CHAPTER III

REVIEW LITERATURE

Candidiasis

Since 1970, the annual death rate due to candidiasis increased significantly in association with change in medical practice, including widespread of therapies, the use of broad-spectrum antibacterial agents with less awareness, the common use of indwelling intravenous devices and the advent of chronic immunosuppressive viral infection such as AIDS (7). Disseminated infection carries a high mortality which approaches 55 % in the case of candidiamia (11). Data from the ongoing National Nosocomial Infectious Surveillance System conducted in the United State showed a 48.7 percent increase in *Candida* bloodstream infections between 1980 and 1981. And not only in AIDS patients, the serious infections are also an increasing problem due to the immunosuppressive nature of surgery, organ transplantation and the treatment of malignancy (19). The genus *Candida* spp. are now the fourth or fifth most common nosocomial blood culture isolate in USA (11). Among various kinds of clinical manifestations in HIV infected patients, oropharyngeal candidiasis is the most common fungal infection (20).

This malady is caused by yeast in the genus *Candida.* The most frequent species is *Candida albicans.* However, there has been reported that the incidence of a non-*albicans Candida* (NAC) is increasing and becomes the most common etiologic agent in some areas such *as C. glabrata, C. parapsilosis, C. tropicalis C. gulliermondii* and *C. krusei* (21-24). *Candida albicans* is a true opportunistic pathogen that is usually present in the intestine, and probably part of the normal flora of the cutaneous and mucocutaneous surface. *Candida* species are frequently isolated from oral cavity and are detected in approximately 31 to 50 % of healthy individuals (25). But because of the fungicidal effect of human saliva, the presence of glycoprotein and saliva immunoglobulin A (IgA), adherence of *Candida* may be prevented. Some hypotheses of how this genus colonize and/or infect have been

stated. It is possible that some small local alterations, such as those derived from smoking tobacco are enough to promote *Candida* infection in the oral cavity. However, the main factors conditioning this development seem to be changes in the normal microbial population (which could either mask/compete for receptors or interfere directly) and/or alterations of the immune system, the rate of colonization increasing with severity of illness and duration of hospitalization (26). Another promoting factor is that elderly people produce little saliva, which is responsible for both washing and pH regulatory effects. Both of these factors can serve to explain the higher frequecy of oropharyngeal colinization and infection in newborns and elderly people. Malnutrition and in general, low social-sanitary conditions also favor oropharyngeal candidiasis. The frequent drug-intake of people who are aging is a risk factor for oropharyngeal candidiasis other than the usual immunodeficiency-derived physiological status should be taken into account. Antibiotics seem to favor *Candidia* selection, but psychotropic drugs might also cause alterlations in the oral microbial population. The primary immunodeficiency, such as neutrophil dysfuntions, cellular immunodeficiencies, humoral immunodefienciencies, complement alterlations or complex combined syndromes can cause alterations in the oral microflora that can lead to an overgrowth of *Candida* and subsequent oropharyngeal inflammation. Particularly if phagocytosis is impaired, the risk of *C. albicans* infection is clearly increased. Radiation therapy can result in a change in oral bacterial flora and promote colonizacion or infection. There has been stated that in some predisposing factor in certain patients such as in diabetic group, their epithelial mucosal surface of the oral cavity is more adhesive for *C. albicans* than in nondiabetic patients. The most frequent patient with the oral infection is the HIV-positive and AIDS patients, probably due to the local environmental changes in the oral cavity. The most prevalent yeast pathogen is *C. albicans*. It has been suggested that HIV infection increases the adhesivity of *C. albicans* to the oral mucosal surface. The frequency of esophageal and orophageal candidiasis among these patients varies between 45 % and 95 % and is considered one of the earliest symptoms of AIDS among HIV-positive patients, as well as an early predictive marker of pulmonary tuberculosis in these patients. The prevalence rates of oropharyngeal colonuzation by *C. albicans* on HIV-positive AIDS patients without evidence of infection range in some series from

50 % to 78 %. *C. albicans* has the highest frequency of isolation (55 %) and the prevalence of other species appears to be lower: *C. tropicalis* (17 %) and *C. krusei* (12 %) (27-29).

Antifungal drugs

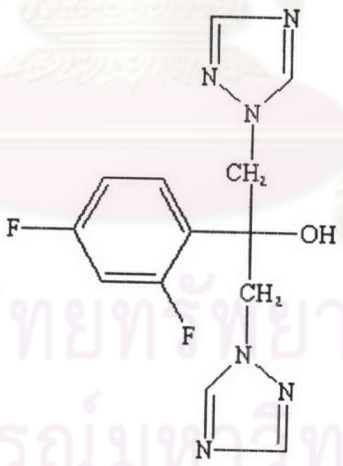
Based on the mechanism of the antifungal drugs, we can divide the drugs into 3 major groups as followed.

1. Antifungal drug inhibiting ergosterol synthesis

The three major groups of antifungal drug, azole polyene and allylamine/ thiocarbamates are directed againt ergosterol. The mechanism of action of antifungal drugs are explained below.

