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RESEARCH ARTICLE

A COMPARATIVE ANALYSIS OF ANTIFUNGAL SUSCEPTIBILITY OF KERATITIS ASSOCIATED FUNGAL BIOFILM AND PLANKTONIC CULTURES.

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Abstract

Biofilm are considered as the most important developmental characteristics in ocular infections. Biofilm eradication is a major challenge today to overcome the incidence of drug resistance and treatment failures in cases of fungal keratitis. This report demonstrates the in vitro ability of biofilm formation by three common keratitis-associated fungal pathogens, namely, *Aspergillus fumigatus, Fusarium solani, and Candida albicans*. Antifungal sensitivity testing performed for both planktonic cells and biofilm revealed the sessile phenotype to be resistant at MIC levels for the planktonic cells and also at higher concentrations. Antifungal susceptibility testing following re-suspension of the sessile form into a planktonic mode demonstrated an intermediate MIC value with development of resistance compared to the original planktonic state. This demonstrates that antifungal resistance acquired by biofilms is permanent and transferred to subsequent generations thereby converting a sensitive phenotype into a resistant form.

Introduction:-

Corneal blindness is a significant health problem across the world. According to the World Health Organization report, it is estimated that ocular trauma and corneal ulceration result in 1.5 to 2 million new patients of corneal blindness annually, posing a major public health concern for developing countries like India (**Whitcher et al., 2001**).

Worldwide, fungi account for around 50% cases of infective keratitis. Srinivasan *et al* from South India reported that 44% of all central corneal ulcers were caused by fungi (**Srinivasan, 1997**). Molds are most common in tropical parts of the world like India and southeastern United States with more than 70 species identified as responsible pathogens. Involvement of new species is also reported regularly. In India, according to previous reports, *Aspergillus sp.* and *Fusarium sp.* are the commonest organisms followed by dematiaceous fungi along with an increasing incidence of Candida keratitis (**Sengupta et al., 2012**).On the other hand, in temperate regions like northern parts of America, *Candida albicans* forms the most common isolate although Fusarium keratitis is increasingly recognized (**Sun et al.,2007**).

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Recent advances have provided evidence that microbial adherence to the human biotic surface constitutes the first step towards development of a biofilm community which is contemplated to be responsible for more than 65% of human infections (**Donlan, 2002**). A biofilm is a structured microbial community of cells enclosed in a matrix of extracellular polymeric substances (EPS), growing on a substrate, and displaying phenotypic features that vary from their planktonic or free-floating counterparts (**Costerton,1995**). Such an existence presumably allows the microbial cells to survive in hostile environments, enhances their resistance to physical and chemical pressures, and promotes metabolic cooperation. Although bacterial biofilms have been extensively studied since the mid 1980s, little attention was paid to medically relevant fungal biofilms until the past decade. Much of our understanding about fungal biofilm formation has been gained through the studies of biofilms formed by Candida species on artificial surfaces (**Kumamoto et al., 2002**) such as stents, shunts, prostheses, implants, endotracheal tubes, pacemakers, and various types of catheters, to name a few (**Shimuzu et al., 2000**; **Ramage et al., 2005**).

The role of fungal biofilms in ophthalmic setting, particularly with reference to infective keratitis, is unknown. However, this may possess a potentially important role in pathogenesis of the disease condition as well as outcome, as biofilm formation tends to induce an antifungal resistance and alter the response to therapy. While our knowledge is restricted to fungal biofilm formation on implanted medical devices and its role in invasive infections, recently, few reports have speculated a similar pathologic mechanism in contact lens related fungal keratitis. In vitro studies have been conducted to demonstrate the formation of fungal biofilm on a contact lens substrate and have also shown a high degree of antifungal resistance (**Imamura et al., 2008; Sengupta et al, 2012**).

In this experimental study we have studied three fungal strains—*Aspergillus fumigatus*, *Fusarium solani*, and *Candida albicans* —isolated from patients with infective keratitis to compare the antifungal susceptibility between planktonic culture, biofilm mode of growth and post-biofilm dispersed planktonic culture(BDPC).

Materials and Methods:-

The fungal pathogens - *Aspergillus sp*, *Fusarium sp*, and *Candida sp* - isolated from cases of fungal keratitis attending the Cornea Services of Priyamvada Birla Aravind Eye Hospital, Kolkata were selected for this study. The three strains have been characterized earlier based on their genetic data. (**Saha et al., 2009**). Briefly, the DNA was isolated from the culture medium and the quality evaluated on 1.2% agarose gel. Species confirmation was done using D1/D2 region of LSU (Large SubUnit: 28SrDNA) based on PCR technique. Ribosomal ITS (internal transcribed spacer) region is amplified by using primers 5'-GGTTGGTTTCTTTTCCT-3' and 5'-AAGTAAAAGTCGTAACAAGG-3'.

