ANTIFUNGAL SUSCEPTIBILITY TESTING FOR CLINICAL ISOLATES USING DILUTION METHODS

Submitted in partial fulfillment of the requirement for the award of Master of Technology in Biotechnology

By

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BONAFIDE CERTIFICATE

This is to certify that this project report is the bonafide work of "Adithya S. (39810001)" carried out the project entitled "Antifungal susceptibility testing for clinical isolates using dilution methods" under our supervision from January 2021 to March 2021.

Internal Guide **External Guide**

Head of the Department

Submitted for Viva voce examination held on

Internal Examiner **External Examiner**

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DECLARATION

I “Adithya S. (39810001)” hereby declare that the project report entitled “Antifungal susceptibility testing for clinical isolates using dilution methods” done my me under the guidance of Dr. P. Durgadevi (External guide) and Dr. L. Inbathamizh (Internal guide) at Vidyasagar Institute of Biomedical Technology and Sciences (A unit of Medical Research Foundation), Sankara Nethralaya, 41 College road, Chennai – 600 006 is submitted in partial fulfillment of the requirements for the award for the Master of Technology degree in Biotechnology.

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ABSTRACT

Fungal eye infections are one of the serious problems, since the progression of the fungal eye infections are very rapid, the need for correct treatment at the correct time is very important. 30 eye infecting fungal samples isolated from the patients have been tested for Minimum Inhibitory Concentration (MIC) for antifungal agents such as Amphotericin B, Natamycin and Voriconazole using dilution method broth microdilution. The MIC values of Amphotericin B and Voriconazole were found to be very low of lesser than 1µg/ml for most of the fungal isolates and for natamycin most of the fungal isolates were resistant and some of them were susceptible only at high concentration ranges. One of the unidentified fungal clinical isolate's DNA has been isolated and PCR was performed for the isolated DNA targeting the ITS region. The PCR product was amplified again for cycle sequencing which was sequenced. The sequencing result was used in the NCBI BLAST to identify the species and antifungal susceptibility testing was performed for it.

Keywords: MIC, Broth Microdilution, Keratitis, Amphotericin B, Natamycin, Voriconazole, Ocular Isolates.

List of Abbreviations

MIC – Minimum Inhibitory Concentration

FSSC – Fusarium solani Species Complex

CLSI – Clinical and Laboratory Standards Institute

EUCAST – European Committee on Antimicrobial Susceptibility Testing

ICMR – Indian Council of Medical Research

PDA – Potato Dextrose Agar

Mbp – Mega base pair

LD_{50} – Lethal Dose 50%

SDA – Sabouraud Dextrose Agar

YNB – Yeast Nitrogen Base

AL buffer – Lysis buffer

AW1, AW2 buffer – Wash buffer 1 & 2

AE buffer – Elution buffer

DNA – Deoxyribonucleic acid

PCR – Polymerase Chain Reaction

dNTP – deoxynucleoside triphosphate

ITS1 & ITS4 – Internal Transcribed Spacer 1 & 2

Exo – Exonucleases

SAP – Shrimp Alkaline Phosphatases

EDTA – Ethylenediaminetetraacetic acid

NCBI – National Centre for Biotechnology Information

BLAST – Basic Local Alignment Search Tool

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1. INTRODUCTION

Fungal eye infections are more severe and can cause prolonged infection than bacterial eye infections. Most of the fungal eye infection is caused by filamentous fungi belonging to the species of *Aspergillus, Fusarium* and for yeast *Candida sp.* Fungal infection can be cause serious damages to the eyes and if not treated properly could lead to loss of vision. Fungal infections are particularly difficult to treat and as the eyes are the very sensitive organ of the human body and treating of fungal eye infections are quite challenging. So, Antifungal susceptibility testing should be performed to find appropriate antifungal for particular fungal infection (Kumar *et al.,* 2018).

Antifungal susceptibility testing is performed to find out the Minimum Inhibitory Concentration (MIC) of an antifungal agent. The antifungal susceptibility testing is performed in large numbers in recent years as the number of invasive fungal infection are increasing day by day. Since the increase in the fungal infections the need for the accurate concentration or dosage required for the antifungal agents is of high necessity. Thus, antifungal susceptibility testing provides the option for testing the antifungal agents in vitro and determining the MIC of the antifungal agents (Berkow *et al*., 2020).

Aspergillus flavus is a saprophytic fungus present in the soil worldwide. They mainly cause disease to plants such maize, peanut and cotton. They can also cause infection in animals and humans (Amaike *et al*., 2011). *Aspergillus fumigatus* is a saprophytic fungus which recycles the carbon and nitrogen present in the environment. This fungus is generally present in soil where it performs these roles. This is a ubiquitous airborne fungus, which produces conidia or spores into the air which humans inhale. Inhalation of these conidia may cause some lung diseases in the immunocompromised individuals, whereas in normal or healthy individual the conidia are removed by the immune systems. *Aspergillus fumigatus* is a weak fungus. But in recent years this *Aspergillus fumigatus* has become a serious and invasive pathogen, which causes fatal infection in immunocompromised individuals. *Aspergillus fumigatus* is responsible for about 90% of the fungal infection caused by the *Aspergillus species* (Latge, 1999). Both *Aspergillus flavus* and *fumigatus* is responsible for the eye infection called keratitis. Keratitis is the inflammation of the cornea of the eye. Keratitis is usually caused by the

settling of the infectious fungal species in the parts of the eye or due to the injury which may lead to the settling of the fungal species in the eye (Cherney, 2017). *Fusarium* a saprophytic fungus and it can cause opportunistic infection in humans. *Fusarium solani* can causes serious and invasive mycoses diseases in immunocompromised individual and rarely even cause death. It is unknown whether the plant infecting *Fusarium solani* species can cause infection in humans. *Fusarium solani* has over 45 different phylogenetic species, together they are called as *Fusarium solani species complex* (FSSC) (Zhang *et al*., 2006). *Fusarium solani* causes endophthalmitis. Endophthalmitis is fungal or bacterial infection of the eye which affects the vitreous and aqueous humors. The fungal organism is usually settled in the eye due to injury. If untreated could lead to loss of vision permanently (Durand, 2013). Fungal infections are controlled by antifungal agents which inhibits the fungal growth.

Amphotericin B is one of polyene macrolide antifungal agent produced by *Streptomyces nodosus*. It causes fungal cell death by altering the membrane permeability through attachment to a sterol site on the cell membrane (Waugh, 2007). Natamycin is a polyene amphoteric macrolide antifungal agent. It works by attaching to the sterols in the fungal cell membrane which increases the membrane permeability which causes leaking and loss of essential cellular nutrients and constituents (8). Voriconazole is a triazole based antifungal agent. It functions by inhibition of cytochrome P450 dependent lanosterol demethylation which a vital step in ergosterol synthesis in fungus and causes to the death of the cell (Saravolatz *et al*., 2003). Fluconazole is an another triazole based antifungal agent. It works by interrupting the conversion of lanosterol to ergosterol by binding to the fungal cytochrome P450 and causes the disruption of the membrane.

Agar dilution is one the most common dilution method used to find the MIC of antifungal agents, antibiotics and other substance which kills or inhibits the growth of the fungi. MIC is defined as the lowest concentration at which the antifungal agent prevents the growth of the fungi under in vitro condition. This method is employed to test the susceptibility, intermediate or resistance to the antifungal agent. MIC values can be used for surveillance and to compare the different antifungal agents to different species

with different susceptibility. It is the primary step in identifying the MIC of a new antifungal agent or antibiotic (Wiegand *et al*., 2008). Broth microdilution is another dilution method. This is the most popular method because of its accuracy of the results. Dilution methods are considered as reference methods for susceptibility testing and are used to evaluate the performance of other methods. The standards for performing antifungal susceptibility testing using dilution methods are given by Clinical and Laboratory Standards Institute (CLSI), European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Indian Council of Medical Research (ICMR) (Wiegand *et al*., 2008). As most of the filamentous fungi and yeast isolates are becoming resistant to the current antifungal agents (Spierer *et al.* 2015, Sun *et al. 2014)*. The development of new antifungal agents or drugs and evaluating the antifungal agent to find the MIC of the antifungal agent is crucial. The MIC values for the antifungal agents will be found using the dilution methods.

2. LITERATURE SURVEY

2.1 ANTIFUNGAL SUSCEPTIBILITY TESTING

Antifungal susceptibility testing is done for invasive fungal organisms. Antifungal susceptibility testing can be used to find the resistances, epidemiological studies and for the comparison of the in vitro activity of the new and currently available antifungal agents.

Antifungal susceptibility testing is used to find the MIC of the antifungal agents. MIC of an antifungal agent is the lowest concentration required to inhibit the growth of the fungal organism. It is measured in the unit µg/ml. MIC can be used for the treatment of the invasive infection caused by the fungal organism (Arendrup *et al*., 2020).

The increasing incident of the invasive fungal infection and their resistance to the currently available treatment options has made necessary for the standardization of the antifungal susceptibility testing. The standardization of the antifungal susceptibility testing will help to make progress in medical mycology. Thus, the current research is focused to overcome the limitations in the current methods.

The development of the antifungal susceptibility testing is of utmost importance's as there are increasing number of patients getting infected to new fungal infection, discovery of new antifungal agents and the fungal organisms are becoming resistant to the present antifungal treatment (Arikan, 2007).

The MIC is categorized as S – Susceptible, I – Intermediate and R – Resistance. The MIC values should be considered along with the other factors such as pharmacodynamics and pharmacokinetics.

2.1.1 Clinical and Laboratory Standards Institute (CLSI) standard susceptibility testing assay

Standards for antifungal susceptibility testing has been provided by CLSI (formerly National Committee for Clinical Laboratory Standards – NCCLS). They provided reference for yeasts such as *Cryptococcus neoformans* and *Candida spp.* and moulds such as *Sporothrixschenckii, Pseudallescheria boydii, Rhizopus spp., Fusarium spp., Aspergillus spp.* They have also given MIC reference for antifungal agents such as

voriconazole, ravuconazole, Posaconazole. Itraconazole, ketoconazole, fluconazole, flucytosine, amphotericin B. But there are no standard guidelines for the other fungal genera and the MIC values of some of the antifungal agents are still under research (Arikan, 2007).

2.1.2 European Committee for Antimicrobial Susceptibility Testing (EUCAST) standard susceptibility testing assay

After the development of CLSI and EUCAST they been developed for providing antifungal susceptibility testing. They provided the MIC reference for broth and broth microdilution methods primarily for *Candida spp.* It does not cover *Cryptococcus spp.* and other yeasts. EUCAST also provided MIC reference for antifungal agents such as flucytosine, Posaconazole, voriconazole, itraconazole, fluconazole and amphotericin B. But the EUCAST method MIC value was lower than the CLSI method (Arikan, 2007).

