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Basis of antifungal resistance and it's mitigation strategies

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Abstract
Fungal infections are emerging now a days causing a wide spectrum of diseases in humans and animals mostly affecting patients with immunodeficiencies or chronic illness. As such now a days we can see that rise in fungal infection in patients suffering from Covid-19. It has become a measure challenge for clinicians to treat invasive fungal infections as there is only limited classes of antifungal agents are available. So this causes the over use of antifungal agents which gives opportunity to fungal pathogens to develop resistance. The main five types of antifungal use nowadays are mainly azoles, echinocandin, polyenes, alamines and pyrimidine analogues. These compounds mainly act through different mechanisms to show there fungicidal or fungistatic effect. In response to this, fungal pathogens have developedcertain mechanisms which enable them to escape or to overcome the effects of antifungals. Due to these mechanisms the resistance against this antifungal compounds as emerged worldwide.

Keywords: Antifungal, azoles, biofilm, drugs, novels, resistance

Introduction
Fungal infections are emerging now a days causing a wide spectrum of diseases in humans and animals mostly affecting patients with immunodeficiencies or chronic illness. Additionally, organ/tissue transplant patients are at high-risk for fungal infections. Factors such as prolonged lung colonization and frequent exposure have proved to greatly influence the severity of these fungal infectionsThe introduction of the first antifungal compounds was in the late 1960s, resistant fungal isolates have been reported in many countries worldwide since then [1]. As such now a days we can see that rise in fungal infection in patients suffering from Covid-19 [2]. Present-day antifungal treatment constitutes five main classes of antifungals, which comprise the: polyenes, imidazoles and trizoles, allylamines, echinocandins and the compound 5-flucytosine. The most common resistance is against azole compounds and mainly reported in Candida species [3]. Ability to form biofilm, alter mitochondrial function, overexpression of E flux pumps and mutation gives them ability to develop resistance. Azole resistant isolates of *Aspergillus fumigatus* are reported in many countries. In over last 10 years there is a rise in Azole and echinocandin resistance in Candida species showing the emergence of multidrug resistance strains [4]. This resistance can transfer to one another through sexual reproduction which further increases the chances of mutation and causing genetic variability. Chromosomal aneuploidy and phenotypic plasticity are further causes of transmission and emergence of resistance. The basic reason emergence of antifungal resistance is inappropriate use of antifungal drugs. The dose and spectrum of action effects the emergence of drug resistance. This is mainly due to inability of early diagnostic techniques as in case of severe or immunocompromised patients there is not adequate time for diagnosis using current methods (histopathology analysis and phenotypic examination of colonies cultured using selective agar plates). These methods are time consuming and due to which broad-spectrum antifungal is used in critically affected or ill patients. So, to overcome resistance certain mechanisms like search for novel antifungals, combination antifungal therapy and antifungal drugs plus non antifungal drugs can be used. This increases the effectiveness of the drug and decreases the chances of resistance.

Antifungal compounds
A substance known as an antifungal agent when it selectively removes the fungal pathogens from a host with no or little harm to the host [5]. The Major antifungal classes and their mechanism of action is given in Table 1 and Fig 1. The mechanism of antifungal resistance is given in Fig 2.

Biofilm

A network of cells that are associated to each other or a surface forms a biofilm. There is production of extracellular matrix by these cells that provides protection against stressful environment [6]. The ability to form biofilm is used by many pathogenic yeast and moulds. This biofilm act as a shield against host defense and antifungal compounds. Further it increases adherence to the host surface. The diffusion of antifungals into the cells is prevented by formation of extracellular matrix. Biofilms formed by *Candida albicans* show very high MIC against multiple antifungals. There was the high amount of extracellular DNA was found in extracellular matrix of *Candida albicans* biofilms, which is thought to contribute to the structure and formation of biofilm [7, 8]. Recent studies showed that various plant derived preparations and compounds that showed an activity against biofilm formed by candida species. A total of plant extracts and 29 essential oils were found to be effective against biofilm forming candida [9]. Moreover, this could be an alternative approach to overcome antimicrobial resistance in fungus. In water distribution network biofilms served as the primary repository for microorganism and are incredibly biologically diverse. Within this networks interkingdom biofilms can be found between fungi and bacteria [10].

