Phylogeny, diagnostics and antifungal susceptibility of clinically relevant Fusarium species

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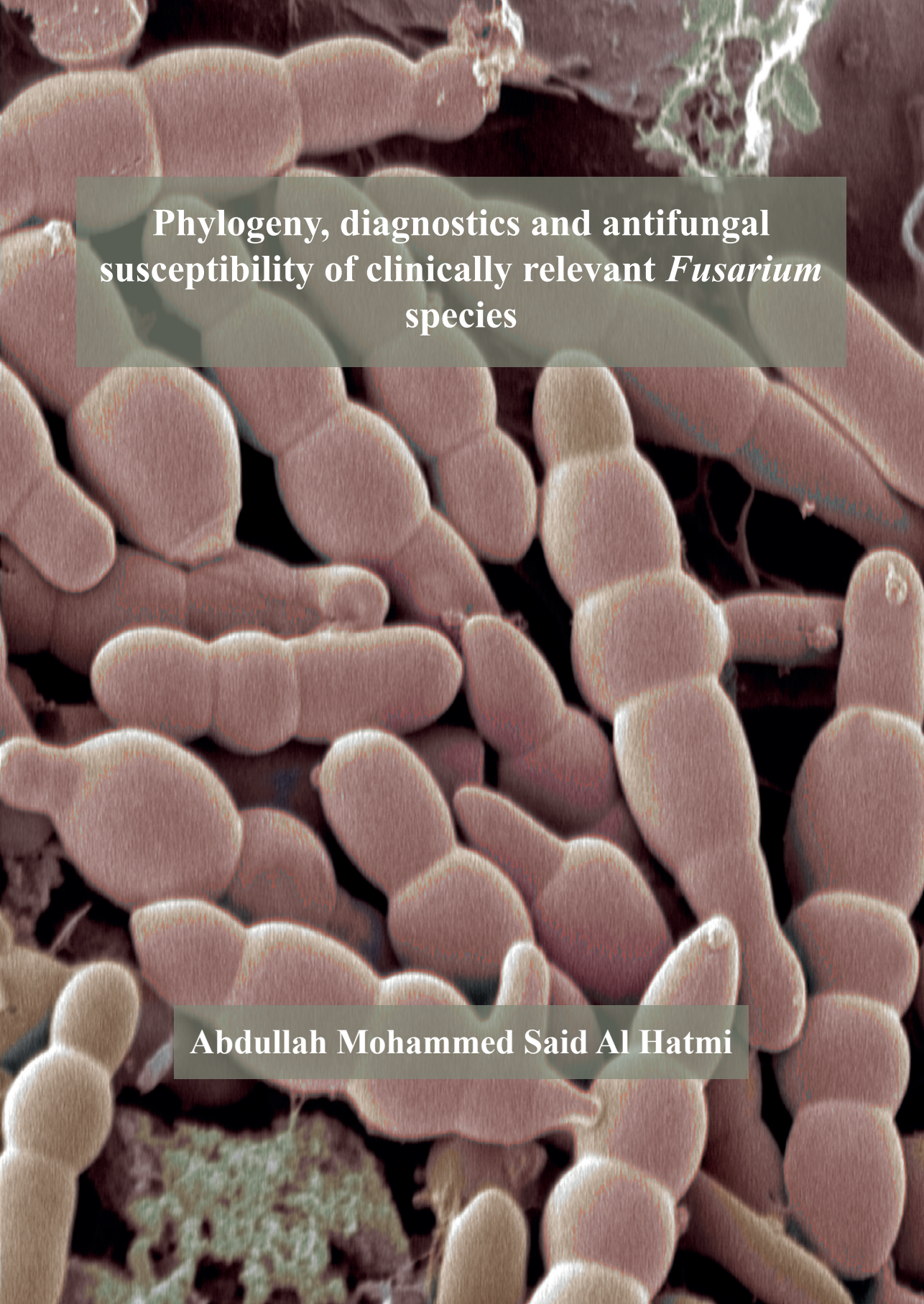
Publication date
2016

Document Version
Final published version



Phylogeny, diagnostics and antifungal susceptibility of clinically relevant *Fusarium* species

Abdullah Mohammed Said Al Hatmi

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ISBN 978-94-91407-43-7

Cover design:
Front : Scanning electron micrograph of multicelled macroconidia of *Fusarium culmorum*. The picture was kindly provided by Jan Dijksterhuis.
Back: Macroscopic and microscopic characteristics of *Fusarium*

Layout: Abdullah Al Hatmi

Printed by: Proefschriftmaken.nl

The printing of this thesis was supported by contributions of:
IBED; CBS-KNAW; Astellas Pharma B. V. Netherlands and Gilead Science B. V. Netherlands.

