Synopsis of the Thesis

on

Studies on virulence factors of pathogenic *Fusarium* spp. isolated from corneal infection

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By

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Introduction:

Keratitis:

Keratitis is the term applied for inflammation of the cornea. Corneal infections are known to be the second most significant cause of monocular blindness rated after unoperated cataract in some developing nations in particular and in the tropics in general. Microbial keratitis is a common, potentially vision-threatening ocular infection that may be caused by bacteria, fungi, viruses or parasites. Emphasizing the importance of corneal ulceration as an important cause of visual loss, many studies have reported the prevalence of microbial pathogens and identified the risk factors predisposing a population to corneal infection in India (Bharathi *et. al*., 2003) and abroad. Keratitis due to filamentous fungi is reported worldwide including Asia, Africa, America and European countries (Bharathi *et al*., 2003, Xie *et al*., 2006, Ritterband *et al*., 2006, Tuft and Tullo 2009). Risk factors are poor contact lens care; overuse of contact lenses, illnesses or other factors that reduce the body's ability to overcome infection, cold sores, genital herpes and other viral infections, crowded, dirty living conditions; poor hygiene.

Fungal keratitis:

A fungal keratitis is an inflammation of the eye's cornea that results from infection by a fungal organism. The principal routes of inoculation are introduction concurrent with a penetrating or perforating wound, either mechanical injury or surgery, and introduction through an epithelial defect. Mycotic keratitis is classified as an ophthalmological emergency. The incidence of this disorder has increased over the last 30 years. Fungi causing keratitis have gained importance in Asian countries and contribute nearly half of the world's fungal keratitis cases (Thomas *et al*., 2013; Ansari *et al*., 2013). In tropical countries, fungi account for up to 67% of corneal infections.

The epidemiology of fungal keratitis varies at different geographical locations. The outcome of fungal keratitis is poor due to delay in diagnosis and difficulty in treatment. In India, high incidence of fungal keratitis is reported from every corner of the country (Manikandan *et al*., 2013, Tewari *et al*., 2013). *Fusarium* keratitis is one of the most common fungal infections of the cornea. *Fusarium* species are implicated in fungal keratitis cases primarily from South India, while *Aspergillus* species are reported from other parts of the country (Ghosh *et al*., 2016). Fungal keratitis accounting for 44% of corneal ulcers in India, 36% in Bangladesh, 37.6% in Ghana, and 17% in Nepal. Prevalence varies by region and is highest in South India (36.7%) but is also common in West India (36.3%) and East India (26.4%). *Fusarium* and *Aspergillus* species are the most common isolates (37.2% and 30.7%, respectively). Males are affected more commonly than females (65-71% of patients are male) (Camila, 2016).

***Fusarium*:**

The genus *Fusarium* is a large group of hyaline fungi that are ubiquitously present in soil, water and air, both in temperate and in tropical climates. *Fusarium* species cause a broad spectrum of infections in humans, ranging from mildly superficial to fatally disseminated. *Fusarium* keratitis is a serious ocular disorder that may lead to vision loss. Because of its risk of permanent loss of vision, corneal infections are considered an ophthalmologic emergency.

(Tupaki-Sreepurna *et al*., 2017). Historically, the genus *Fusarium* has developed into a large genus with multiple species complexes, some of which can cause opportunistic infections in humans. Especially members of the *Fusarium solani, F. oxysporum, F. fujikuroi, F. dimerum, F. chlamydosporum,* and *F. incarnatum-equiseti* species complexes have been involved in human and animal infections. *Fusarium* species are among the most resistant fungi; infections are commonly refractory against treatment with the most known systemic and conventional antifungal agents (Guarro *et al*., 2013, Van Diepeningen *et al*., 2014). *Fusarium* pathogens typically show broad *in vitro* resistance to antifungal agents with a high variability being present within each species (Taj-Aldeen *et al*., 2016, Araujo *et al*., 2015, Al-Hatmi., 2015). In general, members of the *Fusarium solani* species complex (FSSC) are most commonly observed in all clinical infections and show the highest minimum inhibitory concentrations (MICs) against various antifungal drugs. (Taj-Aldeen, S. J., 2017). Many studies report the prevalence of *Aspergillus* spp., followed by *Candida* and *Fusarium spp.* (Suman *et al.,* 2009). However, there is a shift in this prevalence in recent studies where *Fusarium* is now reported as the most common fungal pathogen for keratitis. A 5-year retrospective review of fungal keratitis in Malaysia showed that that the incidence of fungal keratitis has increased each year from 2007 to 2011 by 12.50%, 17.65%, 21.21%, 26.83%, and 28.57%, respectively. *Fusarium* species were the most common fungi isolated, followed by Candida species (Mohd-Tahir *et al.*, 2012; Ranjini & Vandana, 2016)

Virulence factors:

A determinant of pathogenicity is a virulence factor. Virulence factor is a microbial component that damages the host (Casadevall *et al.,* 2007).