Biofilm Formation

It is a multistage procedure which depends on the properties of microorganisms, construction and properties of the colonized materials of the host. Biofilm factor affecting anti-fungal resistance include biofilm growth and extracellular matrix.

Biofilm Growth

Study performed on minimal inhibitory concentration values of nystatin, amphotercin B, fluconazole and chlorhexidine antifungals during early, intermediate and maturation phases of Candida biofilms. It was seen that there was huge increase in resistance with increase in metabolic function of expanding biofilm [11].

Extracellular matrix

Extracellular polymeric substances act as physical barrier that prevents a access of antimicrobial to the cells embedded in the biofilm community. In a study it was seen that the survival rate of the candida cells in biofilms decreased about 20% when EPS was removed (treated with amphotericin B) [12]. In a recent study it was seen that various amino acid metabolism pathway were significantly upregulated in high biofilm forming isolates as compared to low biofilm forming isolates. There was upregulation of AAT1, gene encoding and aspirate amino transferase which play certain role in metabolic pathway in biofilm forming isolates. It suggests that it could be a potential target for antifungal compound development and as a biomarker for these high biofilms forming isolates [13].

Structure target site alteration

There is emergence of resistance in many pathogenic fungi due to mutation in genes encoding antifungal targets. There is alteration in amino acids due to non-synonymous SNPs which further alter the structure and reduce binding affinity of the antifungal to the target. Antifungals like azole compounds act by disruption of ergosterol biosynthesis. This is done by inhibition of sterol, 1, 4 alpha demethylase which is a cytochrome p 450 enzyme. The gene ERG11 in yeast and CY351 in moulds and code this enzyme. If there is some substitution in amino acids which causes structural changes in the active site of demethylase leading to azole resistance. In some cases, it is seen that due to increase transcription of CYP51A there is emergence of azole resistance [14]. In *Candida neoformans* the single amino acids substitution was shown to confer resistance against fluconazole [15]. In isolates resistant for the allylamine (Terbinafine) was created due to substitution in amino acids found in squalene epoxidase which is the target site of terbinafine. In *Aspergillus fumigates* substitution in the *erg*A gene was found to confer resistance to terbinafine. The mutation in FKS1 gene causes resistance to echinocandin. This gene encodes for 1,3 beta glucan synthesis which is one of the main components of fungal cell wall. The resistance to antifungal 5 flucytosine is formed due to mutation in Fcy2 permease which prohibits 5 flucytosine to enter the cells. Due to this mutation there is no conversion of 5 flucytosine to toxic 5 chlorouracil which leads to resistance.

Metabolic Bypass

It is a mechanism by which pathogenic fungi formed resistance to antifungal components by preventing the accumulation of toxic metabolites which is done by inactivation of another enzyme in the pathway. In *Candida albicans* this mechanism has proven to confer the resistance against azole compounds. The mechanism of action of azole compounds is inhibition of 14 alpha leno sterol D methylase which is a key enzyme in ergosterol biosynthesis. Due to the inhibition of this enzymes, there is depletion of ergosterol and accumulation of toxic 1, 4 alpha demethylated sterol in the membrane. The gene Erg 3 encodes for C5 sterol desaturase which catalyses the ergosterol biosynthesis. When this gene is nonfunctional there is no conversion of 14 methylated sterol into toxic sterol [16]. Thus, preventive disturbance of the cellular membrane and probiotic resistance.

Over-expression of E flux pumps

There are specific e flux pumpswhose overexpression gives the ability to fungal pathogens to excrete the antifungal compounds. This resistance mechanism is found in many fungal species as there is increase gene expression of E flux pump protein complexes. There is increase of transportation of antifungals outside the cells. In a study azole resistance to upregulated E flux pump expression was reported for multidrug resistance, CDR1 and CDR2 (Candida drug resistance genes) in man Candida species [17]. In another study exposure of *Aspergillus fumigatus* was done to itraconazole showed upregulation of many unknown putative drugs e flux transporter [1].

