, which is due to the increased resistance in Candida species.

The azoles target ERG11 gene product (cytochrome P450 lanosterol 14α-demethylase). This enzyme is an important part of the ergosterol biosynthetic pathway. The development of azole resistance among fungi may arise due to increased levels of the target product and upregulation of drug efflux controlling genes (Lupettiet al., 2002). The emergence of azole resistance may be due to the involvement of various molecular mechanisms.
There are many mechanisms which may lead to drug resistance, such as changes in cell wall which may interrupt the uptake of drug, alteration in the drug target enzyme ERG 11P (Lanosterol 14α demethylase) in case of azoles or change in cellular content of target enzyme due to site mutation or overexpression of this gene. Several such changes also promote the biofilm formation by these Candida species with increased resistance to antifungal agents (Mishra et al., 2007).

Song and co-workers (2004) cloned ERG11 promoter and monitored the induction of ERG11 gene expression by luciferase assays. They also assessed the effects of pH, carbon source and growth conditions on induction of the ERG11 promoter by azoles. Although, treatment with terbinafine and fenpropimorph also resulted in a delayed induction of ERG11 promoter, these agents target other enzymes of the ergosterol pathway at upstream level.

The deregulation in drug resistant effector gene may be responsible for the development of drug resistance. This may be due to occurrence of point mutations in transcriptional regulators of these genes. However, resistance to antifungal agents can also occur due to point mutations occurring directly in the genes coding targets products (enzymes) of the antifungal agents (Vandeputte et al., 2012).

Wang and co-workers (2005), amplified ERG11 gene of three fluconazole susceptible and 10 resistant C. albicans isolates from different sites. ERG11 gene of these organisms was amplified by PCR. The comparison of ERG11 gene sequences demonstrated mutations at 21 sites among 13 strains including 17 same-sense and 4 missense mutations. The studies suggest that fluconazole resistance of C. albicans may be associated with point mutations of V437I and N440K occurring in ERG11 gene, but not with the point mutations (Wang et al., 2005).

In vitro susceptibility testing is generally done to select appropriate antifungal agent for treatment. The most important use of antifungal such testing is in identifying agents that show resistance. Various mechanisms may lead to development of resistance among Candida species to azole drugs, the most common cause is the induction of the efflux pumps encoded by the multi drug resistant (MDR) genes. The point mutations in the gene encoding for the target enzyme (ERG11) may also be
another cause. The resistance of *Candida* species to echinocandins may be due to acquisition of point mutations in the FKS genes encoding the one of subunit of its target enzyme (Pfaller *et al.*, 2012).

Failures of drug treatment in fungal infections and standardization of antifungal susceptibility testing have emphasized the problem of antifungal resistance and underlying mechanisms. Resistance of *Candida* species and *Cryptococcus neoformans* to flucytosine (5FC) develops during monotherapy. Acquired resistance results from a failure to metabolize flucytosine (5FC) to 5FUTP and 5FdUMP, or from the loss of feedback control of pyrimidine biosynthesis (Bossche *et al.*, 1998). Fluconazole resistance may be associated with earlier use of fluconazole as intermittent therapy or continuous prophylactic treatment. Decreased susceptibility of ergosterol biosynthesis is another mechanism of resistance observed in a number of isolates of *C. albicans*, *C. neoformans* and *Histoplasma capsulatum* following treatment. Mutations have been seen in the CYP51A1 gene of drug resistant *C. albicans* isolates. Over-expression of this gene in *C. albicans* and *C. glabrata* may be responsible for a decreased susceptibility to azoles.

### 2.10 Relation of drug resistance with virulence and pathogenicity

The understanding of the pathogenesis of various fungal pathogens and their virulence factors might help in the development of new antifungal drugs. There are numerous virulence factors present in *Candida* species. The major virulence factors are: adherence to the tissues and host cells, secretion of hydrolytic enzyme, phenotypic switching and morphological dimorphism (Kuleta *et al.*, 2009). Angiolella and co-workers (2008) studied the relation of drug resistance with the virulence and its phenotypic traits in *Candida* species. Fluconazole (FLC,CO23RFLC) and micafungin (FK,CO23RFK) resistant strains have been generated by treating candidal infections with increasing concentration of drug. Homozygous mutation was found in FSK 1 gene and FLC,CO23RFLC strain acquired increased expression of drug resistant efflux pump. The drug resistant strains were more pathogenic in an experimental systemic infection mouse model. This increase in pathogenicity may result due to many physiological and chemical changes occurring in *Candida* species due to acquisition of drug resistance against fluconazole. These changes may involve cellular alterations, changes in polysaccharides content, rapid and extensive hyphae formation, increased adherence to plastic surfaces and capacity to form biofilm. Studies on biochemical and physiological changes would help
in better understanding of virulence and drug resistance in *Candida* species which would in turn, help in developing new antifungal drugs against these fungal pathogens.

