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Molecular Identification and *in-vitro* Antifungal Susceptibility of *Fusarium* sp. Isolated from Corneal Scrape

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ABSTRACT Keratomycosis can have deleterious effect to human eye and may lead to loss of vision. Fungal keratitis caused by a *Fusarium* species was isolated and described. The isolate was identified to be *Fusarium solani* and confirmed by molecular data based on ITS rDNA sequence. The fungal isolate was evaluated for its antifungal susceptibility against five common antifungal agents. The result indicated that miconazole was highly sensitive but fluconazole and ketoconazole were intermediately sensitive. Amphotericin B was found to be effective against the pathogen with MIC value of 4mg/L. Human pathogenic *F. solani* is highly diverse and resistant to many antifungal agents. Therefore, accurate identification and susceptibility profile of this species is very essential for rapid diagnosis and treatment.

INTRODUCTION

Keratomycosis or fungal keratitis can have a deleterious effect on the human eye and are usually associated with significant loss of visual function. Many species of fungi commonly classified as fungal ophthalmic pathogens have been reported to cause eye infections (Eduardo and Alfonso 2008). Among several fungal pathogens, *Fusarium* spp. is considered as the most dangerous filamentous fungi causing various diseases in humans particularly in immunocompromised patients (Nucci and Anaissie 2007). Classification of *Fusarium* at the sub-genus level has been problematic, but increasing reports of *Fusarium* as a human pathogen in ocular infections has generated an interest in finding a more consistent basis for classification. Accurate identification of the etiologic agents causing keratomycosis is critical to rapid diagnosis. It will also help in advancing our understanding of each species and may lead to more identification methods and effective antifungal therapy and improve clinical outcomes. Besides, identification of *Fusarium* by morphological traits is often difficult because mycelial pigmentation, shape and size of conidia are unstable and highly dependent on composition of media and environmental conditions. Molecular technique such as internal transcribed spacer (ITS) ribosomal DNA (rDNA) have become reliable and are highly suitable tool for identifying variable species like *Fusarium* and for assessing genetic variations within populations. The aim of this study was to analyse *in-vitro* antifungal susceptibility and molecular identification of a *Fusarium* sp., causing ophthalmic mycoses.

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MATERIALS AND METHODS

Clinical Observation

A 38 year male, farmer by occupation visited the outpatient wing of eye department of Kasturba Medical College, Manipal University, Manipal with pain, redness in left eye and a history of trauma due to stick injury of one week. Before arrival to the hospital he got treated with ciprofloxacin eye drops and acyclovir ointment by a local practitioner. The symptoms persisted and upon examination he had a corneal ulcer with hyphating margins, satellite lesions and indurations.

Isolation of Organism

Following instillation of local anaesthetic eye drops the patient was positioned near a slit-lamp

for corneal scraping procedure. Using a sterile kimura scalpel, the base of the ulcer scraped for collection of the infective tissue for potassium hydroxide (KOH) microscopy, culture for aerobic bacteria and fungi. The scrape specimens obtained were inoculated on to Sheep Blood Agar (SBA), Sabouraud Dextrose Agar (SDA) in 'c-streaks' and incubated at 37°C and 28°C respectively. In addition non-nutrient agar with *Escherichia coli* overlay was incubated at ambient temperature for *Acanthamoeba*.

Morphological Identification

After 72 h of incubation, SBA was sterile for aerobic bacteria and no *Acanthamoeba* was isolated. The SDA plates indicated growth of filamentous fungi. Microscopic observation revealed thin, slender, branching, hyaline and septate mycelium. Numerous conidia were observed which were septate and sickle shaped. Based on the morphological traits the fungus was identified to be *Fusarium* sp.

Antifungal Susceptibility Test

Antifungal susceptibility testing for the isolate was performed using the microdilution method following the Clinical and Laboratory Standards Institute (CLSI) document M38-A2. Spores suspension of 1 X 10^5 CFU/ml was prepared by counting spores in a haemocytometer and RPMI 1640 supplemented with 2% glucose (Aberkane 2002; Rodriguez- Tudela et al. 2003). A complete inhibition of visual growth at 48 h was considered to be the end point antifungal concentration.