Resistance due to Mitochondrial alterations

In various studies it is seen that mitochondria plays a significant role in formation of antifungal drug resistance. *Candida glabrata* isolates showed increase fluconazole resistance upto 50 micro gram per ml when there was loss of mitochondrial function [18]. In a study on *Aspergillus fumigatus* strains that had mutation in mitochondrial complex 1 showed that these strains were azole resistance. This mutated strain had MIC of 2-4 milli gram per liter itraconazole whereas the parent strain had mic of 0.25 milli gram per litre. From this study it can be concluded that there is a direct relationship between mitochondrial dysfunction and azole resistance [19]. The country wise reporting Azoles-resistant isolates of *Aspergillus fumigatus* is given in Fig 3

and comparison of azole and echinocandin resistance in *Candida spp*. Isolates is shown in Fig 4.

Stress pathways activations

Cellular stress signaling is essential to survive stressful conditions in environment. This mechanism also helps in protecting the organisms against drug induced stressed conditions. In fungus there is a rapid transaction of signals through mitogen activated protein kinase pathways which help to overcome stressful conditions. This pathways signal through successive phosphorylation of protein kinases. To overcome osmotic, oxidative and cell wall related stress the fungi undergo to pathways namely high osmolarity glycerol pathway and cell integrity pathway. When there is damaged to the cell due to antifungal molecules activation of one or more stress response pathways helps cells to respond to damaged and increase its survival.

Membrane homeostasis adjustment

Eukaryotic membranes are complex structures that contain many lipids which contribute to membrane integrity and maintenance. Azoles and amphotericin B act by disturbing cellular ergosterol content which results in membrane destabilization and lysis of cell. In some studies, it is concluded that cell can adjust its membrane homeostasis to make it more rigid and more fluid. The alteration in ratio of phospholipid species helps cells to achieve this goal. It is said that all phospholipid species have different chemical physio properties which contribute to nature of the membrane [20].

The origin of Antifungal resistance development

Due to nature of selection there is emergence of antifungal resistance mechanisms which benefits the organism to adapt to antifungal compounds. These adaptations depend on certain processes that influence genetic and physiological changes. The evolutionary drivers of antifungal resistance include heritable variation, high reproductive output and differential survival. These drivers are further divided and depend on various factors.

Phenotypic plasticity cause increase antifungal drug resistance

As the cells are not able to move away from unfavorable conditions. They have an ability to change their metabolism as a response to changing conditions. When these cells grow and divide in unfavorable environment this process is known as adaptive phenotypic plasticity. In recent study on *Aspergillus fumigatus* clinical isolates expose to itraconazole showed there was a upregulation of specific E flux pumps, differential expression patterns in ergosterol and phospholipid biosynthesis roots [1]. In another study it was found that there was increase in chitin production in *Candida albicans* after exposure to Caspofungin [21].

Mutation Frequencies and Mutation Rates

The genetic variability is usually determined by the rate of spontaneous mutations arise in the organisms. As exposure of antifungal compounds to pathogenic fungi via environment or through patient creates a stressful environment for the fungi. During these stressful conditions maintenance and repair pathway functions at a low level which increases the mutation rate.

Chromosomal Aneuploidy

The major factor that drives anti-fungal resistance is ability to generate genomic variance in gene pool due to the instability of polyploid cells there is rapid generation of genetic diversity within a population. Some pathogenic fungi like *Candida albicans* displace this genomic flexibility which can lead to antifungal resistance. In a study Caspofungin tolerance was found in aneuploid. *Candida albicans* isolates with either loss of chromosome 5 or showed combined monosomy of the left arm and trisomy of right arm of chromosome 5 [22].

Sexual Reproduction: The development and spread of resistance mechanism somewhat depend upon method of reproduction. The asexual conidia can spread new mutations that originated during hyphal growth. In sexual reproduction there is reshuffling of chromosomes which leads to increase in genetic variability which further facilitates adaption to a changing environment [23].

Methods for overcoming antifungal resistance

As there is limited classes of antifungal agents therefore overcoming antifungal resistance becomes the major concern for doctors and scientist. The inappropriate dose of antifungals is one of the major factors for resistance development as it causes fungus to evolved against selective pressure. Moreover, in proper antifungal drug exposure also leads to resistance. The formation of drug resistance depends upon dose and mechanism of action of antifungal agent.