1.1 Azole-base antimycotic agents

Figure 1. The structure of azole antifungal agent (30)



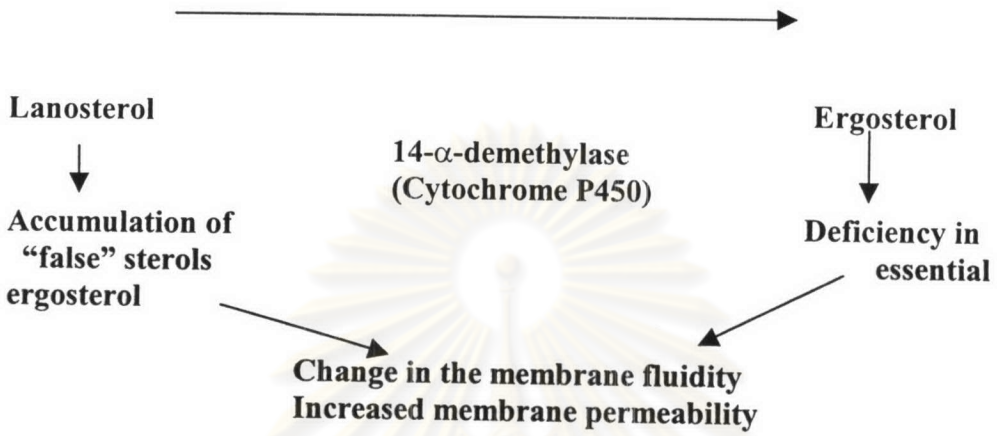
The first report of the antifungal properties of N-substituted imidazoles was published in the late 1960s by RJ. Holt (31). Azole antifungal agents have fungistatic, broad-spectum activity that includes yeast and filamentous fungi. Fluconazole is particularly active against candidiasis and it has become agent of choice for treatment and porphylaxis of systemic candidiasis in oropharyngeal candidiasis because it has an excellent safety profile in immune normal and immunocompromised patients and

it can be administrated by the oral and by the intravenous routes (32). The imidazole derivative compounds have been produced, such as miconazole, econazole, ketoconazole, fluconazole, itraconazole, and voriconazole.

Ergosterol is an important sterol for fungi. It has an essential function in which trace amounts of ergosterol are necessary for the cells to progress through the cell cycle (33). It serves as a bioregulator of membrane fluidity an asymetry and consequently to membrane integrity in fungal cell. Integrity of the cell membrane requires that inserted sterol lacks C-4 methyl groups. There are evidences suggest that the primary target of azole is the heme protein, which cocatalyze cytochrome P-450 dependent 14α-demethylation of lanosterol. The azole acts through an unhindered nitrogen, which binds to the iron atom of the heme, preventing the activation of oxygen which is necessary for the methylation of lanosterol (7). And to interact directly with the apoprotein of lanossterol demethyllase, suggesting that the position of the second nitrogen in relation to the apoprotein may determine the specificity of different azole drugs for the enzyme (2).

Inhibition of 14α-demethylase leads to depletion of ergosterol and acculumation of sterol precursor, including 14α-demethylase set sterols , resulting in the formation of a plasma membrane with altered structure and function (Figure 2). Example alter the activity of several membrane bound enzyme, such as those asscociated with nutreint transport and chitin synthesis and may additionally interfere with nutreint hormone-like function of ergosterol, affecing cell growth and proliferation. It is interesting in this respect that fluconazole causes *C. albicans* to develop large multinucleated cells. It is therefore likely that the fungistatic action of azole is due to a complex multimechanism process arising from changes in membrane sterol composition brought about by the inhibition of P-450 dependent 14α-demethylation of lanosterol (34).

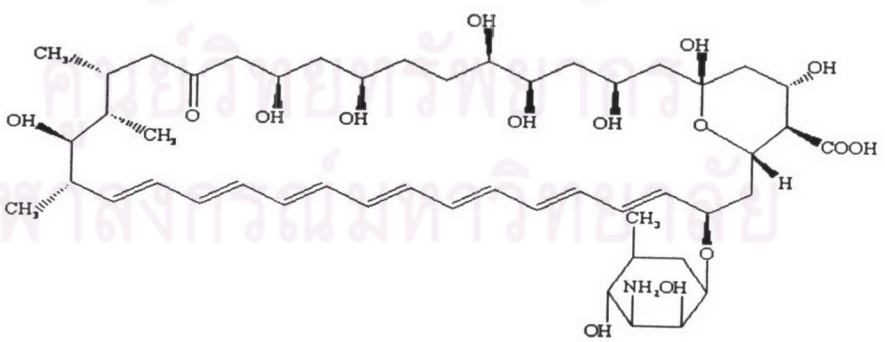
Figure 2 . The action of fluconazole to fungal target enzyme, Cytocrome P-450 dependent 14α-sterol demethylase cause to membrane impair function.



It is to be noted that the azole activity may also vary with the genus treated . And the mammalian cholesterol synthesis is also blocked by azoles at the stage of 14α-demethylation, however the dose requried to effect the same degree of inhibition is much higher than that required for fungi (2).

1.2 Polyenes

Figure 3. The structure of polyene antifungal agents (30).

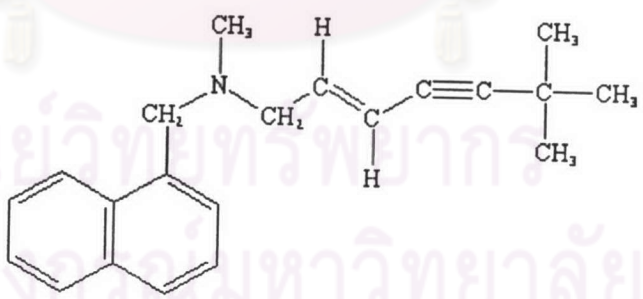


The polyene antibiotics are natural fermentation products, produced by an actinomycete recovered from soil, *Streptomyces nodusus* in Venezuela in 1950s (35). They are fungicidal and have the broadest spectrum of activity in any clinically useful antifungal compound.