The fungal species were collected from mature solid medium culture plate (Sabouraud dextrose agar) and mixed with liquid RPMI 1640 (Himedia, India) and incubated for 24 hrs at 30ºC to obtain the relevant turbidity of 0.5 x 10^4 CFU/ml. The selected isolates were submerged in 96 wells, flat bottom polystyrene plates containing 2 ml of RPMI 1640 medium, inoculated with respective fungal strain as a inoculum dose of 3.5 × 10^6 CFU/ ml and incubated for 72 hours at 30°C. After 72 hours the each well was washed with 1 x PBS repeatedly to remove the non-biofilm cells. Biofilm formation was confirmed under fluorescence microscopy using Concavalin A-Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) conjugate (ConA) (200 l g/ml) counterstaining and XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl-2H-tetrazolium-5-carboxanilide; Sigma) reduction assay using a Multiskan Spectrum-1500 spectrophotometer (Thermo Scientific) as described previously (**Sengupta et al,,2012**).

The following antifungals were used for determination of antifungal susceptibility namely liposomal amphotericin B, voriconazole, ketoconazole, itraconazole, natamycin, and anidulafungin. Antifungal agents were prepared as previously described (**Anil et al., 2002**). Antifungal drug sensitivity tests were performed in accordance with national committee for clinical laboratory standards (NCCLS) guidelines. The following antifungal discs (Hi Media, India) were used which included voriconazole, natamycin, amphotericin B, fluconazole, ketoconazole and anidulafungin. The selective stains were inoculated into SDA media, cultured for 2-5 days and the inoculum was diluted to 0.5×104 CFU/ml with saline water. The inoculum (0.5ml) were plated in SDA media, dried for 15 min at 37°C then antifungal discs were placed on the media surface by following disc diffusion methods. The plates were incubated at 30˚C and the zone diameters were observed for each strain after 48 hrs incubation. The zone diameters were measured at which colonies were remaining normal. The zone diameters were interpreted as proposed by the manufacturer's guidelines. The minimum inhibitory concentration of selective antifungal (voriconazole, amphotericin B, ketoconazole, itraconazole and anidulafungin) (Hi Media, India) were determined according to the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing

(AFST-EUCAST) method (Cuenca-Estrella et al., 2003) using RPMI 1640 supplemented with 2% glucose as the test medium, and an inoculum size of 0.5×10^5 to $2.5\ 1\times10^5$ cells/ml.

The minimum biofilm eradication concentration (MBEC) of the selected antifungals were determined as by Melo et al. The respective antifungal agents were added to the biofilm in serial double-diluted concentrations (0.5 to 500 µg/ml) and total volume of 250 µl with RPMI medium and solutions of each antifungal agent was also prepared in RPMI medium directly, incubated further for 24 hours at 35 ℃. A series of antifungal agent-free wells and biofilm-free wells was also included to serve as positive and negative controls, respectively. After incubation, the remaining biofilm metabolic activity was quantified by the XTT-reduction assay. Testing of these isolates were performed in triplicate.

To evaluate MIC for biofilm derived planktonic cells, biofilms was formed as mentioned above. After 24 hrs and 48 hrs, the biofilms were washed gently and transferred to 2 ml micro centrifuge tube for vortexing for 5 min. Serial dilutions of antifungals were made and transferred to the 96 well plate. The votexed RPMI liquid medium was added to each 96 well plate with 10 µl of antifungal. These biofilm derived planktonic cells (BDPC) were incubated with all the tested antifungal either for 24 or 48 hrs, and MIC was determined as described earlier.

Results:-

Biofilm formation and quantification:-

Fluorescence microscopy demonstrated biofilm formation by all three studied strains that were quantified with the XTT-reduction assay. Mature biofilm formation was observed after 72-hrs incubation for all strains with Candida forming the highest amount of biofilm followed by Aspergillus and Fusarium.

Antifungal sensitivity for planktonic state.

All the three strains were sensitive to most of the antifungals tested by disc diffusion except ketoconazole, which showed intermediate activity against Fusarium (Table I). Estimation of MIC values showed that Voriconazole was most effective antifungal against Aspergillus, amphotericin B was most effective in Fusarium whereas ketoconazole and itraconazole showed lowest MIC against *Candida sp* (Table 2).

Mean biofilm eradication concentration and antifungal sensitivity of post biofilm dispersed planktonic state:-

At biofilm state mean biofilm eradication concentration was measured and found ineffective upto 64µg/ml for all selected antifungals against the isolated fungal species. Antifungal susceptibility test was also performed after biofilm breakdown where the MIC's showed an intermediate value, lower compared to biofilm state but not attaining the values in the planktonic state, (Table 3) for all the tested antifungals.