Early Diagnosis

The diagnosis of deep sheathed fungal pathogen in tissue or organ is almost impossible. This is a major reason which causes high mortality and morbidity during mycosis. Moreover, this delay in diagnosis further leads to delay in appropriate therapy. Currently the methods involve in diagnosis of fungal infections include histopathology analysis and phenotypic examination of colonies culture using selective agar plates. Histology analysis of biopsy samples is not reliable as they lack specificity sensitivity and taxonomic information. On the other hand, the second method requires the culture of samples on non-selective media to maximize the microbial count for 24 hours than they are cultured on genus specific agar plates. As these methods consumes lots of time there is a delay in identification of pathogenic organism. Therefore, it becomes very difficult to treat the patients suffering from life threatening fungal infections. In such circumstances the clinicians have to choose between time consuming confirmatory tests that may delay the therapy versus using broad spectrum antifungal which increases the risk of developing drug resistance.

From these shortcomings the diagnosis of fungal infections it is cleared that there is urgent requirement for early and effective diagnostic techniques. There are some techniques which target specific components rather than the whole cell. The non-culture-based techniques that detects cellular components such as mannan and galactomannan. Nucleic acids and beta glucan various serological test like immunodiffusion, complement fixation and ELISA are there for the diagnosis of endemic mycosis. Histoplasmosis can be easily diagnosed using complement fixation and immunodiffusion test.

Novel Antifungals

Large numbers of researchers and industrial laboratories are now a days screening synthetic and semi synthetic chemical libraries, natural extracts from plants, marine life and already existing medical compounds for their antifungal properties. There are several new antifungal medicines which are in

clinical and re clinical development phase. By various mechanism of action like inhibiting cell wall biosynthesis, in habiting proteins and amino acids synthesis, inhibiting electron transport chain and inhibiting sphingolipid biosynthesis. Recently plant derived nanoparticle to overcome the threat of antimicrobial resistance and biofilm forming in fungi. Recently a novel antifungal compound named 1, 2, 3 triazole has been discovered which is considered as the most powerful antifungal effect [24]. Moreover, this compound also exhibits antibacterial, anti-inflammatory, antiviral, antidiabetic and antitubercular properties [25].

Combined Antifungal Therapy

There are certain advantages of using combination therapy in place of single antifungal therapy. The main advantage is there is decrease chances of formation of resistance and for tolerance, enhances spectrum of activity and increase rate and extend of fungal killings. It is seen that azoles act in synergic way when combined with terbinafine providing good result against Candida.

Antifungal drugs in combination with non-antifungal drugs

It is seen that combination of antifungal drugs with known antifungal drugs shows benefits against fungal infection. Certain non-antifungal drugs like protein pump inhibitors, antiarrhythmic drugs, cholesterol lowering agents, immunomodulators, anti-neoplastic drugs and anti-parasitic agents can be used in a combination with antifungal drugs. As it is seen that cyclosporine along cannot inhibit fungal growth but when given in combination with fluconazole it increases its susceptibility due to E flux pump deletion. Hematopoietic growth factors such as granulocyte colony stimulating factors or granulocyte macrophage colony stimulating factors has seen activity of the antifungal function of phagocytes and it increases the efficacy of antifungal agents which is found that fractional carbon dioxide laser therapy in combination with topical antifungal such as terbinafine is effective in treatment of onychomycosis [26].

Antifungal Stewardship

The main goal of antifungal stewardship programme used to preserve the future effectiveness of antifungals and improve patient's outcome. The key factors and points that are taken in consideration includes optimal antifungal dose, route of administrations and duration of a therapy [27]. Further there should be proper guidelines and diagnostic test that guide us when therapy should be start and stop. Therapeutic drug monitoring is done to optimize the treatment response and minimize its side effects (Fig 5). These drug monitoring can help us to choose between alternative agents, formulations or routes of administration in those cases where optimal drug concentration cannot be obtained by one drug. Moreover, it is important in monitoring and managing patients that are undergoing long term therapy and are at a risk of developing resistance.