From the 1950s, polyene antifungal agents represented the standard of therapy for systemic fungal infection before the discovery of azole. These drugs, which included amphotericin B and nystatin, are amphipatic, having both hydrophobic and hydrophilic properties. These feature of amphipatic structure is believed to be important in it mechanism action (35). It has been reported on the susceptible organisms, eg. yeast, algae and protozoa. These eukaryotes contain sterol in their outer membrane while resistant organisms do not (7). The polyene action is through the intercalation into membrane. Forming a channel through which cellular components, especially potassium ion, leak of vital cytoplasmic components and there by destroying the protein gradient within the membrane and as a consequence by the death of the organism. The action of amphotericin B on fungal cells is believed to involve more than one mechanism such as there are reports that it involves in oxidative damage to cell but the mechanisms are not well known. (35).

1.3 Allylamines

Figure 4. The structure of allylamines antifungal drug (30).



The allylamines such as naftifine and terbinafine and thiocarbonates such as tolnafine and toliclate have been developed as a new class of ergosterol biosynthetic inhibitor by inhibiting the conversion of squalene to 2,3-oxidosqualene by the enzyme squalene epoxidase, SE. (36). Among the SE inhibitors, terbinafine is the most potent agent against a wide variety of pathogenic fungi and has a primary fungicidal action

(37).. This drugs has good activity against at least some azole resistant *C. albicans*. This inhibition coincides with accumulation of the sterol precursor squalene and the absence of any other sterol intermidiate. The fungal cell death is related primarily to accumulation of the sterol precursor squalene and the absence of any other sterol intermidiate. Fungal cell death is related primarily to the acummulation of squalene rather than ergosterol deficiency because high level of the squalene may increase membrane permeability, leading to disruption of cellular organization . In addition, this drug may inhibit the expoxidase through a naphthalene moiety common to both types of drug. The resulting ergosterol depletion and squalene accumulation effect membrane structure function, such as nutrient up take (38, 39). For the terbinafine, there are report that this drugs has good activity against at least some azole resistant *C.albicans* strain and appears highly active against *C. neoformans* (40).

1.4 Pyridines and Pyrimidines (7)

The pyridines (buthionate and pyriferox) and the pyrimidines (triarimol and fenarimol) inhibit lanosterol demethylase and are used extensively as antifungal agents in argriculture but are not used in medicine.

1.5 Morpholines (2).

It discovered in the 1970s with exception of amorofine, which is used in nail infection, are agricultural fungicidal. The morpholines (such as fenpropimorph and amoroifine) inhibit two enzymes in the ergosterol biosynthetic pathway, c-14 sterol reductase and c-8 sterol isomerase. Interesting, the morpholine from propimorph also inhibit cholesterol biosynthesis in mamalian cells, but it affect the dimethylation of lanosterol rather than sterol reductases or isomerase.

2. Antifungal drug active against fungal cell walls

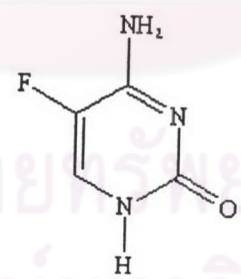
The fungal cell wall contains compounds, such as mannan, chitin, and α- and β- glucans, that are unique to the fungal kingdom. Since these components are not found elsewhere in any organisms, they have been identified as possible targets that provide selective toxicity advantages. A compound that have the ability to affect the cell walls of fungi have been discovered and described over the past 30 years (41).

Ther are three groups of compounds such as aculeacines, echinocandins and papulacandins, that are specific inhibitor of fungal 3-β-glucan synthase. For echinocandins, which are lipopeptides, have fungicidal against *Candida* and *Aspergillus* both *in vitro* and *in vivo* (42).

When *Candida albicans* culture was treated with cell wall antibiotics revealed highly distored, wrinkgle and collasped cells. Dividing cells failed to separated properly and aggreats of enlarged and elongatiom forms. β - glucan inhibitor act as specific noncompetitive inhibits of β- (1-3) glucan synthetase. The inhigition of β - (1,3) – glucan synthetase results in cytological and a ultrastructural changes in fungi characterized by growth as pseudohyphae, thickened cell wall and buds tailing to reported from mother cells and cells also become osmotically sensitive. Suggest that the specific site of the leakage was a tip of bud and this site had been known as the active locus for glucan synthesis (43-44).

3. Antifungal drug inhibiting Nucleic acids.

Figure 5. The structure of flucytosine agent.(30)



Flucytosine (5-FC) has an entirely distinct mode of action from the azoles. 5-FC is a water soluble, fluorinated pyrimidine analog with inhibitory activity against many yeasts that was discovered in 1957. 5-FC is used in combination with the antifungal drugs such as amphotericin B and fluconazole such as in meningitis and dissiminated candidiasis.

5-FC is taken up into the cell by a cytosine permease it has two primary mechanisms of action. The first is conversion by cytosine deaminase into 5-fluorouracil (5-FU) with subsequent conversion through several intermidiateds into 5-

fluorouridine triphosphate and incorporation in to fungal RNA with resultant inhibition of protein synthesis and the conversion by uridine monophosphate pyrophospharylase into 5-fluorodeoxyuridine monophosphate (F-dUMP) which inhibits thymidylate synthetase and DNA synthesis. 5-FC is fungal specific because mammalian cells have little or no cytosine deaminase (7, 45).

Antifungal resistant

1. Polyene resistance

The report of amphotericin B resistance are limited, but it appear that severely immunocompromised pateints are at the highest risk. The previous exposure of polyene and cytotoxic chemotherapy contribute to the development of amphoterin B resistance such as in *Candida* spp. and *Cryptococcus neoformans* (33).

The primary polyene resistance has been found in *Pseudallescheria boydii ,Fusarium,Trichosporon beigellii* (46). The primary resistant has emerged in parallel with the increase in the number of invasive infectious due to the so-called emerging fungi. The secondary resistance has been found in yeasts causing infections in patients with cancer. Furthurmore, yeast isolates from patients undergoing myelosuppressive chemotherapy or bone marrow transplantation were seem to have significantly higher MICs to amphotericin B than colonizing isolates from immunocompetant patients (47).