2.2 FUNGAL ORGANISM INFECTING EYES

Eye infection can be caused by variety of organisms including bacteria, virus and fungi. Fungal eye infections are rare but if suscept becomes really hard to cure it. There are two types of fungal eye infection one is keratitis which is the infection of the cornea of the eye and the second is the endophthalmitis is the infection of the vitreous of the eye. Endophthalmitis is of two types: exogenous endophthalmitis is the direct fungal infection of the eye and endogenous endophthalmitis, here the vitreous is affected by the bloodborne pathogen which reaches the vitreous of the eye. Some of the common fungi which causes this infection are the *Aspergillus spp., Fusarium spp., and candida spp.*

2.2.1 Aspergillus spp.

Aspergillus was first identified in 1729 by Pier Antonio Micheli. *Aspergillus* is an asexual spore forming fungi. *Aspergillus* consist of 837 species. Aspergillus is mostly found in the oxygen rich surfaces. *Aspergillus* can cause serious infection in humans and animals and they are also a common food contaminant. *Aspergillus* can also cause neonatal infections. *Aspergillus* primarily cause lung infections such as pneumonia and paranasal sinus infections. The symptoms may include breathlessness, chest pain, cough and fever. The individuals which weakened immune systems are more

susceptible to these fungal infections. The first incident of fungal keratitis is believed to be caused by the *Aspergillus species* in 1879, where a farmer got hit in the eye by an oat chaff. Agricultural works are most common factor for the *Aspergillus* related keratitis (Erdem *et al*., 2017). Recently, *Aspergillus* keratitis is also reported to appear after surgical procedures such as keratoplasty, laser assisted in situ keratomileusis and keratotomy.

2.2.1.1 Aspergillus flavus

Aspergillus flavus is pathogenic and saprophytic fungus which is found almost anywhere. *A. flavus* is an opportunistic fungus which causes infection in animals and humans, many in immunocompromised individuals, they cause aspergillosis. They commonly cause infection to crops. They have a hyphal growth and the colonies form yellowish-green colour. *A. flavus* can grow in quite high temperatures than other fungi organism. They can grow at a temperature range of 12-55ºC. *A. flavus* causes non-invasive pneumonia, sinusitis, cutaneous infection, neutropenia, hepatocellular carcinoma and hepatitis. *A. flavus* is responsible for the 80% of the keratitis caused by the fungi. *A. flavus* keratitis is usually treated by antifungal agents such as caspofungin, Posaconazole, voriconazole, itraconazole, amphotericin B. But some of the *A. flavus* strain has been resistant to voriconazole, itraconazole and amphotericin B.

It has been shown that *A. flavus* isolated from the keratitis patients showed significant production of aflatoxin which is not generally found in *A. flavus* isolated from the environment. This may be due to the normal eye temperature 33 to 34ºC. But the exact process is unclear (Leema *et al*., 2010). About 75% of keratitis caused by *Aspergillus sp.* belongs to *A. flavus* (Manikandan *et al.,* 2013).

2.2.1.2 Aspergillus fumigatus

Aspergillus fumigatus is a ubiquitous fungus present in soil and in compost heaps. This fungus recycles nitrogen and carbon. The spores of this fungus become airborne very readily. *Aspergillus fumigatus* is the common species in *Aspergillus* which causes diseases in immunocompetent individual.

This fungus is capable of growing even at temperatures of 50ºC and the spores can survive even in temperatures up to 70ºC. The spores present in air almost everywhere

and are constantly inhaled by humans and are easily removed from the body by the body's immune system but in immunocompetent individuals this might cause infections such as pulmonary aspergillosis and may even cause death, thus this fungus is called as opportunistic fungus. *Aspergillus fumigatus* has genome size of 29.4 mbp. Currently *Aspergillus fumigatus* is treated using imidazole, itraconazole and voriconazole.

In *Aspergillus fumigatus* infection of keratitis autophagy plays the innate immunity activation and primary defense mechanism against the fungal infection. This can reduce the clear the fungal organism and the infection (Li *et al*., 2020). About 11.5% of keratitis caused by *Aspergillus sp.* belongs to *A. fumigatus* (Manikandan *et al.,* 2013).

2.2.2 Fusarium spp.

Fusarium is a filamentous fungus which is also called has hyphomycetes. It is present in soil and in plants in symbiotic relationship. They produce mycotoxins in some cereal plants which when consumed by animals and humans are affected by it.

Fusarium spp. cause opportunistic infections in humans. The common infections caused in normal human beings are keratomycosis – an infection of the eye's cornea and onychomycosis – an infection of nails. In immunocompetent individuals they cause some invasive fungal infection. Some of the *Fusarium species* which causes fungal infection are *Fusarium solani, Fusarium oxysporum, Fusarium verticillioides, Fusarium proliferatum* etc.

2.2.2.1 Fusarium solani

Fusarium solani is a filamentous fungus of the division Ascomycota. It is commonly present in the soil. This fungus can cause keratitis in humans. When grown in PDA agar this fungus forms a cottony and white colony and grows rapidly. They first produce hyphae and then conidiophores. The conidiophore branches to produce conidia. Some of the strains of this fungus have phialides which produce macroconidia. They can also produce chlamydospores under some growth condition. These chlamydospores have are brown and round with rough walls and of size 6-11µm. *Fusarium solani* is ubiquitous fungus and are found in symbiotic relationships with the roots of the plants. They are usually found at a deep of 80cm. *Fusarium solani* has 5-13 chromosomes with a genome size of 40Mbp.

Half the infection caused by the *Fusarium spp.* is by *Fusarium solani*. They can cause endophthalmitis, fungemia, skin infection, osteomyelitis, corneal infection and fusariosis. *Fusarium solani* can form biofilms on contact lens, this however, can be prevented by soaking the lens in polyhexanide biguanide overnight. *Fusarium solani* can be treated with amphotericin B and natamycin.

Fusarium solani is a very tough fungus and a multidrug resistant mould which makes it difficult to treat. Endophthalmitis caused by Fusarium solani has been successfully treated by natamycin and voriconazole (Andres *et al*., 2018). About 44.7% of keratitis caused by *Fusarium sp.* belongs to *F. solani* (das *et al.,* 2014).

2.2.3 Candida spp.

Candida is a genus of yeast and are ubiquitous. Some of the species lives by endosymbiotic relationship with humans. This endosymbiotic *Candida* can cause diseases if the mucosal linings are broke or in immunocompromised individuals. Most common *Candida* infection is the candidiasis which affects the mouth which forms white patches in the tongue and other parts of mouth and throat. They can also cause systemic infections such as candidemia which is the infection of the bloodstream. Some of the common antifungal used to treat *Candida* are fluconazole, clotrimazole, miconazole and amphotericin B. *Candida* forms creamy, white and round colony in the culture. *Candida* can ferment sucrose, maltose and glucose into gases and acid.

2.2.3.1 Candida albicans

Candida albicans are opportunistic pathogen causing infection in immunocompromised individuals usually found in human gut. *Candida albicans* is one of the hospitals acquired diseases. About 60% of gastrointestinal tract contains *Candida albicans*. They can cause candidiasis and invasive candidiasis. If invasive candidiasis is left untreated it could lead to death. *Candida albicans* can cross blood-brain barrier. *Candida albicans* can be treated using clotrimazole, nystatin, fluconazole, echinocandin and amphotericin B.

They are dimorphic fungi which can grow as both filamentous fungi and yeast. The change in the colony morphology is due to the change in environmental conditions. The ability of the *Candida albicans* to switch from yeast to filamentous also has virulence

effect, as hyphal forms can escape body's macrophage. They can be grown in lab and even in vivo. *Candida albicans* has a genome size of 16mbp in haploid stage and 28mbp in diploid stage. About 40.5% of eye infection caused by *Candida sp.* belongs to *C. albicans* (Motukupally *et al.,* 2015).

2.2.4 Eye infections

Eye infections occurs when microorganisms such as virus, fungus and bacteria infect the parts of the eye. Organism can infect the cornea, vitreous (the gel like fluid present inside the eye), outermost layer of the eye (white parts of the eyes), eyelids and oil glands of the eyelids which causes keratitis, endophthalmitis, conjunctivitis, blepharitis, stye or hordeolum respectively.

2.2.4.1 Keratitis

Keratitis is the inflammation of the eye's cornea. Symptoms include red eye, photophobia, impaired eyesight and pain (Mahmoudi *et al*., 2018). They can be caused by variety of organism such as: Viral - Herpes simplex virus, Bacteria - *Pseudomonas aeruginosa* and *Staphylococcus aureus*, Fungal - *Fusarium solani*, *Candida albicans* and *Aspergillus fumigatus*, Amoebic - Acanthamoeba, Parasitic - *Onchocerca volvulus.*

Some of the commonly used antibiotics for bacterial keratitis is ofloxacin, moxifloxacin, gatifloxacin and levofloxacin and for viral keratitis aciclovir. Fungal keratitis can be treated using natamycin, amphotericin B and voriconazole (Mahmoudi *et al*., 2018).

2.2.4.2 Endophthalmitis

Endophthalmitis is the inflammation of the vitreous of the eye which is the gel like fluid present in the interior cavity of the eye. This might happen after cataract and intraocular surgery. If not treated properly it could lead to loss of vision or even eye. Symptoms include swelling, redness and pain. Endophthalmitis can be of two types exogenous and endogenous. Exogenous endophthalmitis is caused by the direct inoculation of the organism in the vitreous and endogenous endophthalmitis is caused by the organism which are transported to the vitreous in the bloodstream (Shwu-Jiuan Sheu, 2017). Endophthalmitis can be caused by variety of organisms: Bacteria - *Pseudomonas aeruginosa, Cutibacterium acnes, Staphylococcus pneumoniae, Staphylococcus epidermidis, Staphylococcus aureus and Neisseria meningitidis*, Virus - Herpes simplex

virus, Fungi - *Fusarium spp.* and *Candida spp.*, Parasite - *Toxocara* and *Toxoplasma gondii.*

Due to rapid progression of the infection and poor prognosis of the infection, treatment should be started very quickly. Some of the usually used antibiotics for bacterial endophthalmitis are vancomycin and ceftazidime and for fungal endophthalmitis voriconazole, fluconazole and amphotericin B (Shwu-Jiuan Sheu, 2017).

2.3 ANTIFUNGAL AGENTS

Antifungal agents are also called as antimycotic agents is a fungicide which is used to prevent and treat mycosis such as keratitis, endophthalmitis, aspergillosis, candidiasis etc. Antifungal can be systemic and local. Systemic includes intravenous and oral and local are topical administration.

Antifungal are of 3 classes polyenes, azoles, allylamines and echinocandins. Polyene functions by binding to the ergosterol present in the fungal cell wall and results in the leaking of the cell's organic molecules and ions which results in the death of the cells. Some of the examples of polyenes are rimocidin, nystatin, natamycin, hamycin, filipin, candicidin, Amphotericin B etc. Azoles functions by inhibiting the conversion of the lanosterol to ergosterol by blocking the action of lanosterol 14α-demethylase. Azoles can be further subdivided into three category thiazoles, triazoles and imidazole. Some the examples of azoles are abafungin, voriconazole, terconazole, ticoconazole, sulconazole, sertaconazole etc. Allylamines functions by blocking the activity of squalene epoxidase another enzyme required for ergosterol synthesis. Examples of allylamine are terbinafine, naftifine, butenafine and amorolfine. Echinocandins functions by blocking the activity of 1,3-β-glucan synthase which is responsible for the production of glucan. Some of the examples of echinocandins are micafungin, caspofungin, anidulafungin etc.