Table 1: Major antifungal classes and their cellular targets

Chemical class	Compounds	Target pathway	Molecular target
Azoles	Fluconazole Itraconazole Voriconazole Posaconazole Isavuconazole Ketoconazole	Ergosterol biosynthesis	14α-sterol demethylase
Echinocandins	Anidulafungin Caspofungin Micafungin	1, 3 β-glucan biosynthesis	1, 3 β-glucan synthase complex catalytic subunit
Polyenes	Amphotericin B Nyastatin Natamycin	Ergosterol biosynthesis	Ergosterol in fungal membranes
Allylamines	Terbinafine, Naftifine	Squalene epoxidase	Squalene epoxidase
Pyrimidine analogs	Flucytosine	Pyrimidine salvage pathway	DNA/RNA molecules

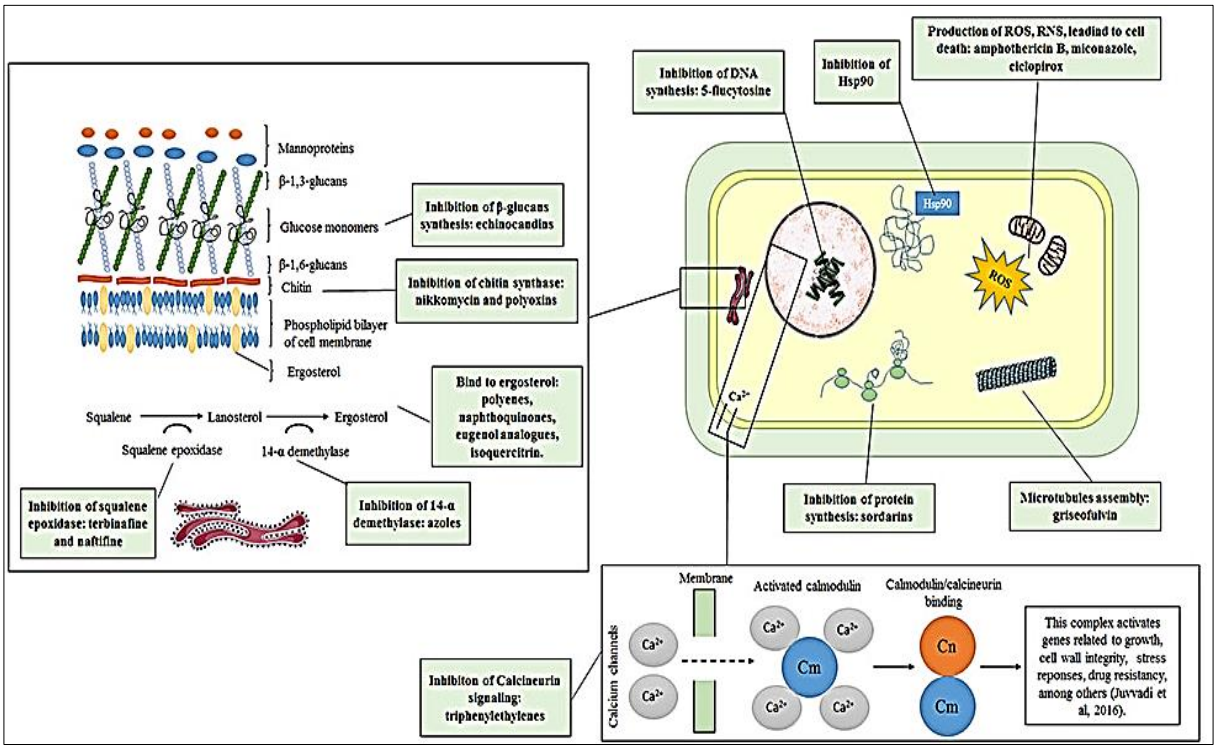

Fig 1: Mechanism of Action of various antifungal compounds [11]