- **Enzymes:** Production and secretion of hydrolytic enzymes, such as proteases and lipases are very important virulence factors. These enzymes play a role not only in nutrition but also in tissue damage, dissemination within the human organism, iron acquisition and overcoming the host immune system, and strongly contribute to fungal pathogenicity. Proteases from *Aspergillus* spp. causing keratitis have been reported only from *Aspergillus flavus*. The exoproteome analysis of a keratitis causing *A. flavus* showed that nearly 50% of the exoproteins possess catalytic activity and one of these, an alkaline serine protease (Alp1) is abundant and present in multiple proteoforms. (Selvam *et al*., 2015; Leema *et al*., 2010). Extracellular enzymes such as lipase, deoxyribonuclease (DNase), α-amylase, protease, cellulase and pectinase produced by the *Fusarium* isolates causing keratitis. *Fusarium* proteases have been documented in rabbit model for keratitis. However, detailed characterization regarding the types, number and amount of proteases are lacking from keratitis causing *Fusarium* isolates (Mythili *et al*., 2014)
- **Cell wall component:** Fungal cell wall consists of three main components; lipids, proteins and carbohydrates (glucan, mannan and chitin). The carbohydrate content of the cell wall varies depending upon the type of fungal species. β-glucans typically comprise 50-55% of the cell wall polysaccharide content (Kogan *et al.,* 2007). Increased virulence with increased chitin content and with glucan-containing covalently attached phospholipid, also high phospholipids content of cell walls as a characteristic of highly virulent strains.

- **Mycotoxin:** Trichothecene are mycotoxins produced mostly by members of the *Fusarium* genus. Inhibition of protein synthesis is thought to be the fundamental mechanism of TC toxicity. The toxin binds to the peptidyl transferase, which is an integral part of the 60S ribosomal subunit of mammalian ribosome. Zearalenone is one of the most common *Fusarium* mycotoxins in the temperate regions of America, Europe and Asia. ZEA is a nonsteroidal, estrogenic mycotoxin produce by *Fusarium* species (Wood, 1992; Scientific committee on food, 2000).
- **Pigments:** Naphthoquinone shows many biological activity, including phytotoxic, insecticidal, antibacterial, and fungicidal properties (Albrecht and Heiser, 1998; Baker *et al.,* 1990). In addition, some of them produce cytotoxic and anticarcinogenic effects (Vogel *et al.,* 1975).

Models for keratitis study:

Various infection models have been developed to study and understand the mechanism of Keratitis disease. Mouse model of corneal fusariosis was developed that permits the evaluation of fungal infection and pathogenesis (Wu *et al.,* 2004). Rat model to study *F. solani* keratitis showed appropriate dose to develop infection (Zhu *et al.,* 2011). Rabbit model was developed to study the protease profile of keratitis causing *Aspergillus* spp. and *F. solani* (Gopinathan *et al.,* 2001). *Ex vivo* models have been used to study keratitis caused by *Candida*, *Fusarium,* herpes simplex virus, *S. aureus,* and *P. aeruginosa.* The mouse corneal organ culture developed for *Candida* infection was used to study fungal adhesion mechanisms and drug screening. *Fusarium* infection was modelled using human corneal buttons stabilized in an artificial anterior chamber (Refractive Technologies, Cleveland, OH). Recently, rabbit corneas were used in an *ex vivo* model for *Pseudomonas* and *Staphylococcus* keratitis. In that study, human and rabbit cornea *ex vivo* keratitis models were compared. (Pinnock *et al*., 2017; Harman *et al*., 2014)

Rationale:

Keratitis incidences due to *Fusarium* are increasing worldwide and it is becoming leading cause of infection. It has become multidrug resistance and very few reports are there documenting virulence factors of pathogenic *Fusarium* species. Extensive studies comparing levels of virulence factors among various isolates have not been reported. Also, comparison of *in vitro* results with infection model are lacking. So there is a need to identify and comparative studies of different virulence factors of *Fusarium* causing keratitis.