Strain of *C. albicans* acquiring resistance to amphotericin B and azole have been communicated in patients receiving treatment with these antifungal.

In 1994, Law and colleages reported the rate of amphotericin B resistance from *Candida* spp. isolated from patients with AIDS. The result showed that 1 of 948 isolates were resistant and 12 isolates were intermidiate resistant (11).

2. Flucytosine resistance

Primary resistant to 5-Fc is common in certain yeast and molds Non-albicans *Candida spp.* and dimorphic fungi , have high rates of 5-FC resistance. The study of 348 *Candida* isolates,only 12 (3.4%) isolates were flucytosine resistance.

One study showed primary flucytosine resistance in *C. albicans* ranging from 6.5% of isolates in Europe to 33% in the USA with a general prevalence around 9%

Because 5-FC resistance develops frequently , the drug should never be used as a single agent to treat either yeast or mold infection (47).

3.Azole resistance

There are many reported of fluconazole resistance because this drug has become important in treatment of mucosal candidiasis in AIDS patients.

Because fluconazole become the antifungal agent of choice in the treatment and porphylaxis of oropharyngeal candidiasis in early 1990s, fluconazole resistant was subsequently described in up to 41 % of the patients in the following year.

Maenza and co-workers reported a cross-sectional study of the frequently develop oral candidiasis in HIV patients. *Candida* colonize the mounts of 64 to 84 percent of the patients and causes symptomatic disease up to 46 percent and the prevalence of fluconazole resistance has been estimated to be 21 to 32 percent in symptomatic patients and up to 14 percent in asymptomatic patients (48).

And one study reported the susceptible rate during a 2-years period. From total 851 strains, 523 (61.5 %) strains were susceptible, 11 strains (1.3 %) showed intermediated susceptibility and 317 (37.2 %) were resistant to fluconazole while 403 (47.3 %) were susceptible, 43 (5.1 %) intermediately susceptible and 405 (47.6 %) resistant to itraconazole (49).

The risk factors for fluconazole-resistant candidiasis in HIV patein was study by Maenza in 1996 (50). The case-control study was performed in 25 pateints and control who had treatment-responsive with resistant infections had lower CD4 counts and a longer duration of antifungal therapy than the matched control. So the advanced immunosuppression and previous exposure to oral azole were found to be risk factor in development of resistance. The factor that may contribute to clinical antifungal drug resistance was shown in the table 1.

Table 1. The Risk factor of drug resistant in organisms (6)

Antifungal drug	Localisation of infection	Fungal pathogen	Risk factors
Fluconazole	Oropharyngeal candidiasis	*Candida* spp	HIV infection Fluconazole use Low CD4 cell count Prior use of antituberculous drugs and antibiotic
Fluconazole	Candidaemia	*Candida krusei*	Acute leukaemia Fluconazole use Severe neutropenia
Fluconazole	Candidaemia	*Candida glabrata*	Haemathological malignancy and solid tumors Permanent central venous catheter Abdominal portal of entry Neutropenia Fluconazole use Amphotericin B use Antibiotics
Amphotericin B	Invasive infections	*Fusarium*	Haemathological malignancy Severe and prolonged neutropenia Broad spectrum antibiotics
Amphotericin B	Invasive infections	*Scedosporium prolificans*	Acute leukaemia Severe and prolonged neutropenia Broad spectrum antibiotics
Amphotericin B	Disseminated disease	*Aspergillus terreus*	Acute leukaemia Severe and prolonged neutropenia

Antifungal Susceptibility Testing (51, 52)

Basically, *in vitro* susceptibility tests are meant to provide (i) a correlation between *in vivo* activity and therapeutic outcome, (ii) a measure of the relative activities of two or more antifungal agents, and (iii) a means to monitor the development of drug resistance. It must also be recognized that there are inherent limitation with all *in vitro* susceptibility tests because the MIC is not a physical or chemical measurement. A second consideration is that the interaction between the microorganism and antifungal agent is artificial, and the persistence or progression of an infection can occur despite the administration of appropriate antifungal therapy. susceptibility testing dose not reproduce *in vivo* condition, in part because (i) host factor are absent, (ii) the agent concentration is essentially static (pharmacokinetics), and (iii) the test inoculum size is probably at variance with the infection load. The determination of the *in vivo* efficacy of antifungal agents thus takes into account both host defense and intrinsic antimicrobial activity. As a result, a distinction is made between clinical resistance observed *in vivo* and microbiological resistance observed *in vitro*. This distinction is important because life-threatening systemic fungal infection are often disease of the immunocompromised, in whom the underlying condition and iatrogenic factor contribute more to the final outcome than antifungal therapy. Nevertheless, despite the fact that antifungal susceptibility testing may not always identify those patients who will respond to antifungal therapy, *in vitro* microbiological resistance can often be used to predict therapeutic failure.

1. NCCLS M27 Method for Yeast Susceptibility Testing.

Beginning in the 1980s, extensive planning, interlaboratory cooperation for testing antifungal susceptibility in 1997. This first big step has allowed investigators to each other. The NCCLS subcommittee on Antifungal Susceptibility Testing published the M27-A2 reference method for broth dilution susceptibility testing of *Candida* spp. and *C. neoformans* to allow the development of standard breakpoints to guide therapy and to decrease interlaboratory variability. The NCCLS M27-A2 reference method is the result of a series of collaborative studies that focused on standardizing variables that influence in vitro susceptibility testing and MIC endpoint determination for yeasts, including incubation duration and temperature, medium

composition, and inoculum size. This protocol has progressed from a document that examined the role of variables in standardization through proposal, tentative approval, and approval. Adherence to the M27 method has been shown to provide greater than 90 % intralaboratory and interlaboratory reproducibility. Standardized testing methodology should ultimately result in the development of breakpoints, with an improved understanding of the relationship between *in vitro* testing and *in vivo* outcomes.