### 2.11 Overcoming drug resistance against fluconazole in *Candida* species

As discussed earlier, many genetic alterations may be associated with the emergence of drug resistance. Some possible mechanisms of drug resistance like prevention of entry of drug in cell, degradation or modification of antifungal drug in cells does not exist in fungi. Organism may develop resistance against antifungal drugs due to repetitive use of these drugs. There is a need to study the number of fungal pathogens showing increased resistance on continuous basis. The possible way to overcome resistance is use of new antifungal drugs, regular continued studies of isolated fungal pathogens on regular basis and their susceptibility patterns against fluconazole may help in the task of overcoming antifungal drug resistance (Yang *et al.*, 2001).

To overcome drug resistances, there is great need for developing new strategies currently undertaken to find alternative therapy targets and antifungal agents. Discovery of novel antifungal agents can be achieved by screening various natural or synthetic chemical compounds from various sources. In addition, discovery of novel potential antifungal targets are required to be explored through genome sequencing approaches for better understanding the biology of pathogenic fungi (Vandeputte *et al.*, 2012).

### 2.12 Ergosterol pathway

The ergosterol biosynthetic pathway is a special branch of the mevalonate pathway, which is specific and unique in fungi (Fig-2.4). In this pathway there are several steps that act as targets for antifungal drugs. The antifungal agents like azoles, allylamines, thiocarbamates and morpholines inhibit the synthesis of ergosterol at different levels. Enzyme (1,3)-β-glucan synthase is not found in mammalian cells, which acts as target for echinocandins and pneumocandins. Inhibition of ergosterol pathway at different levels may provide a clue to find novel drugs (Matusevičius *et al.*, 2008).

Sterols are constituents of the cellular membranes that are essential for their normal structure and function. In mammalian cells, cholesterol is the main sterol found in various membranes (de Souza and Rodrigues 2009) but in eukaryotic cells, other essential lipids beside sterols also play an
important role in structural and signaling functions. The selection process of dominant sterol in eukaryotes is not well defined. Ergosterol has protective role against mechanical and oxidative stress (Dupont et al., 2012).

Osumi and co-workers (1978) studied the effect of [Methyl-14C] methionine on the growth of yeast cells under aerobic and anaerobic conditions and studied biosynthesis of ergosterol pathway after the methylation of the side-chain. Their work suggested that major pathways varied and depended on several conditions including oxygen supply and other factors.

The role of mutations corresponding to sterol alteration with antifungal resistance has not been thoroughly investigated in Candida albicans. The clinical strains of C. albicans resistant toazole and amphotericin B have mutation in ERG3 gene which encodes sterol delta (5, 6)-desaturase. Besides this deletion of ERG11 gene (lanosterol 14alpha-demethylase gene) may be possible when ERG3 is not functional but under aerobic conditions (Sanglard et al., 2003).

Georgopapadakou and co-workers (1992) studied the relationship between sterol biosynthesis inhibition, membrane integrity, and cell growth inhibition in Candida albicans against five squalene epoxidase inhibitors such as; thiocarbamates tolnaftate, tolciclate, naftifine, terbinafine and SDZ 87-469. All the compounds inhibited sterol.
Ergosterol pathway in *Candida albicans* (Contd.)

Reaction: dimethylallyl-pyrophosphate + dimethylallyl-pyrophosphate + delta3-isopentenyl-PP - pyrophosphate + geranyl-PP

Synonyms: Geranyl-diphosphate synthase, Prenyltransferase
Ergosterol pathway in Candida albicans (Contd.)
Besides ergosterol, some other sterols found in eukaryotic microorganisms such as fungi and protozoa are important. Ergosterol and other 24-methyl sterols are the part of metabolic pathways and essentially required for growth and viability of parasites. Several drugs are available which inhibit sterol pathway including those used to treat fungal diseases and treatment of high cholesterol in humans (de Souza and Rodrigues 2009).
2.13 Ergosterol pathway inhibitors

Use of clinically safe ergosterol pathway inhibitors may offer an alternative in overcoming resistance in *Candida* species. Among the currently available antifungal agents, statins have been shown to have limited antifungal effects which are primarily fungistatic not fungicidal. Statins inhibit 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase. These compounds, however, may produce some side effects viz myopathy and asymptomatic increase in hepatic transaminases. The development of myopathy in patients increases, particularly when these compounds are given in combination with other agents which share common metabolic pathways (Ballantyne et al., 2003, Bellostan et al., 2004).