Objectives:

1. Morphological and molecular identification of pathogenic clinical *Fusarium* isolates
2. Quantification of virulence factors of pathogenic *Fusarium* spp.*in vitro*
3. Development of *ex vivo* infection model for fungal keratitis and testing and expression analysis of fungal virulence factors, in different phases of infection

Results:

Morphological and molecular identification of pathogenic clinical *Fusarium* isolates: Total 25 isolates were used in this study. Twenty two pathogenic fungal isolates were collected from corneal scrapping of keratitis patients and 3 non-pathogenic *Fusarium* isolates were obtained from Microbial type culture collection, Pune. Potato dextrose agar was used as growth medium. Morphological identification was done using Lactophenol cotton blue staining. For molecular identification fungal isolates were grown in PDB for 8 day and then DNA was isolated. PCR amplification of two conserved region ITS (Internally transcribed spacer) and TEF (Translation elongation factor) was carried out followed by agarose gel electrophoresis and sequencing. The obtained sequence was blasted using NCBI BLAST, *FUSARIUM* ID and MLST database. From n=25 isolates, 3 isolates were identified as *F. sacchari*, 4 isolates were identified as *F. delphenoides* and 16 isolates belonged to FSSC and 1 isolate identified as *F. equiseti*. The nucleotide sequences have been submitted to genebank.

Quantification of virulence factors of pathogenic *Fusarium* spp. *in vitro*:

Protease: Isolates were grown in PDB for 8 days and culture filtrate was acetone precipitated and precipitates were dissolved in 1x PBS (Phosphate buffered saline). Azocasein assay was carried out for specific activity determination. The activity varies among isolates of same species. Specific activity ranges from 24.03±0.57 units/mg (Cc 52) to 223.6±18.8 units/mg (Cs 1) of protein. Among FSSC isolates, specific activity ranges from 38.87±2.93 units/mg (Cc 50) to 223.6±18.8 units/mg (Cs 1) of protein. Among *F. sacchari* isolates, specific activity ranges from 24.03±0.57 units/mg (Cc 52) to 158.8±3.18 units/mg (Cc 167) of protein. Among *F. delphenoides* isolates, specific activity ranges from 37.65±1.93 units/mg (Cc 26) to 154.1±0.80 units/mg (CSH 4) of protein. Characterization was done at different pH and with different inhibitors using azocasein assay and gelatine zymography. pH range pH 3 to pH 10 was used. PMSF (Phenylmethanesulfonyl fluoride- inhibitor of serine protease), Pepstatin A (inhibitor of aspartyl protease) and EDTA (Ethylenediaminetetraacetic acid- inhibitor of mettalo protease) were used as inhibitor at final concentration of 5mM, 1μM and 10 mM, respectively for both azocasein assay and gelatin zymography. Optimum pH for all majority of isolates was pH 7.4 and for two FSSC isolate and one *F. sacchari* isolate optimum pH was pH 3. Majority of proteases produced by all three species are serine and mettalo proteases followed by aspartyl proteases. However, results of azocasein assay and gelatin zymography assay do not completely comply with each other. On the basis of specific activity determination two isolates were selected, one with highest activity (CS 1) and one with lowest activity (Cc50) for real time expression study. Real time expression study was carried out for FSSC isolates only as whole genome sequence of only *F. solani* is available. Total 15 genes were selected using Uniprot KB and those who were positive for signal p were included in study. Nine high molecular weight (C7Z7U2, C7ZNV5, C7YY94, C7Z436, C7Z7Y4, C7YPA2, C7Z0E6, C7ZFW9 and C7YY94) and 6 low molecular weight (C7YQJ2, C7YVF3, C7Z6W1, C7Z8P9, C7YSA1 and C7YSV9) proteases were selected. Only seven genes C7Z7U2, C7ZNV5, C7YQJ2, C7YVF3, C7YY94, C7Z436 and C7Z6W1 were getting expressed in both the isolates. Relative expression of all 7 gene is lower in isolate Cc50 and higher in CS 1. Among all seven genes maximum expression was observed in C7ZU2, which code for tripeptidyl

peptidase. To understand types of proteases secreted by *Fusarium* isolates exoproteome analysis is being carried out for one representative isolate from each species.