2.3.1 Amphotericin B

Amphotericin B is an antifungal agent used in treating fungal infections such as cryptococcosis, coccidioidomycosis, candidiasis, blastomycosis and aspergillosis. Some of the common side effects include headaches and fever. Allergic responses may lead to anaphylaxis. Amphotericin B was isolated from *streptomyces nodosus* in

1955. Currently amphotericin B is available in the following formulations as intravenous and oral. There are two amphotericin are available amphotericin A and B. Amphotericin A is almost similar to amphotericin B, but only amphotericin B is used as amphotericin A has very little antifungal activity.

Amphotericin B works by binding to the ergosterol present in the cell of the fungus. After binding to the cell membrane, it causes pores in the membranes which results in the leaking of the organic compounds and ions eventually the cell dies. Since both human cells and fungal cells contain sterol in their cell membrane, amphotericin B also cause pores in the human cells which may be lethal. Although, human cells only have cholesterol, while fungal cells have ergosterol which is the primary binding cite of amphotericin B, it might cause some effect in human cells also. Bacteria are unaffected by amphotericin B as their cell wall does not contain any sterols. Due to this toxic effect usage of amphotericin B is limited (Janout *et al*., 2015).

2.3.2 Natamycin

Natamycin also known as pimaricin is an antifungal agent used in treating fungal eye infections such as stye, conjunctivitis and keratitis (Patil *et al*., 2017). It belongs to the polyene class of antifungal agents. Natamycin is also used as preservatives in food industry (Qiu *et al*., 2015).

Allergic reactions may occur, but it's not clear. Natamycin was discovered in 1955 by fermentation of the bacteria Streptomyces. Natamycin is used to treat fungal organism such as *Penicillium, Fusarium, Cephalosporium, Aspergillus and Candida*. They are available in the forms of eye drops and cream. Natamycin is only little or nothing is absorbed in the intestinal tract thus it cannot be used for systemic infections. LD_{50} of natamycin from animal studies have shown to be 2.5-4.5g/Kg (Patil *et al*., 2017).

Natamycin functions by binding to ergosterol of the fungal membrane and causing leakage of organic molecules and ions which leads to the death of the cell. Natamycin also blocks the amino acid and glucose transport proteins which results in the cells inability to transport nutrients across the cells. Natamycin is very effective even very low concentration as less as 10ppm (Qiu *et al*., 2015).

2.3.3 Fluconazole

Fluconazole is an antifungal agent used for variety of fungal infection. It is available in injection and oral administration. Fluconazole belongs to a class of azole antifungal agents. It is a first-generation azole antifungal agent and it is quite different from other first generation azole antifungal agents as it has triazole ring instead of imidazole ring.

Some of the side effects include seizures, liver problem, rash, diarrhea and vomiting. It functions by affecting the fungal cell membrane by preventing the conversion of lanosterol to ergosterol (Berkow *et al*., 2017). Fluconazole is available from 1988. Fluconazole is used for treating dermatophytes, dimorphic fungi, *cryptococcus neoformans* and *candida spp.*

2.3.4 Voriconazole

Voriconazole is an antifungal agent which is used for a variety of fungal infections such as candidiasis and aspergillosis and fungal organism such as *Candida, Aspergillus, Fusarium* and *Scedosporium* (Sheu *et al*., 2015). It can also be used for fungal infections of central nervous system. It is available in injection and oral administration. Some of the side effect include hallucination, headache, rash, abdominal pain, nausea, vision problems, periostitis, bone fluorosis, stevens-johnson syndrome, squamous cell skin cancer, phototoxicity, damage to pancreas, liver and kidney and hypersensitivity reaction (Sheu *et al*, 2015).

It belongs to the second generation triazole class of antifungal agents and it works by affecting the fungal cell membrane (Sheu *et al*., 2015). Voriconazole is available from 2002 (levine *et al*., 2016). Bioavailability of voriconazole is 96% when orally administrated.

2.4 DILUTION METHODS

Agar dilution is one of the methods used to find the MIC of drugs. This method can be used to test the effectiveness of a new antibiotic. Agar dilution methods are simple, cheap and accurate and can be performed for number of organisms.

Broth microdilution is another method to find the MIC of the antifungal agents. The accuracy of this method is more than agar dilution and this method has an advantage

of using more than one antifungal agents for a single test. The in vitro susceptibility testing is carried out to find whether the fungal organisms has become resistant to the commonly used antifungal agents. These methods are also useful to compare the old and new antifungal agents. Using these methods, we can provide whether the fungal is susceptible or intermediate or resistant to the particular antifungal agents (Ohikhena *et al*., 2017). These results provided to the physicians will help them to choose over the range of antifungal agents available to treat the patients (Tenover, 2009).

Due to the increased incidences of the fungal eye infections and to provide the correct antifungal treatment MIC should be known. The MIC values can be found be using dilution methods by following the guidelines provided by Clinical and Laboratory Standard Institute, European Committee for Antimicrobial Susceptibility Testing and Indian Council of Medical Research. The MIC values can be very useful as the lowest concentration used for inhibiting the growth, mutant formation can be prevented.

3. Aim and Scope

AIM

The purpose of the antifungal susceptibility testing is to find the MIC of antifungal agents for different fungal organisms causing infection in eye.

OBJECTIVE

- To culture the fungal clinical isolates.
- To prepare the antifungal agent's concentrations according to the guidelines provided by ICMR.
- To harvest the spores from the cultured fungal clinical isolates for performing susceptibility test.
- To preform antifungal susceptibility testing using broth microdilution method to find the MIC for the fungal clinical isolates.
- To identify the unknown clinical isolate by isolating DNA and by performing sanger sequencing

SCOPE

Finding the MIC using the dilution methods for the antifungal agents such as amphotericin B, natamycin, voriconazole and fluconazole can be very useful for the effective treatment for the fungal infection and as the lowest concentration required for killing the fungus or inhibiting its growth is used, the side effects caused to the patients by the antifungal agents can be reduced.

4. MATERIALS AND METHODS

4.1 FUNGAL SPECIMEN SELECTION AND GROWTH

Different fungal organisms which are responsible for the infection of eyes are selected. These fungi are responsible for the eye infection such as keratitis and endophthalmitis. These fungal species are grown on SDA media.

4.1.1 Lactophenol Cotton Blue staining

Transparent tape is cut and placed between the thumb and the index finger with the sticky side out. The tape is pressed against the colony of interest. A drop of lactophenol cotton blue is placed on the glass slide and the tape is placed on the slide, smooth the tape and view it under the microscope (Leck, 1999).

4.1.2 Slide Culture Technique

Square agar blocks cut from SDA plate and placed each on each side of the slide. Needlepoint inoculum of the fungal spores are inoculated at all the four edges of the agar block. A coverslip is placed over the agar blocks and the whole set up is incubated. After the visible growth of the fungus appeared, the coverslip is removed from the block and placed in the lactophenol cotton blue stain in another slide and viewed under a microscope (Kali *et al.,* 2014).

4.2 PREPARATION OF MEDIA

YNB media (HiMedia) is used for inoculating the fungal organism for dilution method procedures (Zaragoza *et al*., 2011)

4.2.1 Media preparation for agar dilution

26.8g of YNB media is dissolved in 400ml of distilled water, then 20g of glucose is added as carbohydrate source and filter sterilized (prepared according to the manufacturer instruction) and then 6g of agar is dissolved in 400ml of distilled water and melted and used as gelling agent.

4.2.2 Media preparation for broth microdilution

10.05g of YNB media is dissolved in 150ml of distilled water and then 7.5g of glucose is added as carbohydrate source and filter sterilized (prepared according to the manufacturer instruction).

4.3 PREPARATION OF ANTIFUNGAL AGENTS

The required amount of the antifungal agents for the required volume and concentration can be calculated using the formula:

Weight(mg) = Volume(ml) x Concentration(µg/ml)/Assay potency(µg/mg)

After calculating the required weight for the required volume. The amount of the antifungal agent should be taken more than the required weight. Then the required volume for the extra amount of weight taken should be calculated by the formula:

Volume(ml) = Weight(mg) x Assay potency(µg/mg)/Concentration(µg/ml)

The concentration range for water insoluble antifungal agents: Amphotericin B (HiMedia), Natamycin (Sigma-Aldrich) and Voriconazole (Sigma-Aldrich) is 0.0313 to 16 µg/ml and for water soluble antifungal agents: Fluconazole is 0.125 to 64 µg/ml (ICMR, 2019).

4.3.1 Preparation of antifungal agents for agar dilution

The concentration selected is 1280 µg/ml. The further calculation is made to derive the required weight and volume of the antifungal agent and the solvent respectively.

Amphotericin B = 6.83mg in 4ml of DMSO

Natamycin = 5.86mg in 4ml of DMSO

Voriconazole = 5.27mg in 4ml of DMSO

Fluconazole = 5.5mg in 4ml of water

After dissolving the required amount of the antifungal agents in the desired solvents, the antifungal agents are further diluted to the required concentration mentioned above by the following method (12):

Table 4.1: Dilution procedure for preparing the varying concentration of antifungal agents

Step	Concentration (µg/ml)	Source	Volume (ml)	Diluent (ml)	Intermediate Concentration (µg/ml)	Final Concentration at 1:10 dilution in agar (µg/ml)
	1280	Stock	-	-	1280	128
1	1280	Stock	2	2	640	64
2	1280	Stock	1	3	320	32
3	1280	Stock	1	7	160	16
4	160	Step 3	2	2	80	8
5	160	Step 3	1	3	40	4
6	160	Step 3	1	7	20	2
7	20	Step 6	2	2	10	1
8	20	Step 6	1	3	5	0.5
9	20	Step 6	1	7	2.5	0.25
10	2.5	Step 9	2	2	1.25	0.125
11	2.5	Step 9	1	3	0.625	0.0625
12	2.5	Step 9	1	7	0.3125	0.0313

4.3.2 Preparation of antifungal agents for broth microdilution

The concentration selected is 1600 µg/ml for water insoluble antifungal agents and 1280 µg/ml for water soluble antifungal agents. Further calculation is made to derive the required weight and volume of the antifungal agent and the solvent respectively.

Amphotericin B = 4.27mg in 2ml of DMSO

Natamycin = 3.67mg in 2ml of DMSO

Voriconazole = 3.3mg in 2ml of DMSO

Fluconazole = 3.9mg in 3ml of water

After dissolving the required amount of the antifungal agents in the desired solvents, the antifungal agents are further diluted to the required concentration mentioned above by the following method (CLSI, 2018):

Table 4.2: Dilution procedure for preparing varying concentrations of water insoluble antifungal agents

Tube No.	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9	Tube 10
Add drug amount (ml)	-	From stock 0.5	From stock 0.5	From stock 0.5	From tube 4 0.5	From tube 4 0.5	From tube 4 0.5	From tube 7 0.5	From tube 7 0.5	From tube 7 0.5
Add solvent (ml)	-	0.5	1.5	3.5	0.5	1.5	3.5	0.5	1.5	3.5
Intermediate concentration(µg/ml)	1600	800	400	200	100	50	25	12.5	6.25	3.13
Add drug from above row (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
YNB media (ml)	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9
Concentration(µg/ml)	32	16	8	4	2	1	0.5	0.25	0.125	0.0625
Add drug from above row to microtiter well (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Add inoculum to well (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final concentration (µg/ml)	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.0313

Table 4.3: Dilution procedure for varying concentration of water-soluble antifungal agents

Tube No.	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9	Tube 10
Add drug amount (ml)	From stock 2	From tube 1 1	From stock 1	From tube 3 1	From tube 3 0.5	From tube 3 0.5	From tube 6 1	From tube 6 0.5	From tube 6 0.5	From tube 9 1
YNB media (ml)	2	1	7	1	1.5	3.5	1	1.5	3.5	1
Intermediate concentration(µg/ml)	640	320	160	80	40	20	10	5	2.5	1.25
Add drug from above row (ml)	1	1	1	1	1	1	1	1	1	1
YNB media (ml)	4	4	4	4	4	4	4	4	4	4
Concentration(µg/ml)	128	64	63	16	8	4	2	1	0.5	0.25
Add drug from above row to microtiter well (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Add inoculum to well (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final concentration (µg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125

4.4 PREPARATION OF INOCULUM

The fungal colonies grown in the SDA agar are harvested and added to 5ml of distilled water containing 0.1% tween-20. Then the inoculum is checked for absorbances at 625nm using spectrophotometer and the inoculum is adjusted for 0.5 McFarland standards for an absorbance range of 0.08 to 0.13 which would give an inoculum of 1-2.5X10^5 cells/ml which is used for the dilution methods (EUCAST, 2020).