There are a number of drugs which interfere with the sterol pathway in fungi. Sterols are normal constituents of the cellular membranes and also help to their normal growth. Sterols are produced in fungi, mammalian cells and trypanosomatids. Biophosphonates are such inhibitors which acts on enzyme farnesyl diphosphate synthase in ergosterol pathway (Zhang et al., 2009). The effect of such inhibitors on the growth of trypanosomatids and structural organization of protozoans body have been well studied (de Souza and Rodrigues 2009).

Aminobisphosphonate drugs inhibit growth of amoebas *Dictyostelium discoideum* by osteoclast-mediated bone resorption. The aminobisphosphonate (100 m M alendronate and 30 m M YM-175) inhibit the conversion of mevalonate into sterols. One out of three enzymes such as isopentenyl diphosphate (IDP) isomerase, farnesyl diphosphate (FDP) synthase and squalene synthase serve as the target for this inhibition. The initial precursor squalene of sterol biosynthesis was inhibited in extracts of wild-type amoebas by alendronate or risedronate (Joannae et al., 2000). The studies suggest that FDP synthase is the intracellular target for the bisphosphonate drugs.

Alendronate is an aminobisphosphonate compound mainly used for the treatment of osteoporosis and some other disorders of bone resorption. This compound has been known to act by inhibiting the formation of GGPP (Geranyl-pp) and is a potent inhibitor of cytosolic trans-prenyltransferase (FPP synthase) enzyme (Keller and Fliesler 1999). The drug, Fosamax, which is used in treatment of osteoporosis and many other infections targets isoprenoid biosynthesis, which is an important target for drug discovery. By this pathway organisms produce a number of smaller molecules naturally.
Isoprenoid biosynthesis is a target for drug discovery. Inhibition studies of FPP synthase, an enzyme which condenses IPP and DMAPP to the sesquiterpene farnesyl diphosphate (FPP) involving a carbocation mechanism is inhibited by bisphosphonates. These drugs are also inhibit sterol biosynthesis in protozoa and inactivate γδ T-cells to kill tumor cells, thus, are very important in oncology (Oldfield 2010). Recent studies on parasites have revealed that bisphosphonates are widely used in the treatment of benign and malignant diseases led by increased bone resorption and also as antiparasitic agents (Docampo and Moreno 2001).

Lentz and co-workers (1999), demonstrated the usefulness of some novel compounds for topical application as well as by systemic use. These workers included five dichlorinated 8quinolinols (2,5,6-, 3,5-, 3,7- and 4,5-dichloro-8-quinolinol) were tested against Candida albicans and C. tropicalis in liquid medium Sabouraud's dextrose broth supplemented with and without bovine serum. The 5,6-, 3,5-, and 3,7-dichloro-8-quinolinols proved more effective than the control, 5-fluorocytosine in the study. The cytotoxicity tests of these compounds using baby hamster kidney (BHK) cell proved to be more cytotoxic than the control.

Certain compounds such as calcineurin inhibitors cyclosporine A (CsA) and tacrolimus (FK506) which are calcineurin inhibitors acts synergistically with azoles providing potential fungicidal activity. Other antifungal agents such as terbinafine and fenpropimorph target other enzymes at other levels in the ergosterol biosynthetic pathway. These agents also exhibit synergistic antifungal activity against wild-type C. albicans when used in conjunction with CsA and FK506. The combination of these drugs also exhibit synergistic effects against other Candida species such as C. glabrata and C. Krusei. These species rapidly acquire resistance to azoles. The effectiveness of non-azole antifungal agents targeting ergosterol biosynthesis can be increased by inhibition of the calcineurin signaling pathway (Onyewu et al., 2003).