Discussion:-

Antifungal sensitivity tests have developed greatly in the recent years; various methods such as agar dilution, disc diffusion, concentration gradient and liquid medium dilution are applied in various laboratories in India. We used disc diffusion methods following NCCLS guidelines (2002) where the test mediators, inoculum concentration, incubation temperature for different mycelial fungus are clearly defined. This study showed that in planktonic form of growth, *Aspergillus fumigatus* and *Fusarium solani* were more sensitive to voriconazole compared to other tested azoles, natamycin and anidulafungin. while Amphotericin B was effective against Candida as well as showed good sensitivity against both Aspergillus and Fusarium. However, due to poor penetration in cornea and requirement of high dosage to ensure the adequate concentration in eye, it is not preferred as first line of management in cases of fungal keratitis **(Chang et al, 2006)**. Natamycin is the standard of care in many countries especially in developing countries where natamycin was effective in superficial infection and not in severe cases. Lalitha et al. reported that voriconazole had a lowest MIC against Aspergillus while fluconazole and miconazole showed resistance against maximum number of species. Ketoconazole and itaconazole showed invitro sensitivity against Candida rather than other filamentary fungi (**Lalitha et al, 2007**). However, as showed in this study that resistance of Fusarium and Aspergillus to azoles had increased greatly. Simultaneously, it is important to remember that all the results of antifungal sensitivity tests in vitro are completely not coincident with the therapeutic effects in vivo as the immune defence of hosts also plays important roles in the occurrence of fungal infection. If the pathogen was sensitive to an antifungal treatment with this medication will be effective up to 90% of the patients and in case of resistance therapy

it will be effective in 60% of patients [15]. One of the factors that may play a role in such discordance may be due to existence of invivo biofilm community in these recalcitrant cases of infective keratitis.

One of the defining characteristics of biofilms is their increased resistance to antimicrobial agents. Fungi have been reported to be up to 1000-fold more resistant to antifungal agents than planktonic free floating cells, yet this recalcitrance to antimicrobial therapy has yet to be fully elucidated (**Ramage et al., 2011,Xu et al., 2000**). Antifungal resistance is both complex and multi-factorial. It can be inducible in response to a compound, or an irreversible genetic change resulting from prolonged exposure. Specifically, these include alterations or over-expression of target molecules, active extrusion through efflux pumps, limited diffusion, tolerance and cell density, which are all characterized mechanisms utilized by fungi to combat the effects of antifungal treatment (**Jabra Rizk et al., 2004**).

Planktonic cells generally rely on irreversible genetic changes to maintain a resistant phenotype, whereas biofilms are able to persist due to their physical presence and the density of the population, which provides a resistant phenotype irrespective of defined genetic alterations (**Kuhn et al., 2002; Kuhn et al., 2004**). The results of our study demonstrate that all the studied organisms were sensitive to the tested antifungals in planktonic mode while high degree of resistance was acquired when they attained biofilm state. Interestingly, this study showed that the post biofilm dispersed planktonic cultures were also resistant to the tested antifungals which implies a permanent genetic alteration incurred during biofilm formation which is responsible for converting a non resistant pathogenic strain to a resistant phenotype.

Conclusion:-

To conclude, biofilm formation by Aspergillus, Fusarium and Candida isolated from cases of infective keratitis confers antifungal resistance which is permanent and persists even after breakdown of the biofilm, probably explaining that such resistance is acquired due to a combination of factors which include both morphological and genetic changes. This may be one of the key factors responsible for failure of antifungal therapy in majority of cases of infective keratitis. Further studies need to be performed to identify the genetic changes responsible for generation of resistant fungal phenotypes.

TABLE I

Fungi	Ampho B	Natamycin	Ketoconazole	Itraconazole	Anidulafungin	Voriconazole
A. fumigatus	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
C. albicans	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
F. solani	Sensitive	Sensitive	Intermediate	Sensitive	Intermediate	Sensitive

Antifungal Susceptibility Testing By Disc Diffusion

TABLE II

Organism	Ampho B		Voriconazole		Itraconazole		Ketoconazole		Anidulafungin	
	Disc	Broth	Disc	Broth	Disc	Broth	Disc	Broth	Disc	Broth
A.fumigatus	2	2.5	0.6	0.12	2	1.5	4	2.5	1	1
F.solani	1	1	4	8	8	12	8	8	8	12
C.albicans	2	3	16	16	0.32	0.2	0.6	1.2	1	2

Comparative data of antifungal susceptibility (mic) using disc diffusion and microbroth dilution technique. All measurements are in µg/ml

TABLE III

Organism	Ampho B		Voriconazole		Itraconazole		Ketoconazole		Anidulafungin	
	MBEC	BDPC	MBEC	BDPC	MBEC	BDPC	MBEC	BDPC	MBEC	BDPC
A.fumigatus	>64	16	>64	8	>64	32	>64	16	>64	24
F.solani	>64	12	>64	24	>64	32	>64	24	>64	>64
C.albicans	>64	24	>64	32	>64	12	>64	24	>64	8

Comparative data of mean biofilm eradication concentration (mbec) and biofilm dispersed planktonic cell (bdpc) mic values. All measurements are in µg/ml

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