4.5 AGAR DILUTION

After the antifungal agents are prepared according to the necessary varying concentration, 1ml of the intermediate concentration is mixed with 4.5ml of the YNB media and 4.5ml of melted agar which would give the final required concentration. Then it is mixed well and poured into the plates and allowed to solidified. The fungi are then inoculated on different spots in the agar plates. The plates are prepared for all the varying concentration for amphotericin B, natamycin, voriconazole and fluconazole. The plates are incubated at 35ºC for 48 hours and checked for growth.

4.6 BROTH MICRODILUTION

The antifungal agents prepared for the varying concentration and 0.1ml of each intermediate concentration is added to the wells of the microtiter plates from lowest concentration to highest concentration. 0.1ml of the inoculum prepared is added to all the wells of the microtiter plates and the plates are incubated at 35ºC for 48 hours and checked for growth.

4.7 FUNGAL DNA ISOLATION

The fungal DNA isolation was performed according to Qiagen DNA isolation kit. The fungal colonies are mixed with sterile water 100µl contained in 1.5ml centrifuge tube and then 20µl and 200µl of proteinase K and AL buffer respectively was added to the tube and mixed well by vortex. Then the tubes are incubated at 56ºC for 30 minutes. After the incubation, 200µl of ethanol was added and mixed well by vortex. Then the contents in the 1.5ml centrifuge tube are transferred to spin column and the spin column is centrifuged at 8000rpm for a minute and the filtrate obtained in the spin column is discarded. 500µl of AW1 buffer was added to the spin column and centrifuged at

8000rpm for a minute and the filtrate contained in the collecting tube is discarded and then 500µl of AW2 buffer was added and centrifuged at 14000rpm for 3 minutes. The collecting tube attached to the spin column is discarded and the filtrate of the spin column is fixed to a fresh 1.5ml centrifuge tube. Now 200µl of AE buffer or distilled water added to it and it is incubated in the room temperature for a minute and then centrifuged at 8000rpm for a minute. The isolated DNA was quantified using Qubit fluorometer.

4.8 PANFUNGAL PCR

PCR was performed for the isolated fungal DNA targeting the ITS (Internal Transcribed Spacer) region. The following is the reaction mix for panfungal PCR:

Table 4.4: Panfungal PCR reaction mix

Milli Q water	25µl
dNTP	8µl
Buffer	5µl
Taq polymerase	0.3µl
ITS1 forward primer	1µl
ITS4 reverse primer	1µl
DNA template	10µl

Table 4.5: Panfungal PCR cycle conditions

	Temperature	Time	Cycle
Initial denaturation	95ºC	5 minutes	
Denaturation	95ºC	30 seconds	30 cycles
Annealing	55ºC	1 minute	
Extension	72ºC	1 minute	
Final Extension	72ºC	5 minutes	

The panfungal PCR product quality is checked by agarose gel electrophoresis.

4.9 EXO-SAP TREATMENT

5µl of the panfungal PCR product is mixed with 0.5µl of exonucleases (Thermo scientific) and 1µl of shrimp alkaline phosphatase (Fermentas). After this step, the mixture is placed in thermal cycler for the following reaction:

Table 4.6: Thermal profile for Exo-SAP treatment

Temperature	Time
37ºC	15 minutes
85ºC	15 minutes
4ºC	Hold

4.10 BIGDYE TERMINATOR CYCLE SEQUENCING PCR

After performing the thermal profile for Exo-SAP treatment the product is used for PCR according to Big Dye Terminator V3.1 Cycle Sequencin Kit (Applied Biosciences, USA) following as per manufacturer protocol. Following is the reaction mix for the bigdye terminator cycle sequencing PCR:

Table 4.7: Reaction mix for bigdye terminator cycle sequencing

Ready reaction mix	2µl
Sequencing buffer	1µl
Forward primer	1µl
Reverse primer	1µl
Milli Q water	3µl
Amplified PCR product	2µl

Table 4.8: PCR conditions for bigdye terminator cycle sequencing

	Temperature	Time	Cycle
Initial denaturation	96ºC	1 minutes	
Denaturation	96ºC	10 seconds	25 Cycle
Annealing	50ºC	5 seconds	
Extension	60ºC	4 minutes	

4.11 PURIFICATION OF CYCLE SEQUENCED PCR PRODUCT

The cycle sequenced PCR product is mixed with 50µl ice cold ethanol, 10µl of Milli Q water, 2µl 125mM EDTA and 2µl of 3M Sodium acetate and incubated at room temperature for 15 minutes. After incubation the mixture is centrifuged at 12000rpm for 20 minutes, the supernatant is discarded and washed with 200µl of 70% ethanol for 3 times and centrifuged at 12000rpm for 10 minutes for each wash. The washed product is given for sequencing to identify the organism.

4.12 ANALYSIS OF SEQUENCING

The sequence of the PCR amplified DNA was deduced with the help of the ABI prism 3100 AVANT (Applied Biosystems, USA) genetic analyzer that works based on the principle of sanger dideoxysequencing. The amplified product with the dye at the terminated 3’ end was subjected to capillary electrophoresis by an automated sample injection. The emitted fluorescence from the dye labels on crossing the laser area were collected in the rate of one per second by a CCD at particular wavelength bands (virtual filters) and stored as digital signals and the computer for processing.

4.13 BLAST ANALYSIS

Amplified product was confirmed by DNA sequencing and the results obtained using NCBI BLAST tool (BLAST: Basic Local Alignment Search Tool (nih.gov)) was done to confirm the sequenced data and the percentage of homology was determined.

5. RESULTS AND DISCUSSION

5.1 FUNGAL SPECIES

These are some of the fungal isolates out of 30 fungal clinical isolates used for susceptibility testing which causes serious eye infections grown on SDA for which the