Buurman and co-workers (2004) tested a class of pyridines and pyrimidines against C. albicans strains lacking the Cdr1p and Cdr2p efflux pumps and found C. albicans sensitive to these compounds. Quantitative analysis of sterol intermediates that accumulated during growth inhibition in their study demonstrated the accumulation of lanosterol, suggesting that the inhibition of growth of fungus was due to decrease in ergosterol biosynthesis. These compounds have been shown to be possessing antifungal activity against C. albicans via lanosterol demethylase (Buurman et al., 2004).
2.14 Diagnosis of *Candida* infections

Different *Candida* species exhibit variability in resistance against antifungal agents. Therefore, blood culture system may be very useful in diagnosis of candidemia (Horvath et al., 2007). Fluorescence in situ hybridization (FISH) can be used as screening tests to differentiate *C. albicans* from non-albicans *Candida* species recovered from blood stream infections. The introduction of this test for the initial identification of pathogenic yeasts isolated from blood may prove useful in hospital environment (Alexander et al., 2006). The determination of serum D-arabinitol levels may also play an important role in diagnosis of candidiasis. Serial serum D-arabinitol/creatinine ratio (DA/Cr) measurements may be useful for diagnosis and management of disseminated candidiasis (Walsh et al., 1994). Nucleic acid techniques, such as plasmid profiling, various methods for generating restriction fragment length polymorphisms, and the polymerase chain reaction (PCR) has revolutionized the diagnostic procedures (Yi-Wei et al., 1997). The use of PCR-based methods to detect pathogens directly in the clinical samples, without the need for culture, have been proved more useful in rapid diagnosis. Other important tools include; direct detection of genes or gene mutations responsible for development of drug resistance. The automation of diagnostic procedure and availability of user-friendly software makes these technologies more widely available. Real-time polymerase chain reaction assays have also been developed for the detection of *Candida* species (McMullan et al., 2008). These assays perform well and help in the diagnosis of candidemia. However, the expected clinical outcomes and economic value of these assays remains to be ascertained.

Borst and co-workers (2003) developed amplified fragment length polymorphism (AFLP) analysis as an identification tool for pathogenic *Candida* species. These workers observed a misidentification rate of 6%. The AFLP is universally used, and the results can be easily stored in a general database. Therefore, AFLP may prove to be a good method for the identification of pathogenic *Candida* species.

2.15 Management of Candidal infections

The knowledge of various factors such as environment, exoenzymes, infection sites are essential for preventing candidal infection and the risks associated with these. This might further strengthen efficient management of these infections (Coutinho et al., 2009). In burn patients, *Candida* infection has a significant mortality ranging from 14% to 90%. The early coverage with auto graft tends to decrease the spread of systemic *Candida* infection among these patients. The patients who have an
artificial dermis as a part of their wound management develop systemic Candida infection. Careful monitoring of burn patients receiving broad spectrum antibiotics is required (Cochran et al., 2002). In case of Candida associated symptomatic urinary tract infections (UTIs), the choice of suitable antifungal agent depends upon certain factors such as current clinical status of the individual, site of infection and the pharmacokinetics of the antifungal agent to be used. Fluconazole is preferred drug for the treatment of UTIs due to Candida species as this drug is clinically safe, achieve high concentrations in the urine and its availability in oral and intravenous formulation. Flucytosine is another drug which maintains high concentrations in urine and exhibits broad activity against Candida spp. The use of this agent is however limited because of its toxicity. Amphotericin B may also be useful for treating Candida UTIs with low doses but in selected patient groups. Combination antifungal therapy involving irrigation of the renal pelvis using nephrostomy tube may be useful in treatment of candidal UTIs (Fisher et al., 2011).

The occurrence of candidal infections after prosthetic graft implantation due to acute aortic dissection is rare. For treatment of these infections, surgical resection and prolonged antifungal therapy throughout life is needed in combination as surgical interventions carries high mortality rates. However, in some cases Candida prosthetic graft infection can be managed with antifungal therapy only. Such treatment strategy can be applied with some caution and close watch up for longer time (Motloch et al., 2011).

Invasive candidal infections are of major concern as they produce manifestations similar to that of septic shock (40-60%). Insights into pathophysiology and the available antifungal agents and prophylaxis have contributed a lot in the improvement and prognosis of severe candidal infections. Lower initial risk patients, pre-emptive therapy should be a management strategy that considers the possible risk factors and the dynamics of Candida colonisation. Azoles prophylaxis is effective and must be restricted only to highly selective groups of patients which are at high risk (Eggimann et al., 2003).