Genomic DNA Isolation, PCR Amplification and Sequencing

Total genomic DNA was extracted from mycelia of the fungus grown on potato dextrose agar medium by using CTAB method (Cai 2006). DNA amplification was performed by PCR. The PCR was set up using the following components: 5µL Buffer (10X), 3µl $MgCl_2$ (25mM), 1µL dNTPs (10mM), 1.5µL Taq Polymerase (5U), 1.5µL Forward primer (10µM), 1.5µL Reverse Primer (10µM), 3µL DNA template and 34.7µL distilled water. The PCR condition was run in such a way, where initial denaturation was at 95°C for 5 min. Denaturation, annealing and elongation were done at 95°C for 1min, 52°C for 30 sec and 72°C for 1 min respectively in 45 cycles. Final extension was done at 72°C for 10 min and hold at 4°C forever. For amplification of ITS-rDNA region ITS4 and ITS5 primers were used according to the method described by White et al. (1990). The PCR product, spanning approx. 500-600bp was checked on 1% agarose electrophoresis gel. It was then purified using quick spin column and buffers (washing buffer and elution buffer) according to the manufacturer's protocol (QIA quick gel extraction kit Cat No. 28706). DNA sequencing was performed using the above mentioned primers in an Applied Biosystem 3130xl analyzer.

Sequence and Phylogenetic Analysis

The sequence was annotated using sequin software and submitted to NCBI GenBank database. BLAST homology search was performed to find the closest homologs and sequence analysis was done by comparison of ITS rDNA sequences of 24 other pathogenic *Fusarium* spp., obtained from GenBank database. The sequences were aligned using clustal W and phylogenetic analysis was carried out by Molecular Evolutionary Genetic Analysis software version 4 (MEGA4). The evolutionary tree was reconstructed using the Neighbor-Joining (NJ) method. The optimal tree with the sum of branch length = 0.18683191 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

RESULTS

The fungus was isolated from corneal scrape of a patient suffering from severe eye pain. It showed rapid growth in SDA medium with profuse branching after 72 h of incubation. The colonial morphological traits of the isolate were white cottony to creamy aerial mycelium. Microscopic observation of the fungus revealed thin, slender, branched and septate hyphae with production of numerous macro and microconidia which suggest that the fungus belong to the genus *Fusarium*. Further identification was carried out by considering the partial 18S, ITS1, 5.8S, ITS2 and partial 28S genes. Based on BLAST search anal-ysis of the sequence, the

fungus was found to be the closest homolog to *Fusarium solani*. The sequence of the fungus has been deposited in GenBank database with accession number HM145945.1. Table 1 shows the result of antifungal susceptibility profile of the fungus agai-nst five antifungal agents. The isolate showed resistance against miconazole (75mg/L) and intermediate sensitivity to keto-conazole and flu-conazole with MIC values of 8 mg/L and 16 mg/L respectively. Amphotericin B was found to be most sensitive (4 mg/L) followed by itra-conazole (6 mg/L).

Table 1: Antifungal susceptibility of *F. solani* isolate

	MIC (mg/L)
Antifungal	*48 h*
Amphotericin B	04.0
Miconazole	75.0
Itraconazole	06.0
Fluconazole	16.0
Ketoconazole	08.0

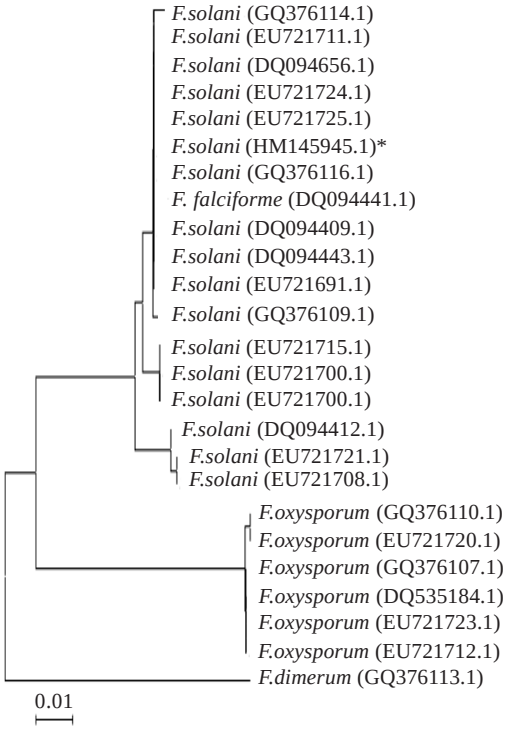

Fig. 1. Phylogenetic tree showing evolutionary relationships of 25 human pathogenic *Fusarium* spp. Accession numbers are given in parentheses. The tree was generated NJ method using MEGA4. * Indicates accession number of *F. solani* under study