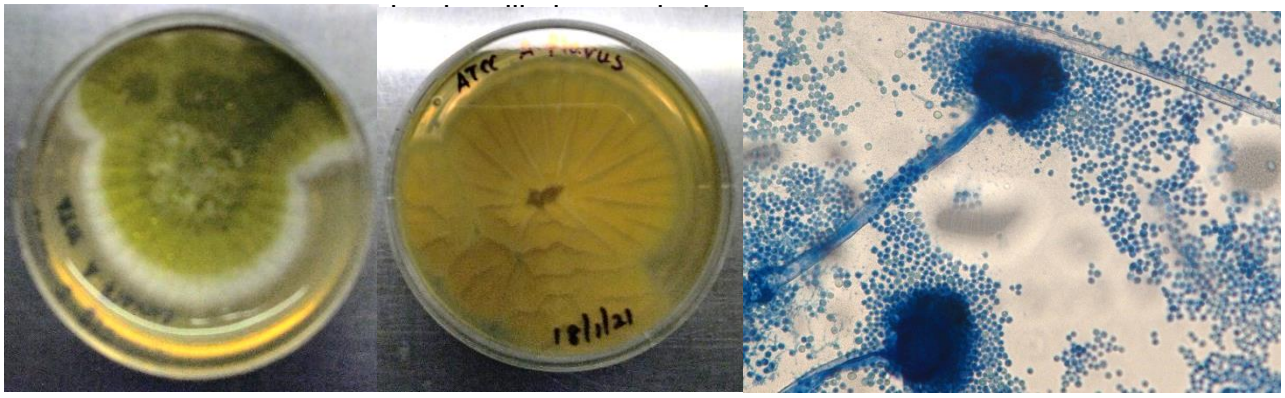

Fig.5.1: Microscopic 40x and culture of Aspergillus flavus

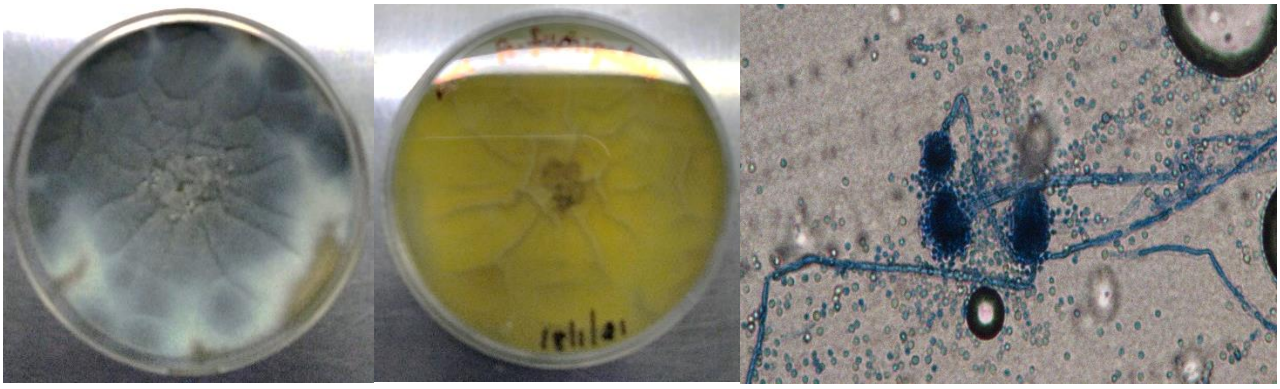

Fig.5.2: Microscopic 40x and culture of Aspergillus fumigatus

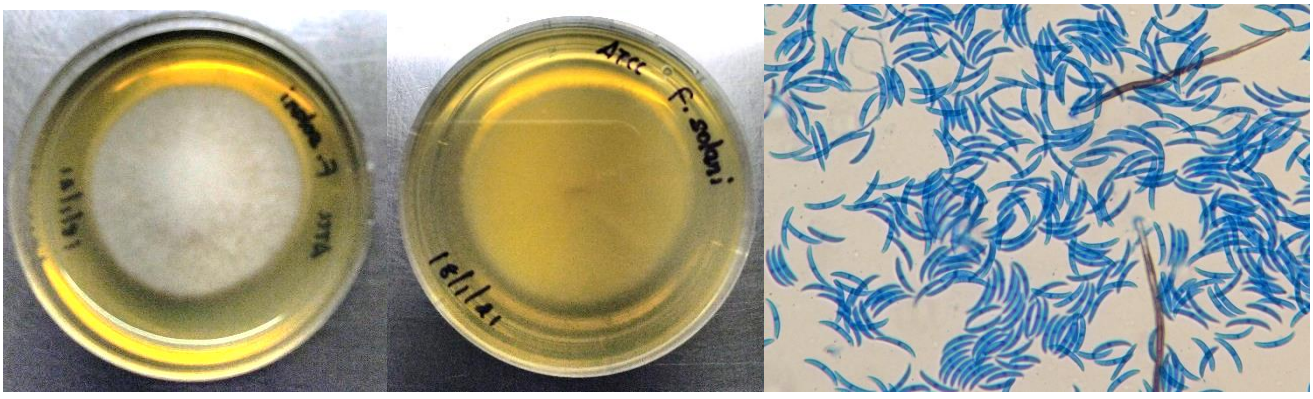

Fig.5.3: Microscopic 40x and culture of Fusarium solani

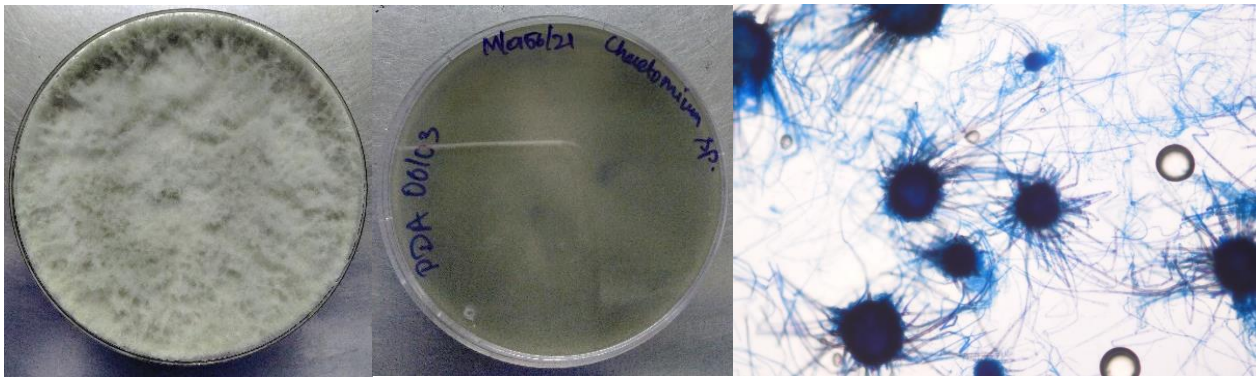

Fig.5.4: Microscopic 40x and culture of Chaetomium sp.

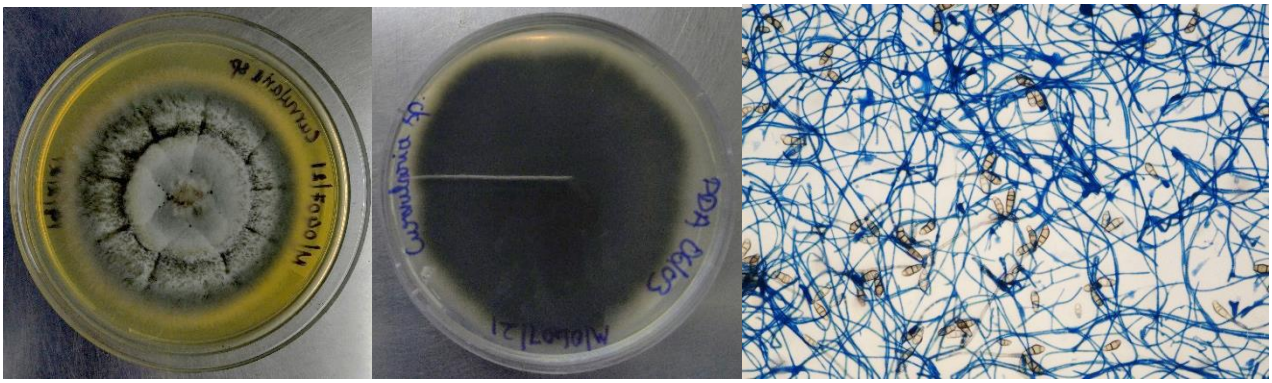

Fig.5.5: Microscopic 40x and culture of Curvularia sp.

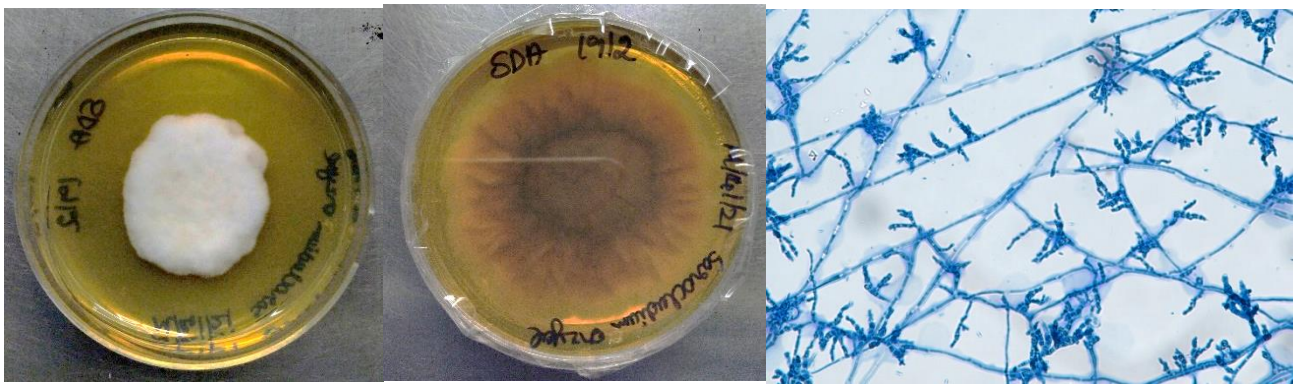

Fig.5.6: Microscopic 40x and Culture of Sarocladium oryzae

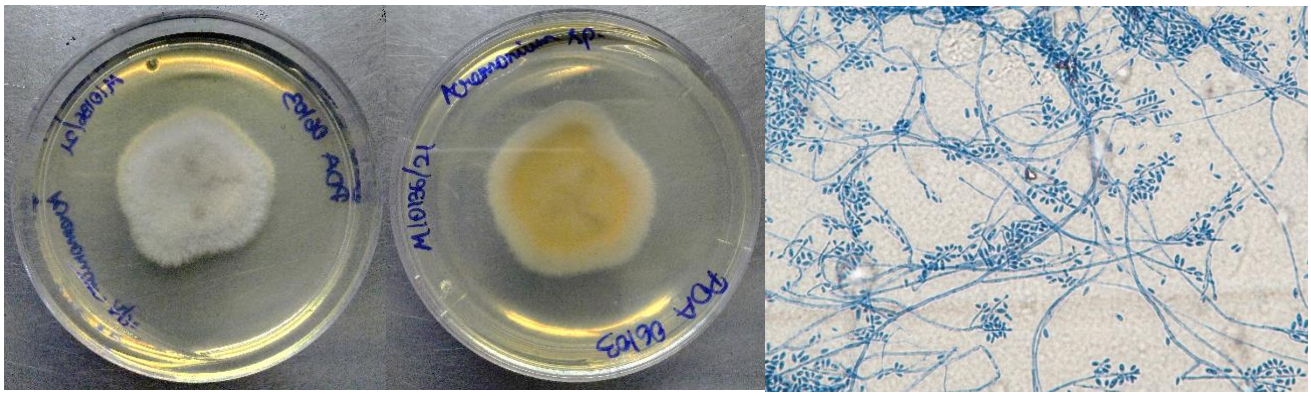

Fig.5.7: Microscopic 40x and Culture of Acremonium sp.

5.2 PREPARATION OF DRUG DILUTIONS

The antifungal agent's amphotericin B, Natamycin and voriconazole were prepared according concentrations and calculations in table 4.3.

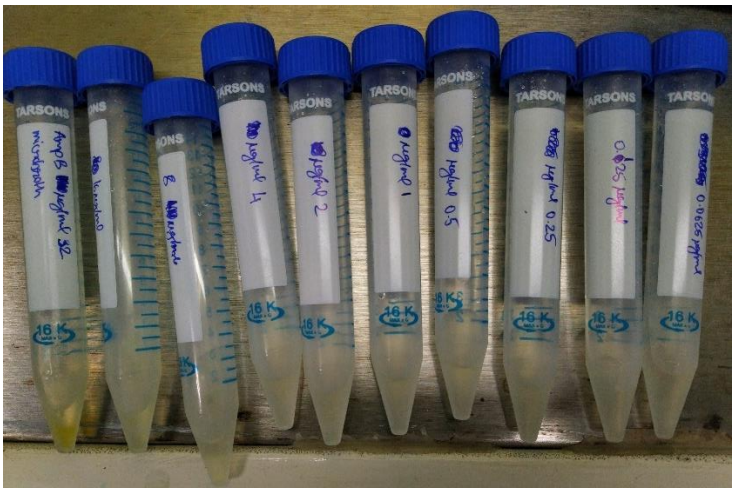

Fig.5.8: Amphotericin B dilutions ranging from 16µg/ml to 0.0313µg/ml from left to right.

Fig.5.9: Voriconazole dilutions ranging from 16µg/ml to 0.0313µg/ml from left to right.

Fig.5.10: Natamycin dilutions ranging from 16µg/ml to 0.0313µg/ml from left to right.

5.3 AGAR DILUTION

Agar dilution method was first performed for Amphotericin B for the recommended range of concentrations. But had no growth in any plates. But the positive control plates had growth indicating that there is no problem with the inoculum. The same problem persisted even during the second attempt. Thus, another dilution method broth microdilution method was used to find the MIC.

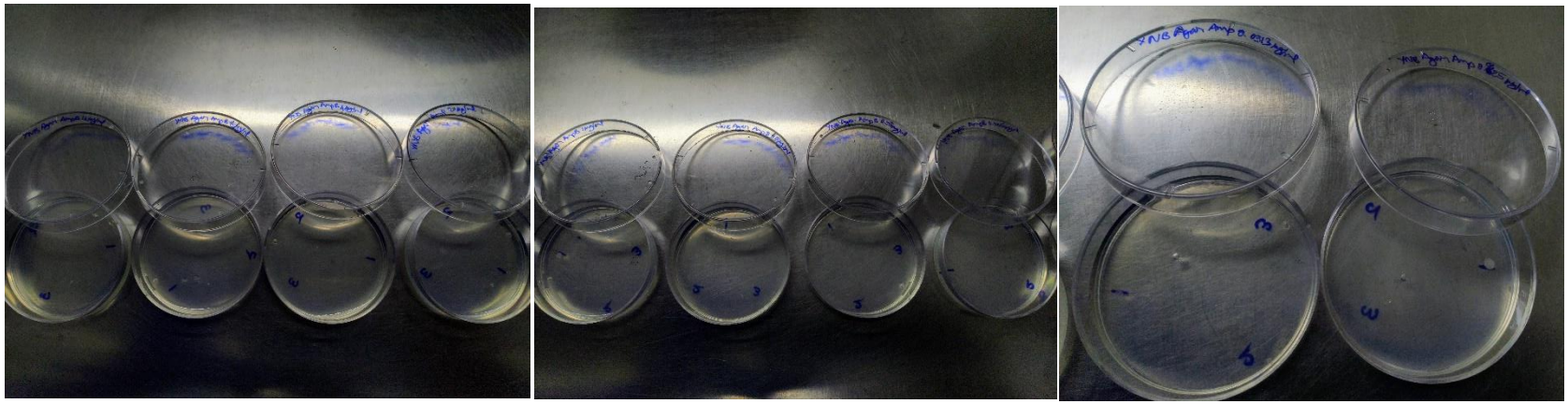

Fig.5.11: Agar dilution performed of Amphotericin B concentration range starting from 16µg/ml to 0.0313µg/ml from left to right.