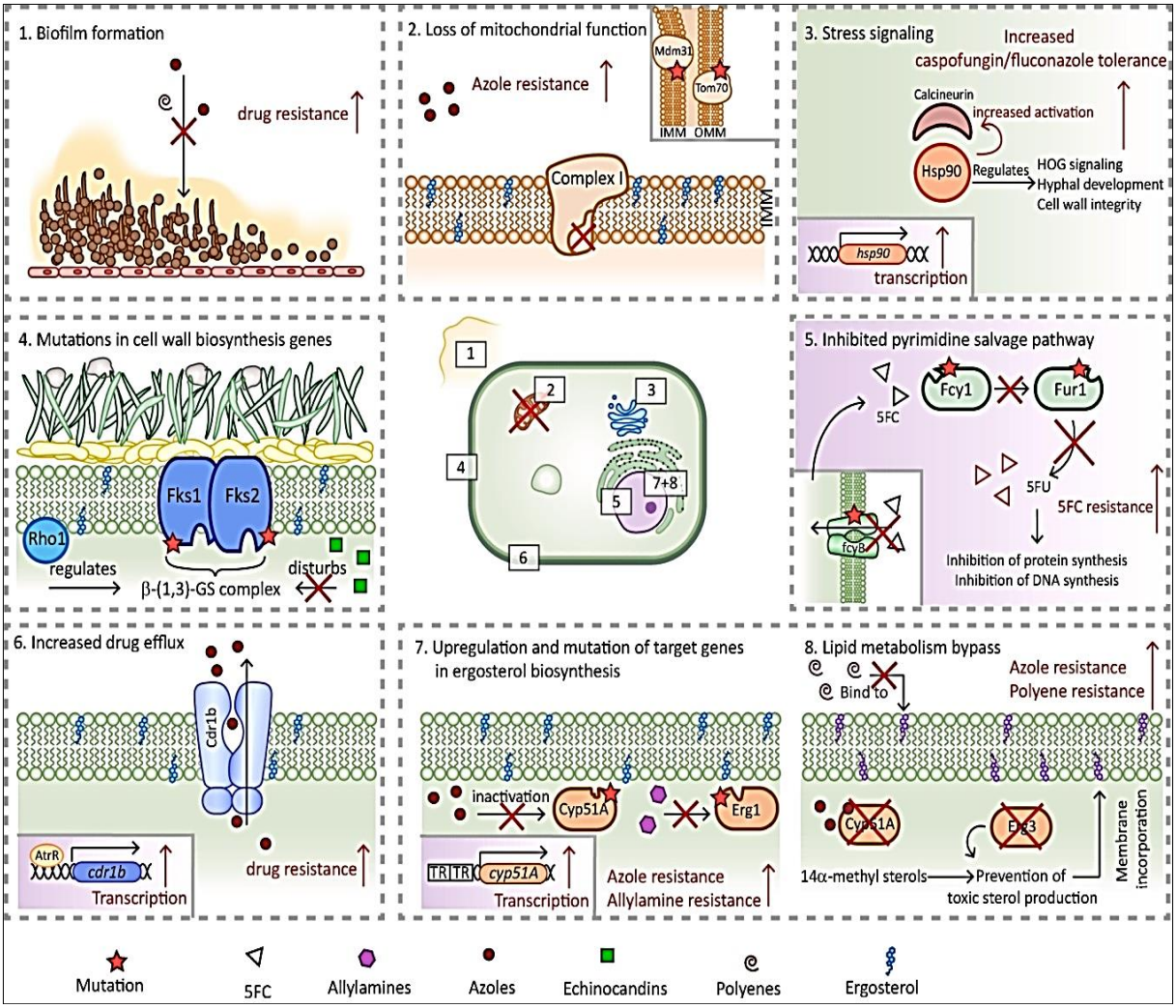

Fig 2: Mechanism of resistance to various antifungal compounds [1]