Evolutionary relationships of the isolate with 24 other pathogenic *Fusarium* spp. obtained from GenBank database is depicted in Figure 1. Altogether the analysis involved 25 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 395 positions in the final dataset. The tree showed two major separate groups one consisting of *F. oxysporum* and other *F. solani*. The presence of the fungus (HM145945.1) under study within the *F. solani* group validates the classification at sub-genus level and precise its identification as etiologic agent causing keratomycosis. The *Fusarium* spp., used for the analyses were obtained from GenBank database and most of them were keratomycotic in nature isolated from corneal scrapes, contact lens and immunocompromised patients.

DISCUSSION

Fusarium is highly diverse and ubiquitous fungi widely distributed in nature as saprotrophs and pathogens causing diseases in plants and animals. During recent years, there has been gradual increase of keratomycosis caused by *Fusarium* in humans (Guarro and Gene 1995). The most common pathogens reported to be an etiological agents of human infection are *Fusarium solani* and *Fusarium oxysporum* (Nucci and Anaissie 2002). Among several fungi causing fungal keratitis, *Fusarium solani* keratitis may completely destroy an eye in a few weeks, since the infection is usually severe and perforation, deep extension and malignant glaucoma may supervene (Sharma et al.1993). Therefore, rapid identification of this species is necessary for quick diagnosis and treatment. Identification to genus level of *Fusarium* has been based on the study of their morphological characteristics. Moreover, isolates involved in human infections usually do not produce the characteristic morphology that allows its identification. Besides, recognition of *Fusarium* to species level is laborious and time consuming. On the other hand, molecular techniques have been developed to identify *Fusarium* species. In this study molecular tools based on ITS rDNA have been used to identify fungus from corneal scrape as *Fusarium solani.* The ITS region is attractive to be used in diagnostic tests, because it is a multicopy gene, maximising the sensitivity of the PCR and a lower number of pathogens would

be necessary to yield sufficient DNA for a positive recognition of the pathogen (Eduardo and Alfonso 2008). This is principally important in the ophthalmic field, where extremely small volume samples are usually available for the diagnostic workup.

Fusarium spp. are resistant *in vitro* to many of the antifungal compounds licensed to treat fungal infections, and among them, *F. solani* is considered the most resistant. Therefore, it is very important to carry out antifungal susceptibility test of this species for rapid diagnosis and efficient administration of appropriate antifungal therapy. Several antifungal agents have been used for treatment of fungal keratitis but *Fusarium* spp., have been found to be resistant to many of them. Many isolates have been found to have lower MICs of amphotericin B, voriconazole, posaconazole and at the same time these drugs have been shown to be ineffective against *Fusarium* infection (Anaissie 1992; Alastruey-Izquierdo et al. 2008). It is often found that antifungal agents cannot be administered based on identification alone since susceptibility profile is isolate dependent, so antifungal susceptibility testing should be performed for any *Fusarium* involved in an invasive fungal infection. The antifungal susceptibility profile of the isolate indicated that most of the antifungal agents were resistant or intermediately sensitive. The isolate showed resistance to miconazole which was paradoxical to the findings of Reuben et al. (1989) who reported that several clinically isolates of *Fusarium* spp. showed activity to miconazole *in vitro*. But in many instances it has been shown that flucytosine and imidazole were practically resistant universally. Such findings agree with the report that optimal treatment for *Fusarium* spp. has not yet been established. In the present study, the isolate was found to be sensitive to amphotericin B. Similar results were obtained by several workers against fungal keratitis caused by *Fusarium* spp. (Pujol et al. 1997; Alastruey-Izquierdo 2008). Thus it may be concluded that accurate identification and susceptibility profile of clinical *Fusarium* spp., is very essential for rapid diagnosis and treatment.

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