5.4 BROTH MICRODILUTION

The MIC value for various clinically isolated fungus has been obtained by performing broth microdilution for amphotericin B, natamycin and voriconazole.

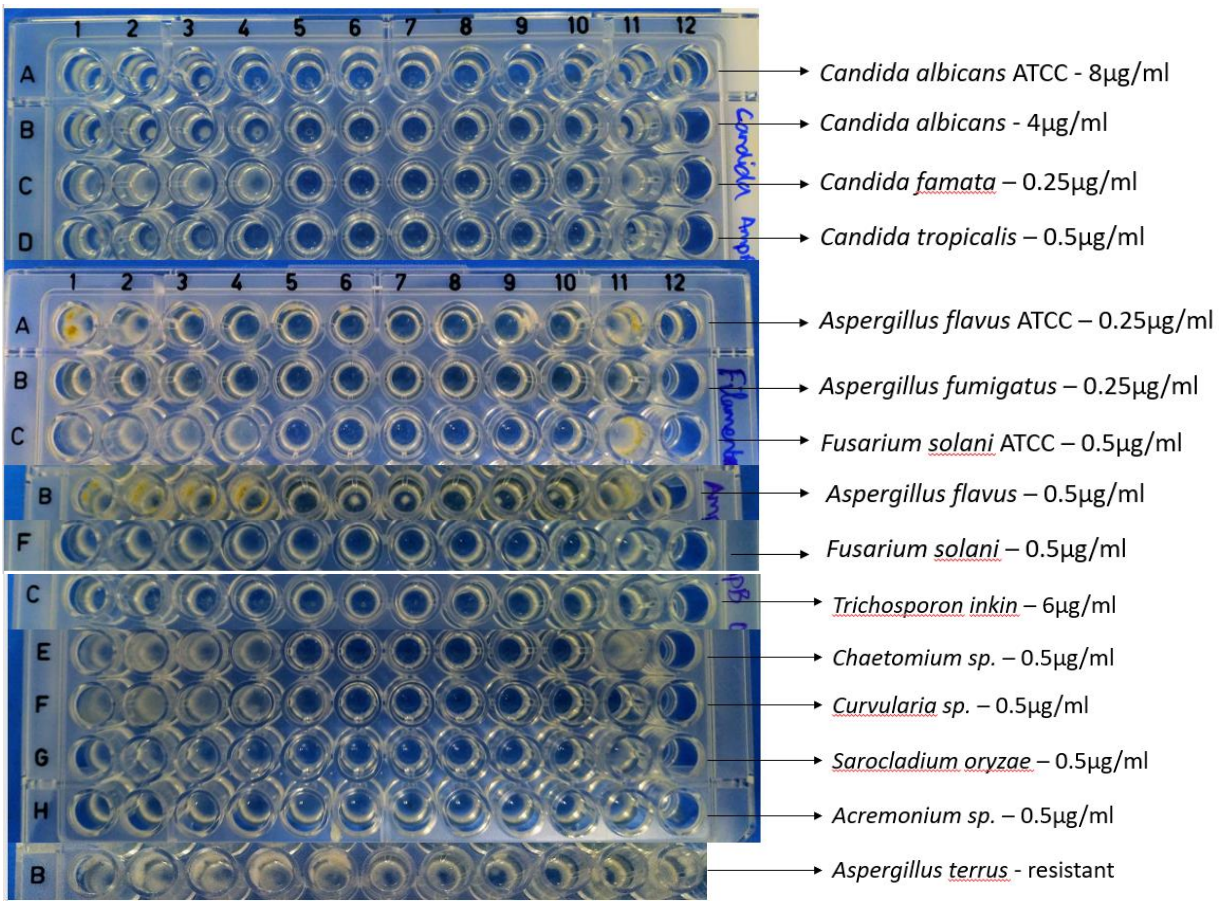

Fig.5.12: Amphotericin B microdilution results for various fungal isolates with its susceptibility concentration (Each well has increasing concentration of antifungal agents 1 – 0.0313µg/ml, 2 – 0.0625µg/ml, 3 – 0.125µg/ml, 4 – 0.25µg/ml, 5 – 0.5µg/ml, 6 - 1µg/ml, 7 - 2µg/ml, 8 - 4µg/ml, 9 - 8µg/ml, 10 - 16µg/ml and 11 - positive control without any antifungal agents)

Table 5.1: Amphotericin B susceptibility table (R – Resistant and S – Susceptible). This table represents the concentration range at which the particular fungi growth is inhibited or whether it is resistant till the concentration range tested and the lowest susceptible concentration is considered as MIC.

	0.0313µg/ml	0.0625µg/ml	0.125µg/ml	0.25µg/ml	0.5µg/ml	1µg/ml	2µg/ml	4µg/ml	8µg/ml	16µg/ml
Aspergillus flavus ATCC 9643	R	R	R	S	S	S	S	S	S	S
Aspergillus flavus 334	R	R	R	R	S	S	S	S	S	S
Aspergillus flavus 435	R	R	R	R	R	R	R	R	R	R
Aspergillus flavus 586	R	R	R	R	R	S	S	S	S	S
Aspergillus flavus 428	R	R	R	R	R	R	S	S	S	S
Aspergillus terrus 557	R	R	R	R	R	R	R	R	R	R
Aspergillus keratitidis 2061	R	R	R	R	R	S	S	S	S	S
Aspergillus fumigatus ATCC 204305	R	R	R	S	S	S	S	S	S	S

Aspergillus fumigatus 442	R	R	R	R	R	R	S	S	S	S
Fusarium solani ATCC 36031	R	R	R	R	S	S	S	S	S	S
Fusarium solani 327	R	R	R	S	S	S	S	S	S	S
Fusarium solani 464	R	R	R	R	R	R	S	S	S	S
Fusarium solani 570	R	R	R	R	R	S	S	S	S	S
Fusarium solani 538	R	R	R	R	R	S	S	S	S	S
Fusarium solani 576	R	R	R	R	R	S	S	S	S	S
Fusarium sp. 157	R	R	R	R	S	S	S	S	S	S
Fusarium sp. 216	R	R	R	S	S	S	S	S	S	S
Fusarium sp. 90	R	R	R	R	R	R	S	S	S	S
Fusarium sp. 618	R	R	R	R	R	S	S	S	S	S

Fusarium solani 49	R	R	R	R	S	S	S	S	S	S
Sarocladium oryzae 141	R	R	R	R	S	S	S	S	S	S
Acremonium sp. 136	R	R	R	R	S	S	S	S	S	S
Chaetomium sp. 156	R	R	R	R	S	S	S	S	S	S
Curvularia sp. 407	R	R	R	R	S	S	S	S	S	S
Candida albicans ATCC 90028	R	R	R	R	R	R	R	R	S	S
Candida albicans 414	R	R	R	R	R	R	R	S	S	S
Candida tropicalis 246	R	R	R	R	S	S	S	S	S	S
Candida famata 354	R	R	R	S	S	S	S	S	S	S
Trichosporon sp. 81	R	R	R	R	R	R	R	R	R	R
Trichosporon inkin 383	R	R	R	R	R	S	S	S	S	S

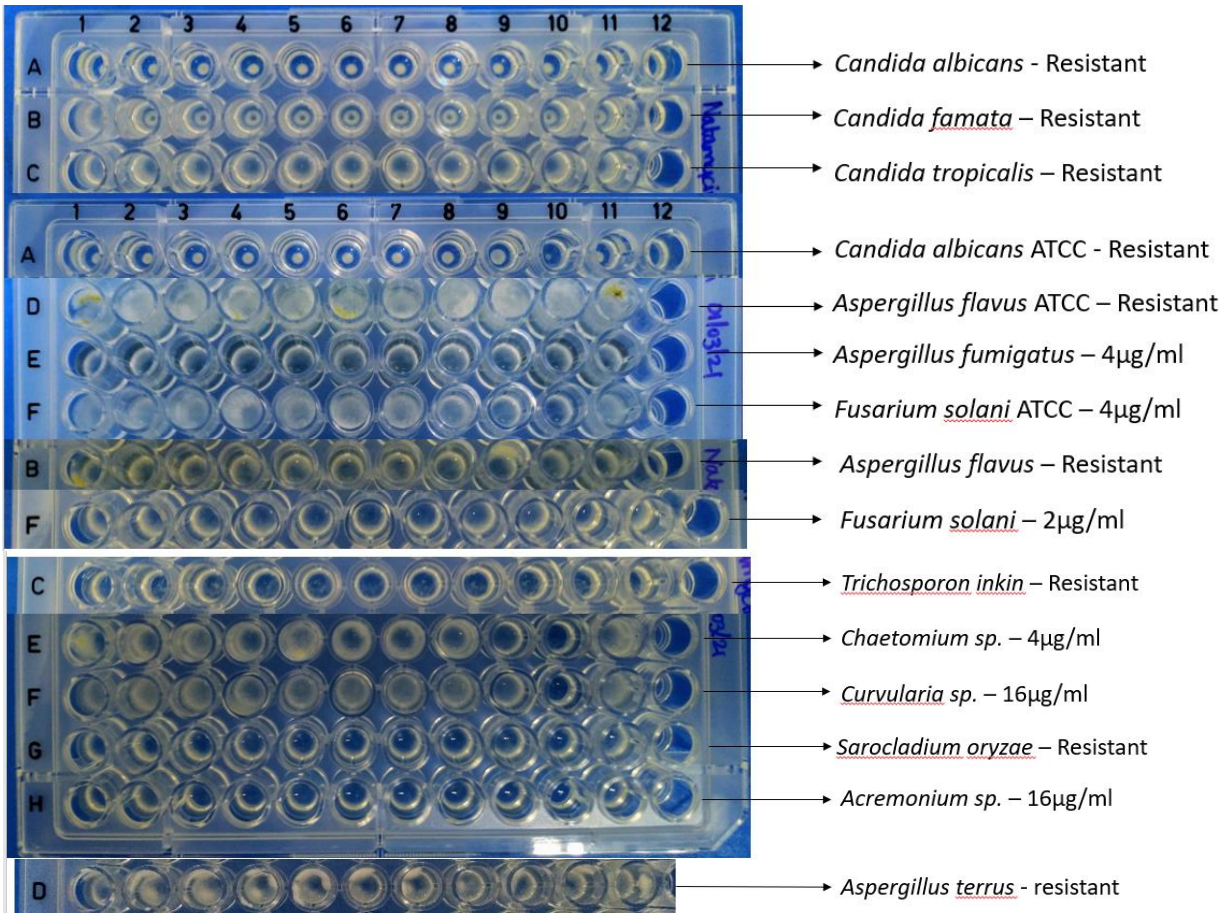

Fig.5.13: Natamycin microdilution results for various fungal isolates with its susceptibility concentration (Each well has increasing concentration of antifungal agents 1 – 0.0313µg/ml, 2 – 0.0625µg/ml, 3 – 0.125µg/ml, 4 – 0.25µg/ml, 5 – 0.5µg/ml, 6 - 1µg/ml, 7 - 2µg/ml, 8 - 4µg/ml, 9 - 8µg/ml, 10 - 16µg/ml and 11 - positive control without any antifungal agents)

Table 5.2: Natamycin susceptibility table (R – Resistant and S – Sensitive). This table represents the concentration range at which the particular fungi growth is inhibited or whether it is resistant till the concentration range tested and the lowest susceptible concentration is considered as MIC.