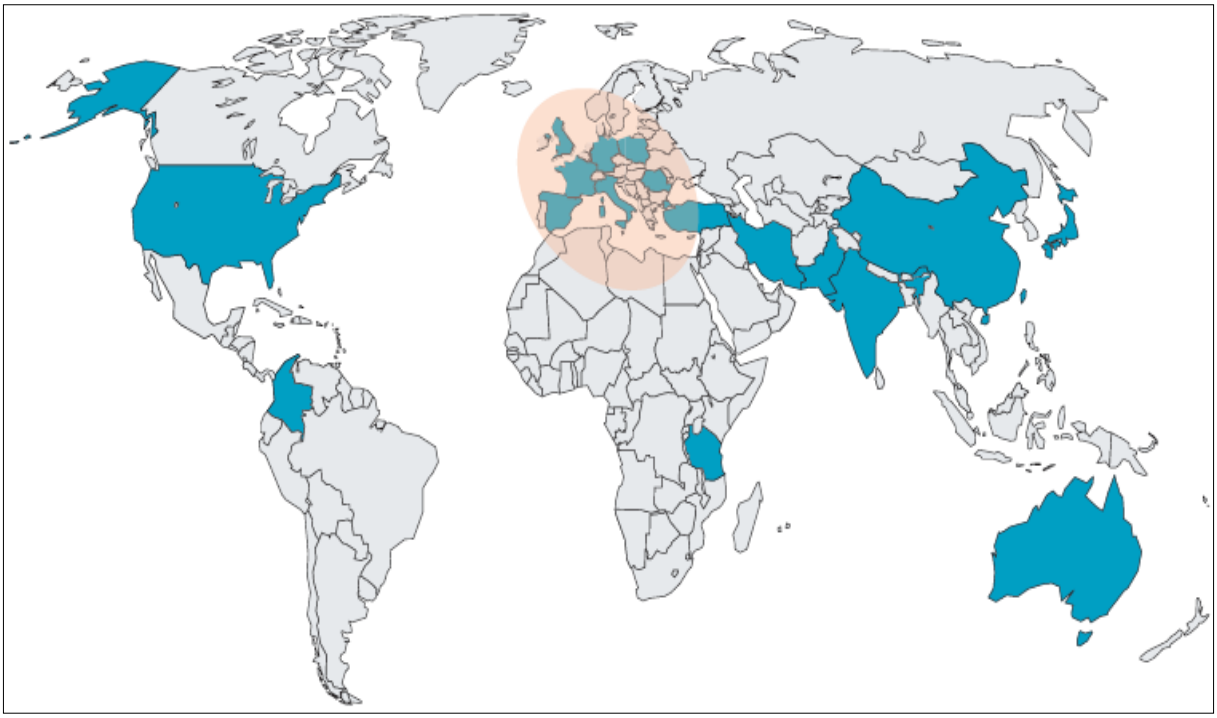

Fig 3: Countries reporting Azoles-resistant isolates of *Aspergillus fumigatus*. Blue – Resistant Oval shaped -Highest burden of resistance (2017) [27]