2.Alternatives to the NCCLS M27 method for Yeast Susceptibility Testing

Modifications and alternative approaches, intended to make the NCCLS methodology more objective, efficient, and convenient, have been proposed. In Europe, the Antifungal Susceptibility Testing Subcommittee has modified the NCCLS M27 microdilution method by using a spectrophotometric endpoint determination after 24 hours of incubation, supplementing the RPMI 1640 medium with 2 percent dextrose, and increasing the inoculum size. Alternative techniques and criteria have particularly focused on improving MIC endpoint determination. This method has been evaluated for intra and interlaboratory agreement and with the newer agents, voriconazole and caspofungin. This methodology and theoretically should produce more reliable results.

3.Colorimetric adaptation

More innovative efforts have proposed using a colorimetric endpoint in a broth microdilution format by including an oxidation-reduction indicator Alamar Blue has been used most extensively and is commercially available in a broth microdilution tray called Sensititre YeastOne (TREK Diagnostics Systems Inc., Westlake OH). Several studies have found good correlation between the NCCLS microbroth dilution method and the YeastOne colorimetric microdilution panel. The sensititre YeastOne method appears to be a suitable alternative procedure for routine antifungal susceptibility testing of *Candida* spp., although species-specific discrepancies have been noted. Two additional colorimetric methods, called the ASTY Colorimetric Microdilution Panel and Rapid Susceptibility Assay (RSA), have also been introduced but have not achieved broad acceptance. The RSA is unique in that it is based on

suppression of glucose uptake by susceptible fungal cells in the presence of antifungal agents. However, there is poor agreement between the RSA and the M27 method for testing fluconazole and itraconazole.

4.Spectrophotometric and other novel adaptations

This simple adaptation of the M27 reference involves agitation of the microtiter plate or resuspension of the well contents using a multichannel pipettor. 5 – FC, or caspofugin, the MIC endpoint is best defined as the lowest concentration of antifungal agent at which the absorbance is resuced to 50 percent in comparison to that for the drug-free growth control. The spectrophotometric method correlation well with another modification of the M27 broth microdilution format that incorporates the addition of the fluorescent dye carboxyfluorescein diacetate (CFDA), used to assess fungal viability. Both the CFDA and spectrophotometric methods and an additional method that quantifies ergosterol content in the fungal cell wall have demonstrated that the low-high phenotype strains of *Candida* are in fact susceptible to the azole antifungal agents. Different vitality-specific and mortality-specific fluorescent dyes have been with flow cytometry for the rapid detection of antifungal susceptibility. Flow cytometric susceptibility testing has good correlation with the NCCLS M27 method.

5.Disk diffusion

The agar diffusion methods(disk diffusion) commonly used for antibacterial testing have also been applied to antifungal susceptibility testing, but they require future validation and standardization. These agar-based methods are simpler, more economic, and discriminate contamination better than broth based testing. The disk diffusion method for testing antifungal agents may give results comparable to those of the M27 method. Disk diffusion may provide a screen for susceptible and resistant isolates, but resistant isolates must be confirmed by MIC testing.

6. Etest

The Etest MIC is read after a 48 hours incubation period as the drug concentration at which the border of the elliptical zone of inhibition intercepts the scale in the antifungal-impregnated plastic strip. The agreement between the Etest ant the NCCLS M27-A2 method varies with different combination of fungal species and antifungal agent. This antimicrobial gradient embedded strip technology is approved in US to determine bacterial MIC, and although approved in Europe, Etest for antifungal agents is not approved currently for clinical use in the United States. The Etest and NCCLS microdilution methods have been compared extensively and shown to produce good agreement.

Antifungal Resistant Mechanism

1. Mechanism of resistance to polyene

Resistance phenomenon to polyene antibiotics, such as amphotericin B and nystatin, is rare , although more than 30 years of clinical use . And the resistance isolates being confine mostly to the less common species of *Candida* such as *C.lusitaniae* , *C.glabrata* and *C.guilliermoudii* (7) and there are reported in *Trichosporon beigellii* , *Pseudallescheria boydii* and *Dermatiaceaus* fungi .The resistance strains of *C.albicans* , *in vitro*. Fryberg suggested that development of resistance occurs by selection of naturally occurring resistance cells,present in small numbers in the population.These naturally resistant cells produce modified sterols that bind nystatin with lower affinity (32).

Ghannoum and Rice concluded the study mechanisms of resistance to polyene followed this (i) growing cell in the presence of incresing concentration of antifungal agent (ii) exposing the cells to a gradient concentration or (iii) creating mutants by one-step mutation with mutagenic agent (32). Hamilton-Miller proposed a biochemical hypothesis that resistance arises due to changes in the sterol content of the cells, sterol content should bind smaller amounts of polyene than do susceptible cells.The deletion of binding of polyene in *C.albicans* mutants could be attributed to (i) a decreas in the total ergosterol content of the cell (ii) replacement of some or all of

the polyene-binding sterols by ones which bind polyene less well (iii) reoriontation,or masking , of existing ergosterol (53, 54).

The study about 27-polyene-resistant *C.albicans* isolates obtained from neutropenic pateints showed that these strain had a 74 to 85 % decrease in their ergosterol content.Thus, decreased ergosterol content may lead to decreaseed susceptible to polyene (55).

Because the polyene act in the ergosterol of fungi. The of cell cycle to the efficiency of drug was studied by compare the polyene susceptibility of exponentail and stationary-phase. The result show that stationary phase cells were more resistant than exponential-phase cells. This result corresponsed to the fact that in the exponentail-phase cells, breakdown and resynthesis of cell wall constituents occurs at a high rate, resulting in improved polyene access to the cell membrane. In contrast,stationary phase cells would be expected to break down and synthesis cell wall at a much lower rate (7).