	0.0313µg/ml	0.0625µg/ml	0.125µg/ml	0.25µg/ml	0.5µg/ml	1µg/ml	2µg/ml	4µg/ml	8µg/ml	16µg/ml
Aspergillus flavus ATCC 9643	R	R	R	R	R	R	R	R	R	R
Aspergillus flavus 334	R	R	R	R	R	R	R	R	R	R
Aspergillus flavus 435	R	R	R	R	R	R	R	R	R	R
Aspergillus flavus 586	R	R	R	R	R	R	R	R	R	R
Aspergillus flavus 428	R	R	R	R	R	R	R	R	R	R
Aspergillus terrus 557	R	R	R	R	R	R	R	R	R	R
Aspergillus keratitidis 2061	R	R	R	R	R	R	R	R	R	R
Aspergillus fumigatus ATCC 204305	R	R	R	R	R	R	R	S	S	S

Aspergillus fumigatus 442	R	R	R	R	R	R	R	R	R	R
Fusarium solani ATCC 36031	R	R	R	R	R	R	R	S	S	S
Fusarium solani 327	R	R	R	R	R	R	R	S	S	S
Fusarium solani 464	R	R	R	R	R	R	R	R	R	R
Fusarium solani 570	R	R	R	R	R	R	R	R	R	R
Fusarium solani 538	R	R	R	R	R	R	R	R	R	R
Fusarium solani 576	R	R	R	R	R	R	R	R	R	R
Fusarium sp. 618	R	R	R	R	R	R	R	R	R	R
Fusarium sp. 157	R	R	R	R	R	R	R	R	R	R
Fusarium sp. 216	R	R	R	R	R	R	S	S	S	S
Fusarium sp. 90	R	R	R	R	R	R	R	R	S	S

Fusarium solani 49	R	R	R	R	R	R	R	R	R	R
Sarocladium oryzae 141	R	R	R	R	R	R	R	R	R	R
Acremonium sp. 136	R	R	R	R	R	R	R	R	R	S
Chaetomium sp. 156	R	R	R	R	R	R	R	S	S	S
Curvularia sp. 407	R	R	R	R	R	R	R	R	R	S
Candida albicans ATCC 90028	R	R	R	R	R	R	R	R	R	R
Candida albicans 414	R	R	R	R	R	R	R	R	R	R
Candida tropicalis 246	R	R	R	R	R	R	R	R	R	R
Candida famata 354	R	R	R	R	R	R	R	R	R	R
Trichosporon sp. 81	R	R	R	R	R	R	R	R	R	R
Trichosporon inkin 383	R	R	R	R	R	R	R	R	R	R

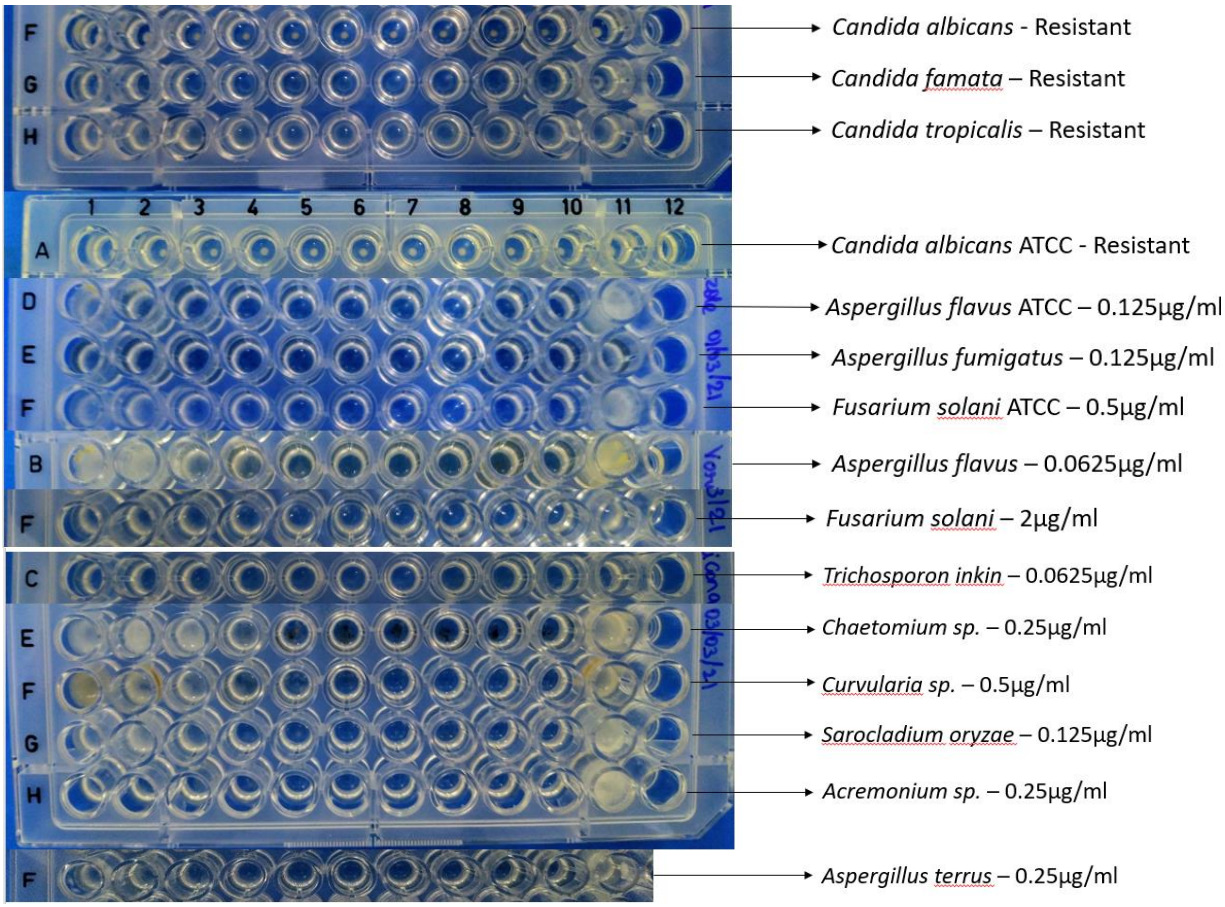

Fig.5.14: Voriconazole microdilution results for various fungal isolates with its susceptibility concentration (Each well has increasing concentration of antifungal agents 1 – 0.0313µg/ml, 2 – 0.0625µg/ml, 3 – 0.125µg/ml, 4 – 0.25µg/ml, 5 – 0.5µg/ml, 6 - 1µg/ml, 7 - 2µg/ml, 8 - 4µg/ml, 9 - 8µg/ml, 10 - 16µg/ml and 11 - positive control without any antifungal agents)

Table 5.3: Voriconazole susceptibility results (R – Resistant and S – Susceptible). This table represents the concentration range at which the particular fungi growth is inhibited or whether it is resistant till the concentration range tested and the lowest susceptible concentration is considered as MIC.

	0.0313µg/ml	0.0625µg/ml	0.125µg/ml	0.25µg/ml	0.5µg/ml	1µg/ml	2µg/ml	4µg/ml	8µg/ml	16µg/ml
Aspergillus flavus ATCC 9643	R	R	R	R	S	S	S	S	S	S
Aspergillus flavus 334	R	S	S	S	S	S	S	S	S	S
Aspergillus flavus 435	R	R	R	S	S	S	S	S	S	S
Aspergillus flavus 586	R	R	S	S	S	S	S	S	S	S
Aspergillus flavus 428	R	R	R	S	S	S	S	S	S	S
Aspergillus terrus 557	R	R	R	S	S	S	S	S	S	S
Aspergillus keratitidis 2061	R	R	S	S	S	S	S	S	S	S
Aspergillus fumigatus ATCC 204305	R	R	S	S	S	S	S	S	S	S

Aspergillus fumigatus 442	R	R	S	S	S	S	S	S	S	S
Fusarium solani ATCC	R	R	R	R	S	S	S	S	S	S
Fusarium solani 327	R	R	R	S	S	S	S	S	S	S
Fusarium solani 464	R	R	R	R	S	S	S	S	S	S
Fusarium solani 570	R	R	R	R	S	S	S	S	S	S
Fusarium solani 538	R	R	R	R	S	S	S	S	S	S
Fusarium solani 576	R	R	R	R	S	S	S	S	S	S
Fusarium sp. 618	R	R	R	S	S	S	S	S	S	S
Fusarium sp. 157	R	S	S	S	S	S	S	S	S	S
Fusarium sp. 216	R	R	R	R	R	R	S	S	S	S
Fusarium sp. 90	R	R	R	R	R	R	R	R	R	R

Fusarium solani 49	R	R	R	R	R	R	S	S	S	S
Sarocladium oryzae 141	R	R	S	S	S	S	S	S	S	S
Acremonium sp. 136	R	R	R	S	S	S	S	S	S	S
Chaetomium sp. 156	R	R	R	R	R	R	R	R	R	R
Curvularia sp. 407	R	R	R	R	S	S	S	S	S	S
Candida albicans ATCC 90028	R	R	R	R	R	R	R	R	R	R
Candida albicans 414	R	R	R	R	R	R	R	R	R	R
Candida tropicalis 246	R	R	R	R	R	R	R	R	R	R
Candida famata 354	R	R	R	R	R	R	R	R	R	R
Trichosporon sp. 81	R	R	R	R	R	R	R	R	R	R
Trichosporon inkin 49	R	S	S	S	S	S	S	S	S	S

Graph 5.1: Antifungal susceptibility testing graph (R – Resistant) the Y-axis represents the concentration range of the antifungals used and the X-axis is the organisms used for testing. The graph shows the concentrations at which the organism growth is inhibited for the 3 antifungal agents.