Glucan: For beta glucan extraction fungal isolates were grown in PDB for 10 days and dry weight was used for extraction. Extraction was carried out using method of Ohno *et al*., 1999. Estimation of total beta glucan was carried out using Congo red assay and for 1,3 beta glucan ELISA (Enzyme linked immumosorbant assay) was carried out. Antibodies used for ELISA were specific for 1,3 beta glucan. Among all the isolates maximum total beta glucan production was seen in *F. delphenoides* isolate Cc 26 (36.131±0.83) and minimum in FSSC isolate Cs 1 (0.850±0.03) and maximum 1, 3 beta glucan production was seen in *F. delphenoides* isolate Cc 26 (6.62±0.83) and minimum in FSSC isolate Cc240 (0.010±0.008). Among FSSC isolates, total beta glucan amount ranging from 0.850±0.03 (Cs 1) to 5.442±0.15 (Cc 240) and 1,3 beta glucan ranging from 0.010±0.008 (Cc240) to 3.701±0.17 (Cc 50). Among *F. delphenoides* isolates, total beta glucan amount ranging from 36.131±0.83 (cc 26) to 2.120±0.07 (CSH 4) and 1,3 beta glucan ranging from 0.139±0.13 (Cc119) to 6.62±0.83 (Cc26). In *F. sacchari* isolates total beta glucan ranging from 1.130±0.09 (Cc61) to 15.256±0.21 (Cc215) and 1, 3 beta glucan ranging from 0.0036±0.0004 (Cc61) to 1.150±0.04 (Cc167). Primarily beta glucan characterization was done using TLC (Thin Layer Chromatography). Further characterization was done by FTIR (Fourier Transform Infrared) spectroscopy. FTIR spectra of standard and sample confirmed the presence of 1, 3 linked beta polysaccharides. 3430 $^{cm-1}$, 2921 $^{cm-1}$, 1000-1200 $^{cm-1}$, 1156-1165 $^{cm-1}$ and 889 $^{cm-1}$ corresponds to -OH group, -C-H bond, Polysaccharide, 1,3 linkage and Beta linked glycosidic bond, respectively. Fluorescence microscopy was carried out to estimate 1,3 beta glucan level using aniline blue dye which specifically binds to 1,3 beta glucan. Cc 26 isolate mycelia showed higher fluorescence and Cs 1 showed less fluoresecence which is in accordance with quantification data. Further, real time expression study was carried out on FKS gene responsible for beta glucan synthesis in *Fusarium*. Two isolates were selected from FSSC group with highest (Cc240) and lowest (Cs 1) beta glucan. RNA isolation was carried out using trizol reagent followed by C-DNA synthesis. Relative expression of FKS gene was higher in Cc 240 and lower in Cs 1 as beta glucan is higher in Cc 240 and lower in Cs 1.

Toxins: Fungus was grown in SDB (Sabouraud Dextrose Broth), PDB, corn and rice media for 20 days in dark for toxin production. Toxin extraction was carried out with ethyl acetate and Acetonitrile: water (3:1) (V/V). Obtained extract was vaporised and dissolved in HPLC grade acetonitrile. Primary characterization was done using TLC. Zearalenone toxin seems florescent blue in presence of long UV light while trichothecene shows brown colour after spraying with 20% H_2SO_4 followed by heating in an oven for 10 minutes. Further, HPLC was carried out for estimation of Zearalenone from samples by using standard ZEA. Peaks of standard ZEA was obtained at 14.1 min and 9.7 min using two different c18 RP (reverse phase) column. The amount of toxin was very less in samples so that peaks obtained were very short. To further confirm the peak of toxin, the samples were spiked with standard and were observed for increased peak height. Out of 22 pathogenic isolates toxin production was seen in six isolates only. Two isolates of *F. sacchari* Cc61 and Cc 215 and four isolates of FSSC Cc240, CSH 5, CSH 9 and CSH 11 showed presence of ZEA toxin in HPLC. Further to confirm the presence of ZEA toxin, mass spectroscopy was carried out. Standard ZEA has molecular

weight of 317 and peak obtained in mass spectrum for standard ZEA at 317.15 and same peak was also observed in samples which were positive for ZEA using HPLC. Hence, the presence of ZEA in samples was confirmed. The maximum production of toxin was seen in PDB and SDB medium. Among all the six isolates, maximum toxin production was seen in Cc 61 (39.8 µg/l) and minimum toxin production was seen in Cc 215 (2.3 µg/l).