The analysis of sterol composition in several clinical isolates of *C.albicans* may be defective in *ERG2* or *ERG3* .Unfortunately, the stability of these mutations or the molecular defects that causes the azole-resistance have not been described for any polyene-resistance mutation (56).

2. **Flucytosine resistance**

Ten percent of *C. albicans* clinical isolates are intrinsically resistant and that 30% will develop seconary resistances.

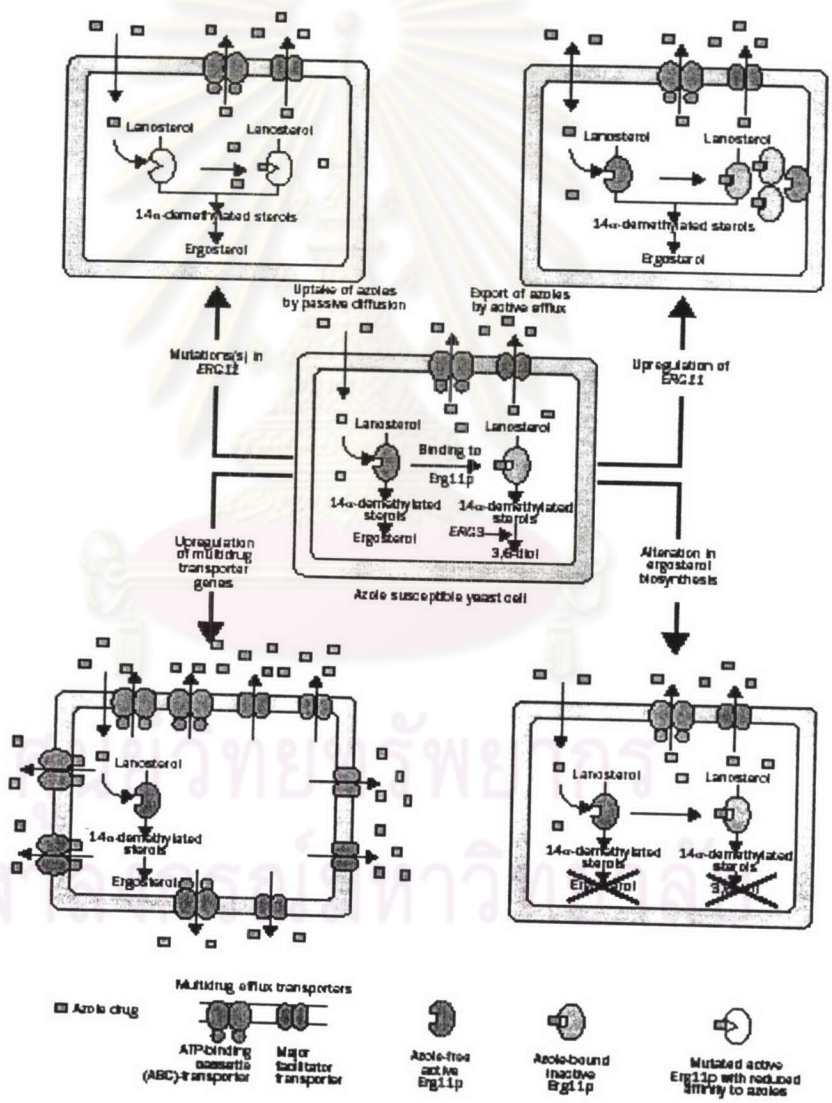
Primary resistance to 5-FC is usually the result of a defect in cytosine deaminase resulting in decreased uptake or less enzyme activity.

And Seconary resistance to 5-Fc in *C.albicans* is due primary to a decrease in the activity of the uracil phosphoriye transferase (UPRTase) which is involved in the synthesis of FUMP and FdUMP(32).

3. Azole resistance

There are many different types of mechanism are known to contribute to azole – resistance as shown in Figure 6.

Figure 6. The azole resistant mechanisms (6)



1. Alteration in the sterol biosynthesis pathway

Inhiobition of 14 demethylase by flucoanzole not only result in ergosteol depletion but also in the accumulation of the methylated sterol 14 α-methylergosta-8,24(8)-dien-3β,6α-diol, which inhibit cell growth. Alteration in the sterol biosynthesi apthway that avoid the accumulation of this growth inhibiting ergosterol in the presence of flucoanzole can cause fluconazole resistant. Inactivation of *ERG3* gene , the enzyme that act at an earlier step than 14 demethylase in the ergosterol biosynthesis pathway, results in altered sterole composition of the membrane and flucoanzole resistance, possibly by accumulation of 14α-methylfecosterol, which aloow growth. Recently it was shown that deletion of the ERG3 gene in *C. albicans* resulted in reduced susceptibility of the muatant to flucoanzole, providing direct genetic evidence that alteration of the sterol biosynthesis pathway can cause fluconazole resistant (57).

2.Alterred azole binding site on target enzyme *CYP 51A1* (14α-demethyloase)

The predominant target enzyme of the azole drug is lanosterol demethylase. The gene encoding this enzyme is designed *ERG 11* in all fungal species. It is involved in an important step in the biosynthesis of ergosterol by demethylase of lanosterol molecule in position 14α (10). The one important mechanism is point mutation in the coding ergion of this enzyme. There are many reports shown the association of mutation of this gene with azole resistance.