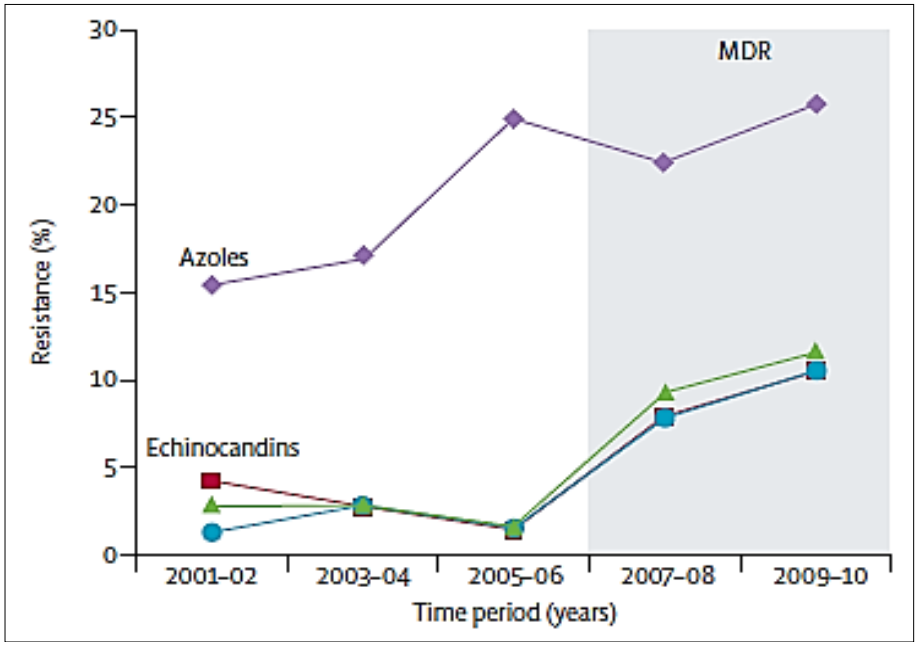

Fig 4: Parallel rise in azole and echinocandin resistance in *Candida spp.* isolates over a 10-year period, showing emergence of multidrug resistant strains. The grey-shaded box shows the time of emergence of substantial multidrug resistance. The three echinocandin-class drugs are shown: red, anidulafungin; green, caspofungin; blue, micafungin [27]

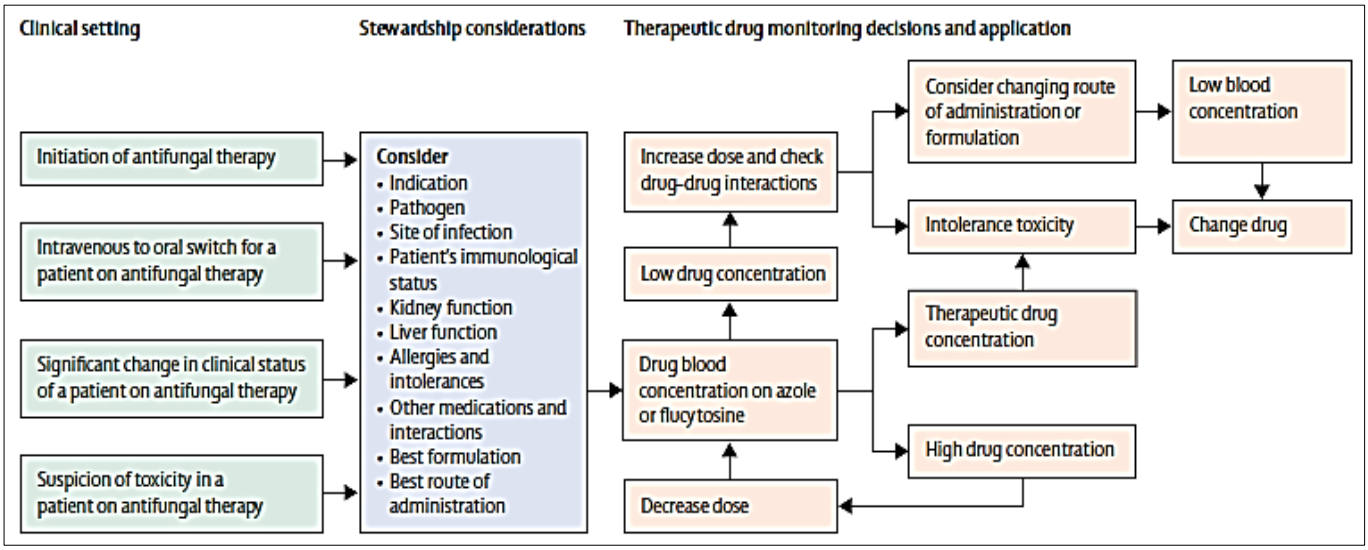

Fig 5: Antifungal Stewardship consideration [27]

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Conclusion

Systemic fungal diseases are difficult to treat as compared to topical fungal diseases. Most of the drug resistance strains of fungi are mostly causing deep mycosis. However, in some strain of superficial mycosis drug resistance is seen nowadays. The solution to this drug resistance problem is use of appropriate drugs against diseases, proper doses and duration of drugs, quick diagnosis and appropriate susceptibility testing should be done. New methods like drug combination specially combining antifungal drugs with other antifungal drugs and with known antifungal drugs can be used to prevent resistance. As there is not appropriate knowledge about the presence of new antifungal compounds in the nature. These compounds can be used to overcome the problem of resistance and will increase the chances survival in patients suffering from systemic fungal diseases. Moreover, with advancement with technologies there is development of new diagnostic techniques which will further help in overcoming antifungal resistance and this new insight will increase the knowledge and help in overcoming the antifungal resistance programme.

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