Pigment: For pigment production fungus was grown in PDB, SDB, YMB (Yeast extract Malt extract Broth) and NB (Nutrient Broth) for 20 days in dark. Pigment extraction was carried out at interval of one day from day two to day 20 using ethyl acetate. Extract was filtered from column containing sodium sulphite to remove moisture from extract and then was vaporised and dissolved in HPLC grade acetonitrile. TLC was carried out primarily. Pigment production was increased day wise and maximum pigment production was seen in PDB followed by SDB. Fungus grown in YMB and NB did not show any pigment production. Primarily characterization of pigment was done using method of Ammar *et al.,* 1979. Two main bands were obtained in TLC and were named as fast orange (F.O.) and slow orange (S.O.) based on their migration pattern in TLC. When TLC was sprayed with 20 % KOH made in ethanol and then heated for 10 minutes at 80˚C, lower band of S.O. turned in to purple band which indicates that S.O. might be anhydrofusarubin and F.O. might be fusarubin. Pigment purification is being carried out to further characterise F.O. and S.O.

Development of *ex vivo* infection model for fungal keratitis and testing and expression analysis of fungal virulence factors, in different phases of infection:

To understand the mechanism of mycotic keratitis caused by *Fusarium* and how fungus penetrate with course of infection *ex vivo* infection model was developed using caprine (goat) cornea. Goat eyeballs were collected from an abattoir in a sterile beaker, washed with 1X PBS and sterilised by incubating in 2.5% povidone iodine for 5 minutes followed by 0.1 % gentamycin for 15 minutes with intermittent PBS washes. Sterile scalpel was used to make an incision in the limbal region of the eyeball and scissors were used to further expand the incision and cut around the middle of the eye, bisecting the eye. The front half (the cornea) was incubated in culture media DMEM (Dulbacco's Modified Eagle's Medium) containing 10 % FBS (Fatal Bovine Serum), 100 I.U. /ml penicillin, 75 µg/ml streptomycin and 35 µg/ml gentamycin. The solid support was prepared using a sterilized solution of agarose (0.5%) and gelatin (0.5%) in PBS. Agarose-gelatin beads were made using surface-sterilized and stretched parafilm in a tube. The cornea was placed on this bead, and the medium was added to the well so that the endothelial layer remained in direct contact with the bead, with the epithelial layer facing the air. Media was changes every 24 hours. The viability of corneal epithelial cells was assessed using the trypan blue assay and the viability of the entire cornea was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Both the assays were carried out at alternate day till 20 days. Morphologically corneas were also observed for opacity. Trypan blue and MTT assay results indicated that corneas were viable till 15 days. For standardization and development of *ex vivo* model 90 corneas were utilised. For infection development in cornea using *ex vivo* model, a scratch was made using sterile scalpel on epithelial layer of cornea. Spores were collected form 10 day old fungi grown on PDA in sterile PBS. Spores were counted using haemocytometer. 10^6 spores were used initially; however, subsequently, all experiments were performed using 932 ± 46 spores. The

progression of infection was monitored visually every day, as well as by keratome sectioning followed by H-E (haematoxylin and Eosin) staining. On day 2, the epithelium was completely disrupted and the fungi could be seen penetrating the upper stroma. On day 4, the fungi were more concentrated in the upper half of the cornea and there was a greater presence of long hyphae compared to day 2. By day 8, half of the stroma was completely disrupted and the lower half also contained fungal hyphae. By day 10, histological processing of the cornea was no longer possible due to complete degradation. Because no well-established virulence factors have been reported in *Fusarium* keratitis, qRT-PCR was restricted to certain proteases. Preliminary gelatin zymography indicated the presence of high molecular weight protease. Relative expression analysis of the proteases C7Z0E6, C7ZFW9, C7Z7U2, C7ZNV5, and C7YY94 was carried out at day 2, 4 and 8, of which C7Z0E6, C7Z7U2, and C7ZNV5 exhibited the highest levels of expression at day 8. Gelatin zymography of infected corneas indicated the presence of more than one protease, and higher activity was seen on days 4 and 8. Two isolates with highest and lowest protease activity has been selected for *ex vivo* infection. Real time expression analysis for all protease encoding genes will be carried out in both the isolates.

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