Marichal and co-worker reported a point mutation in *ERG11* was identified from seven *C. albicans* isolates, of which two were azole – sensitive. In these seven isolates, 12 different amino acid substitutions were identified, of which six have not been described previously this report including A149V D158E E165Y S279F V452A and G465S and 16 silent mutations were found. For 12 nucleotides that D116E, K128T, A149V, Y32H, G450E, R467K and S405R demonstrated that they were important for anazole resistant (58). The other reported study in another fluconazole – resistant isolated of *C. albicans*, DUMC136. They found that 12 nucleotide substitution in *CYP51* gene, resulting in 4 amino acid changes, were

identified in comparision with a sequence from wild type (T315C, T348A, A357G, A883C, T394C, C411T T433C, C658T, A1020G, C1110T, A1440G and T1470C (59).

Stronger evidence for mutation in the *ERG11* gene conferring fluconazole resistantce provided by comparing the sequence of the *ERG11* allels of match pairs of fluconazole-susceptible and resistant isolated obtained at different infection episode from the same patients. An R467K mutation was detected in both *EGR11* allels of a fluconazole resistant *C. albicans* isolates as compared with matched isolates from the same patient with higher sensitivity (60).

A successful approch to demonatrate the involvement of *ERG11* mutation in flucoanzole resistantce has been the heterologous expression of different *ERG11* allels in *S. cerevisiae* and comparing the susceptibility of the strains to fluconazole Kakeya and co-wokers study the mucleotide substitution in Darlington strain of *C. albicans* by gene replaement in *C.albicans* and expression in *S. cerevisiae*. The *CYP51* were sequenced, both alleles contained the histidine for tyrosine substitution at position 132 reported in Darlington by others. And also found a threonine for isoleusine substitution not previously reported in the *C.albicans ERG11*. The encoded I471T change in amino acid conferred azole resistance when overexpress alone and increased azole resistance when added to the Y1321T amino acid sequence in *S.cerevisiae* expression system (61).

The nucleotide substitution that cause amino acid change and contributed azole resistant always located near or in the position of the iron atom in the heme cofactor such as R467K. In general the active site of this enzyme represents a pocket positioned on top of the heme cofactor. Substrate or inhibitor enter the active site through a channel that is accessible only without a shift in an α - helix of the apoprotein. Mutation in the active site pocket, in the channel, and/or in the mobile helix would be predicted to affect the function of the enzyme. (32, 58).

Although, there are many reported the nucleotide substitution can contributed the azole resistance. Cowen and colleages reported no *ERG* substitutions were detected in the result from experimental strain *C.albicans*. So the resistance mechanism may result from the multiple resistant mechanism.

3.Decreased accumulation of Drug and expression of efflux pump.

An inportant mechanism of fluconazole resistance is reduced intracellular aaccumulation of the drug. Inrecent years, it became evidence that fluconazole is actively transport out of the cells in an energy-dependent manner and r\that enhanced drug efflux is caused by overexpression of gene encoding membarne transport protein (63). Eukaryotic cells contain two types of efflux pumps that are known to contribute to drug resistance:

ATP binding cassetle (ABC) transporter and major facilitator (CMF) transportor.

The ABCT are composed of four protein domains : two membrane – spanning domain (MSD) each consisting of six or seven transmembrane spanning segments and two mucleotide binding domains (NBD). The NBD of ABCT bind ATP through an ABC that consists of serves conseved peptide motifs (12). The ATP that is bound to the ABC is used as a source of energy for the ABT. The ATP energy caused transport of the substrated molecule is unknow (32). To date, eight genes for ABC transporters have been identified in C*andida*. An example of an ABC transporter found in both C*andida* and *Cryptococcus* is *CDR1*, which is involve in resistance to fluconazole and other azole (7).

The MF composed primarily of 12 – 14 transmembrane segments and use the proton motive force at the membrane 4 gradient of H+ across the membrane as a source of energy when the proton are pumped into the cell and substrate molucule are pumed out (32). The MF drug efflux proteins associated with the transport of structurally diverse compounds and account for a range of resistance to toxic compounds in microorganisms. An example of MFS protein associated with drug resistance in C*andida* is BEN^{r} (*CaMDR1*) which is implicated in resistance to several drugs, including benomyl, methotrexate and fluconazole. (12).

13 efflux pumps have been described in *C. albicans*. These genes are named *CDR* and have been linked to azole drug resistance. The study to monitered *CDR1* mRNA levels in azole resistant clinical isolated of *C. albicans*, when the *C. albicans* isolated failed to accumulate 3H – labelled fluconazole. The result show fluconazole resistant clinical *C.albicans* isolates exhibited up to a 10 – fold relative increase in

mRNA level for a ABC transporter, *CDR1* gene. In an azole – resistant *C. albicans* isolates not overexpressing *CDR1*, the Ben was massively overexpressed (1). In unrelated study, *C.albicans* FR2 the isolated growth enrichment in fluconazole – containing medium, shown reduced rate of fluconazole accumulation compared with wild type and elevated amounts (2 to 17 fold) of mRNA encoding *CDR1* and *BEN*^{r} (64). In a third study, five *Candida albicans* isolates from recurrent episodes of infection which become gradually resistant fluconazole during treatment. The mRNA levels of *MDR1* gene were enhanced and constitutive high expression of the *ERG 11* gene correlated with a stepwise development of fluconazole resistance (4).

White examined the expression of several genes, in 17 isolates including *ERG16, MDR1, CDR1*. The result show that *MDR1* express was incresed early in the resistance, while the *CDR1* mRNA level was increased only in the final isolates, *ERG16* signal increased toward the end of the serie and increase in mRNA levels of *ERG 16* and *CDR 1* correlated with increase resistance to ketoconazole and itraconazole (65).