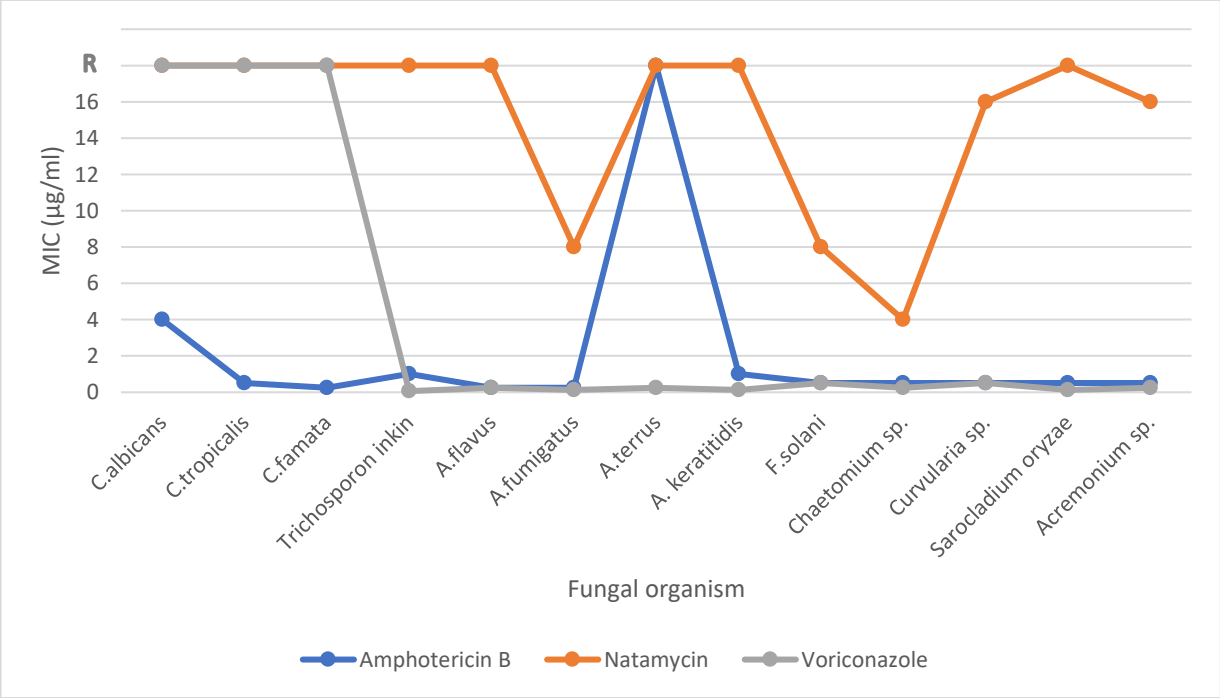

Table 5.4: Total no. of fungal isolate susceptibility distribution between different antifungal agents

	Amphotericin B		Natamycin		Voriconazole	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
Filamentous fungi (Total – 24	22	2	9	15	23	1
Yeast (Total – 6)	5	1	0	6	1	5

Spierer *et al*. 2015, have concluded that 0.2% of amphotericin B had a better effect against *candida species*. The results of this work also had more or less the same

effectiveness against *candida species*. They have also reported that for the same effectiveness for natamycin had to be used at 5% and for voriconazole they also mentioned that they need higher concentrations, likewise in this result all the *candida sp.* were resistant to natamycin and voriconazole. Sun *et al*. 2014, have reported that *Aspergillus flavus* had MIC of more than 32µg/ml for natamycin. As per the ICMR guidelines for natamycin the susceptibility range is from 0.0313 to 16µg/ml, since we had growth till the highest concentration i.e., 16µg/ml *Aspergillus flavus* is resistant to natamycin. They have also mentioned that *Aspergillus terrus* was susceptible at 16µg/ml for natamycin, but in this work there was growth even in 16µg/ml. So, in these findings *A. terrus* considered has resistant to natamycin. Sun *et al*. 2014, have also provided MIC ranges of voriconazole for *Aspergillus sp., Fusarium sp.* and *Curvularia sp.* which also coincides with these results.

5.5 FUNGAL DNA ISOLATION

The DNA was isolated from an unidentified fungus for identification using sanger sequencing method. The isolated DNA was quantified using Qubit fluorometer. The DNA yield was 6.94µg/ml.

Fig.5.15: Unidentified fungal isolate grown on PDA agar slant

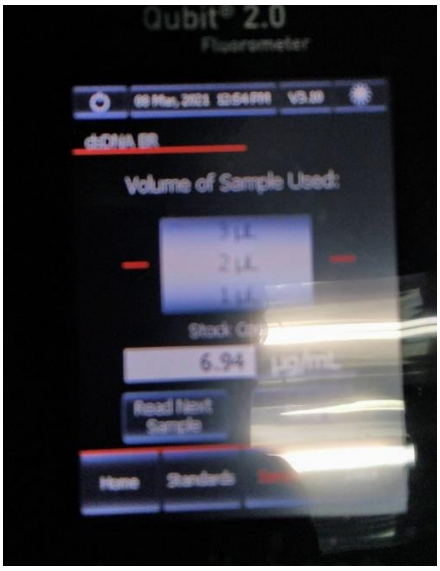

Fig.5.16: The amount of DNA isolated from unidentified fungi and quantified using qubit fluorometer

5.6 PANFUNGAL PCR

The isolated DNA was used for Panfungal PCR by the procedure previously described in the method section 4.8.

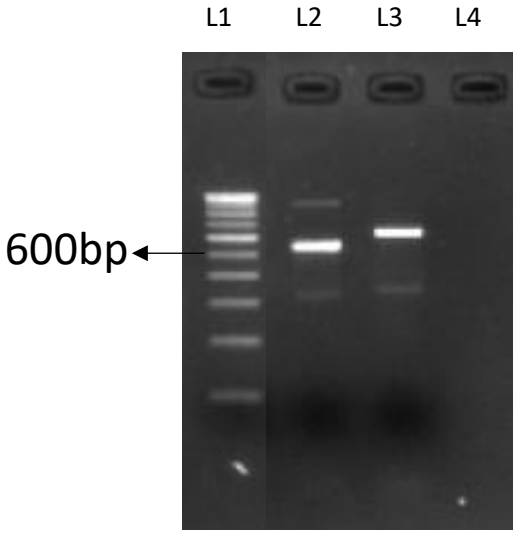

Fig.5.17: Panfungal PCR product (L1-100bp ladder, L2-positive control, L3-Panfungal PCR product, L4-Negative control

The PCR product was mixed with exonucleases and shrimp alkaline phosphatase and then preformed PCR for cycle sequencing and then cycle sequencing PCR product was purified and then it was given for sequencing.

5.7 SANGER SEQUENCING

The sanger sequence result was viewed using the software BioEdit. The total sequence length obtained from targeting the ITS region of the fungal DNA is 627bp.

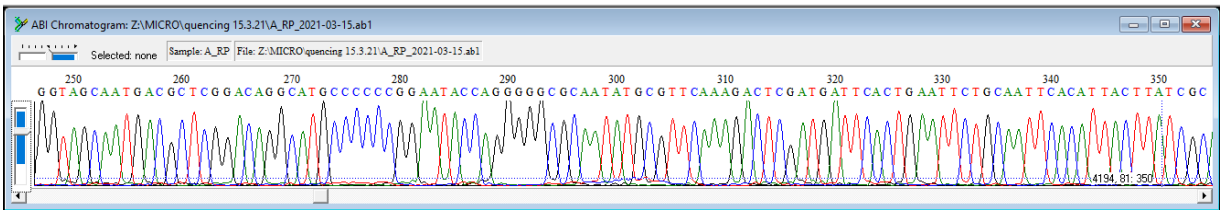

Fig.5.18: Sanger sequencing result analyzed using BioEdit software

The sanger sequencing result was copied in FASTA format and BLAST was performed using the NCBI BLAST platform for identifying the species.

BLAST® » **blastn suite** » results for RID-50DPXB5S01R

Job Title	Nucleotide Sequence ...
RID	50DPXB5S01R Search expires on 03-17 12:41 pm
Program	BLASTN
Database	nt
Query ID	lcl|Query_96509
Description	None ...
Molecule type	dna
Query Length	407

Descriptions

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len
Aspergillus keratitidis strain KAS8109 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Aspergillus keratitidis	739	739	99%	0.0	99.75%	718
Aspergillus keratitidis strain KAS7927 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Aspergillus keratitidis	739	739	99%	0.0	99.75%	717
Aspergillus keratitidis strain BCRC:34221 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Aspergillus keratitidis	739	739	99%	0.0	99.75%	720
Sagenomella keratitidis strain UZ597_17 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	Aspergillus keratitidis	739	739	99%	0.0	99.75%	645
Aspergillus keratitidis strain KAS8116 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Aspergillus keratitidis	734	734	99%	0.0	99.50%	713
Aspergillus keratitidis strain KAS8114 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Aspergillus keratitidis	725	725	99%	0.0	99.01%	716
Aspergillus keratitidis strain KAS8112 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	Aspergillus keratitidis	725	725	99%	0.0	99.01%	716
Aspergillus keratitidis genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: PW3024	Aspergillus keratitidis	721	721	95%	0.0	100.00%	610
Sagenomella sp. internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	Sagenomella sp.	713	713	95%	0.0	99.74%	605
Aspergillus keratitidis isolate F29 ITS5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	Aspergillus keratitidis	710	710	94%	0.0	100.00%	637
Aspergillus keratitidis strain Kas7927 isolate 19053112NU25 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	Aspergillus keratitidis	684	684	93%	0.0	98.96%	585

Fig.5.19: BLAST analyzes of sequencing result

The BLAST analyzes of the sequence gave a 99% sequence coverage for *Aspergillus keratitidis* and came up as the top 7 results and all the 7 results had a 99% sequence coverage. *Aspergillus keratitidis* is a novel species and it belongs to *Aspergillus* subgenus *Polypaecilum* which was first isolated from a keratitis patient by Hsieh *et al*. 2009, they described it as *Sagmonella keratitidis* and later it was termed as *Aspergillus keratitidis* as it belongs to the *Aspergillus* subgenus *Polypaecilum*. This fungus is a xerophile which can grow on environment which has very low water content (Idris *et al*., 2020). The *Polypaecilum* subgenus contains 7 novel species which includes: *Aspergillus baarnensis, A. keratitidis, A. kalimae sp., A. noonimiae sp., A. thailandensis sp., A. waynelawii sp.* and *A. whitfieldii sp.* (Tanney *et al*., 2017). Antifungal susceptibility testing was performed for Aspergillus keratitidis and it was found that is it resistant to natamycin and susceptible to amphotericin B at 1µg/ml and Voriconazole at 0.125µg/ml.

6. SUMMARY AND CONCLUSION

SUMMARY

- Total of 30 fungal isolates infecting the eyes are cultivated for antifungal susceptibility testing.
- The antifungal agent's amphotericin B, natamycin and voriconazole were prepared for the concentration range of 16µg/ml to 0.0313µg/ml according to the ICMR guidelines.
- After the fungal isolates are grown for 48 hours spores are collected and mixed in water contain 0.1% tween – 20 and the absorbance was adjusted at 0.08 to 0.13 at 630nm, so that there are 1-2.5X10^5cells/ml.
- 0.1ml of prepared antifungal agents of each concentration are added to the wells of the microwell plates and 0.1ml the inoculum prepared are added to all the wells containing the antifungal agent and are incubated for 48 hours.
- The concentration range for susceptibility are noted after the 48 hours of incubation.
- DNA of unidentified fungal isolate is isolated and Panfungal PCR, Exo – SAP treatment and bigdye terminator cycle sequencing PCR are performed and the product was sequenced.
- The sequencing results was performed BLAST using the NCBI BLAST platform and the fungal isolate is identified as *Aspergillus keratitidis*, which came up at top 7 hits all with 99% sequence similarity.

CONCLUSION

In this work it was found that yeast clinical isolates are resistant to natamycin and voriconazole thus, amphotericin B has very good activity towards yeast isolates and about 15 of the filamentous fungi used in the antifungal susceptible testing is found to be resistant to natamycin. For filamentous fungi, voriconazole and amphotericin B had MIC value of less than 1µg/ml and unknown fungal isolate have also been identified as Aspergillus keratitidis and antifungal susceptibility test was performed for it and it was sensitive to amphotericin B and voriconazole and resistant to natamycin. So, among amphotericin B and voriconazole, voriconazole is the best antifungal agent.

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