In a cohort study on the systemic candidal infections by Cruciani co-workers 2008 among critically ill infants and observed the role of risk factors associated with the infections. These workers confirmed the associated risk factors (catheter-days) and identified novel risk factors for candidemia in critically ill infants. The risk factors can thus, guide antifungal prophylaxis in intensive care units. Routine prophylaxis strategy should not be followed in all intensive care unit (ICU) patients; the azole resistant strains can be seen for prognosis. Fungal infections can be treated with novel antifungal
agents, including newer azoles (e.g., voriconazole, posaconazole) and echinocandins. However, there is a urgent need to improve diagnosis of systemic candidal infection so that clinicians can intervene during initial stages (Feja et al., 2005).

Novel therapeutic approaches are required to improve the treatment of patients with candidal infections. Advancements in understanding mechanisms of the anti-Candida host response have revolutionized the development of novel immunotherapeutics (Van de Veerdonk et al., 2010). Mills and co-workers 2009 selected any randomized trials which were assessing antifungal therapies for the cases of invasive candidiasis in adult populations. These workers performed a meta-analysis and sensitivity analysis including dosage forms of amphotericin B and fluconazole and compared with other azoles and their mixed treatment. Comparative analysis demonstrated similar results. However, adverse event profiles varied with amphotericin B as this drug exhibited maximum adverse effects.

Anaissie and co-workers (1996) conducted a prospective, randomized, multicenter study to compare efficacy of treatment with fluconazole and amphotericin B for candidal infections. They selected one hundred and sixty-four patients with invasive candidiasis for treatment with either fluconazole (400 mg daily) or amphotericin B (25-50 mg daily; 0.67 mg/kg daily). The clinical response and survival rates were assessed after 48 hours and 5 days respectively. These workers found similar response rates for both fluconazole and amphotericin B (66% and 64%, respectively). Amphotericin B was having more adverse effects than fluconazole, suggesting therefore, that fluconazole was more effective and better tolerated than amphotericin B in treating candidal infections (Anaissie et al., 1996).

Infectious Diseases Society of America has prepared guidelines for the management of patients with invasive and mucosal candidiasis (Pappas et al., 2009). Some novel antifungal agents have been developed and included since 2004 for the treatment of candidemia and other type of candidal infections.

The combination therapy has been proven very effective against fluconazole resistant Candida spp. Huang and co-workers (2008) studied interaction of fluconazole and baicalein (BE) in vitro against 30 fluconazole-resistant Candida albicans strains isolated from clinical cases and observed synergistic activities when tested in combination. However, these drugs when tested singly and in combination using time-kill methods, exhibited weak activity. This synergetic effect of fluconazole
and BE may overcome drug-resistance in *C. albicans* and may be useful in treatment of candidiasis due to this organism.

Tsutomu and co-workers (2011) reported the synergistic activity of lactoferrin and fluconazole against *Candida* spp as this combination inhibited hyphal formation in fluconazole-resistant strains of *Candida albicans*. Variation was however observed in the susceptibility of drug combination among the strains. Iron-chelating function of lactoferrin may be contributing to the synergistic effect of this agent. However, radiolabeled studies suggest that lactoferrin did not alter intracellular concentrations of fluconazole, thus, these synergistic effects may be considered due to the alteration in the uptake of the drug by the fungal cells. These workers suggested that further studies are required to understand exact underlying mechanisms of drug resistance and ways to overcome it with the help of appropriate drug synergism.

Combination antifungal therapy is very attractive step for treating life threatening candidal infections. The *in vitro* and *in vivo* interactions of such therapy is not fully clear because of the limited clinical trials conducted in this regard. Most of the clinical trials exhibit similar observations for combination antifungal therapy vis a vis single drug therapy. Combination of antifungal agent capable of immune modulation would be quite interesting and thrust area for future research (Ostrosky-Zeichner, 2008). Although combinations of novel agents with traditional antifungal agents possess synergism against *Candida* species in clinical trials, yet some cautions are needed in this regard as antagonistic activities have been observed with some of the antifungal drug combinations. Well-controlled and organized clinical trials are required to demonstrate more effective drug regimen. In addition, the studies are also needed to observe the possible side effects and pharmacoeconomic impact of the drug combination regimens (Vazquez *et al.*., 2003).

Turan and co-workers (2011) observed the efficacy of combination therapy of liposomal amphotericin B and voriconazole in the neonates with positive blood culture. These workers demonstrated that in addition to conventional antifungal treatment, voriconazole can be added in treatment of fungal sepsis in neonates. However, more clinical data are required before using voriconazole as a drug of choice in the treatment of neonates.