It is believed that the mutation leading to gene upregulation might be caused by alterations in trans (those involving transcription factors). Using the *Renilla* luciferase reporter system fused to *CDR1* and *CDR2* promoters cloned from azole-susceptible isolates, Micheli and his colleages showed that their expression was enhanced in an azole-resistant strain. In which these genes are constitutively upregulated. Until now, only the *CDR1* and *CDR2* promoters have been dissected systematically for the presence of regulatory elements. A common drug-responsive element (DRE) in both promoters could be experimentally delimited with the consensus 5′-CGGA(A/T)ATCGGATATTTTTTTT-3′, which has no equivalent in eukaryotic promoter databases. This DRE is necessary for *CDR1* and *CDR2* transient upregulation by drugs and for constitutive up regulation in an azole-resistant isolate. However, the detailed pathway resulting in *CDR* gene upregulation and the identity of proteins binding to the DRE have still to be determined (66).

4. expression of the *ERG11* gene

C. albicans upregulates the *ERG11* gene, presumably as a feedback mechanisms to make up for ergosterol depletion (67). Frank and co-workers reported

that even in the absence flucoanzole some fluconazole resisatnt isolates express *ERG11* mRNA at higher level than macthed set susceptible isolates in the presence of drug (68). *ERG11* overexpression has been found in many other fluconazole resistant *C. albicans* . Enhanced gene expression is expected to result in higher exzyme level so need for higher intracellular fluconazole concentrations to inhibit enzyme activity. Lamb and co-workers reported the experiment of overexpression of *ERG11* from *C. albicans* conferred a five-fold enhanced resistant to fluconazole in *S. cerevisiae* as compared to transformants carrying the vector control. Therefore, constitutive *ERG11* overexpression may contribute to fluconazole resistance in clinical *C. albicans* strains (14).

The combination of resistance mechanisms seems to be associated with a high level of azole resistance. Alterations of the target enzymes by several distinct single or multidrug transporters from two different families provide much flexibility in the combination of resistance mechanisms. Molecular epidemiology of azole resistance performed mainly with *C. albicans* isolates demonstrated that the diversity of resistance mechanism combinations was high enough for them to be only very few azole-resistant isolated with identical patterns of *ERG11* mutations and profiles of multidrug transporter genes expression. The relative frequency of resistance mechanisms in a large population of azole-resistant isolates has been investigated in only a few studies. Perea et al. showed that 85 percent of azole-resistant isolated upregulated multidrug transporter genes and that 65 percent contained *ERG11* mutations linked to azole resistance. Overall, 75 percent of the azole-resistant isolates showed combined resistance mechanisms. Sanglad showed the isolates from 18 HIV patient upregulation of multidrug transporter genes, 63 percent contained *ERG11* mutations linked to azole resistance, and 50 percent showed combinations of resistance mechanisms (10, 15).

Another interesting alternative for developing azole resistance has recently been described. It uses the ability of fungal pathogens to build biofilms on synthetic or natural surfaces (69). Biofilms are organized as a dense network of diffentiated cells onto which a layer of extracellular matrix can form. Biofilms can constitute a physical barrier for the efficient penetration of antifungals, which could explain that cells embedded in these structures can become recalcitrant to their action.

Measurement of drug susceptibilities in biofilms of *C. albicans* yielded high MIC values for azoles and amphotericin B as compared to planktonic cells (70, 71). In *C. albicans*, the expression of genes involved in azole resistance (multidrug transporter genes) can also be altered in biofilms and may contribute to the relatively high azole resistance measured in the cell population of these dense structures (72).

The antifungal mechanisms and resistant mechanisms are summarizing in the table below.

Table 2. Antifungal agent : activities against principal modes of action and resistance mechanism of fungal pathogen (10).

Antifungal agents: activities against principal modes of action and resistance mechanisms of fungal pathogens.

Antifungal	Spectrum/comments	Mode of action	Mechanism of resistance observed in clinical isolates
Polyenes			
Amphotericin B	Broad activity against *Candida* spp (except *C. lusitaniae*), *Cryptococcus neoformans* and filamentous fungi (except, of the *Aspergillus* spp, *A. terreus* and *A. flavus*).	Binding to ergosterol and destabilization of cell membrane functions	Alteration in specific steps of ergosterol biosynthesis
Pyrimidines analogues			
5-fluorocytosine (5-FC)	Active against *Candida* spp and *Cryptococcus* spp; however, rapid emergence of resistance can appear when 5-FC is used as monotherapy	Impairment of nucleic acid biosynthesis by formation of toxic fluorinated pyrimidine antimetabolites	Decreased uptake of 5-FC; decreased formation of toxic antimetabolites.
Azoles			
Fluconazole	Active against *Candida* spp and *Cryptococcus* spp, less active against *C. glabrata* and no activity against *C. krusei*; no activity against filamentous fungi	Inhibition of cytochrome P450 14α-lanosterol demethylase	Enhanced efflux by upregulation of multidrug transporter genes.
Itraconazole	Like fluconazole, but enhanced activity against filamentous fungi		Target alterations by occurrence of mutations
Voriconazole	Like fluconazole, but enhanced activity against filamentous fungi, including *Aspergillus* and *Fusarium* spp		Alteration of specific steps in the ergosterol biosynthetic pathway
Posaconazole	Closely related to itraconazole, but more active		
Allylamines			
Terbinafine	Active against most dermatophytes, poor activity against *Candida* spp	Inhibition of squalene epoxidase	Unknown
Morpholines			
Amorolfine	Active against most dermatophytes, poor activity against *Candida* spp	Inhibition of sterol Δ^{14} reductase and Δ^{14} isomerase	Unknown
Echinocandins			
Caspofungin	Active against *Candida* spp with fungicidal activity, moderately active against *Aspergillus* spp, poor activity against *C. neoformans*	Inhibition of the cell wall synthesis enzyme β-1,3 glucan synthase	Unknown