Phylogeny, diagnostics and antifungal susceptibility of clinically relevant *Fusarium* species

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. ir. K.I.J. Maex
ten overstaan van een door het college voor promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op woensdag 21 december 2016, te 14.00 uur

door

Abdullah Mohammed Said Al Hatmi
geboren te Al Hajr, Oman

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Contents

Part III: *In vitro* antifungal susceptibility testing and combinations

Part IV: General discussion, future perspectives, summary in English and in Dutch, acknowledgments, curriculum vitae and list of publications

To my parents, my wife and my son

CHAPTER 1

Chapter 1

General introduction and outline of the thesis

Fusarium: molecular diversity, emerging opportunism and intrinsic drug resistance

- Molecular diversity
- Phylogeny and barcoding
- Clinical aspects and epidemiology
- Application of rapid diagnostic tools
- *In vitro* susceptibility testing of *Fusarium* species
- *In vitro* drug interactions
- Aim and outline of the thesis

Part of the introduction is adapted from:
Plos Pathogens 2016; 2(4):e1005464. doi: 10.1371/journal.ppat.1005464 and Current Clinical Microbiology Reports 2014; 1:10–18.

Historical background of clinically relevant *Fusarium*

The genus *Fusarium* was first described in the early 19th century. In 1935, Wollenweber and Reinking used morphological differences to organize the genus into 16 sections with 65 species, 55 varieties, and 22 forms,[11] but Booth simplified this later to only 14 species.[12] When Leslie and Summerell used morphological and phylogenetic information for reclassification, they ended up with 70 species, most of which formed falcate, multiseptate macroconidia with a beaked apex and a pedicellate basal cell. Microconidia, when present, are one to two-celled and pyriform, fusiform, or ovoid in shape. Both macro- and microconidia are produced in the aerial mycelium on phialides.[13] At present, with the dawn of molecular sequencing, more than 200 species are recognized in 22 species complexes, all differing by morphology, host association, and molecular parameters.[14] Currently, 74 taxonomic species are involved in human infections (Fig 1),[15] as judged from their isolation from clinical samples, and this number is ever expanding. To date, about 36 of the alleged human opportunists have a name, while 38 are still unnamed and can only be identified by multilocus sequence analysis (MLSA). Thus far, 21 species have been described in case reports from proven infections,[16] and more have been isolated from clinical samples.

Phylogeny and barcoding

Morphological characteristics of *Fusarium* species are difficult to evaluate because these are variable within and insufficiently different between species. The *Fusarium* genus, defined in a morphological sense, in the three widely used morphology-based taxonomic description,[13,43,44] appears to be non-monophyletic. These monographs recognize 30, 78 and 70 species, respectively when morphological and sexual criteria are used. In 2013, Geiser and co-authors with clinical and phytopathological expertise have launched a plea for nomenclatural stability with preservation of the name *Fusarium* for all clinically relevant species complexes.[45] However, recently Lombard et al.,[46] moved *F. solani* to the genus *Neocosmospora*, while the *F. dimerum* species complex was transferred to the new genus *Bisifusarium*. Another proposal for *Fusisporium* as genus name for the *F. solani* complex was recently published by Schroers et al.[47] To preserve the nomenclature stability, we adhere in this thesis to *Fusarium* as best known descriptor for the etiological agents observed in the study. Infections are commonly classified by their causative agent (bacterial, viral, parasites and fungal), in this case *Fusarium* spp. cause 'fusariosis'. Hence, when generic names are changed, as a consequence also the disease name is affected.

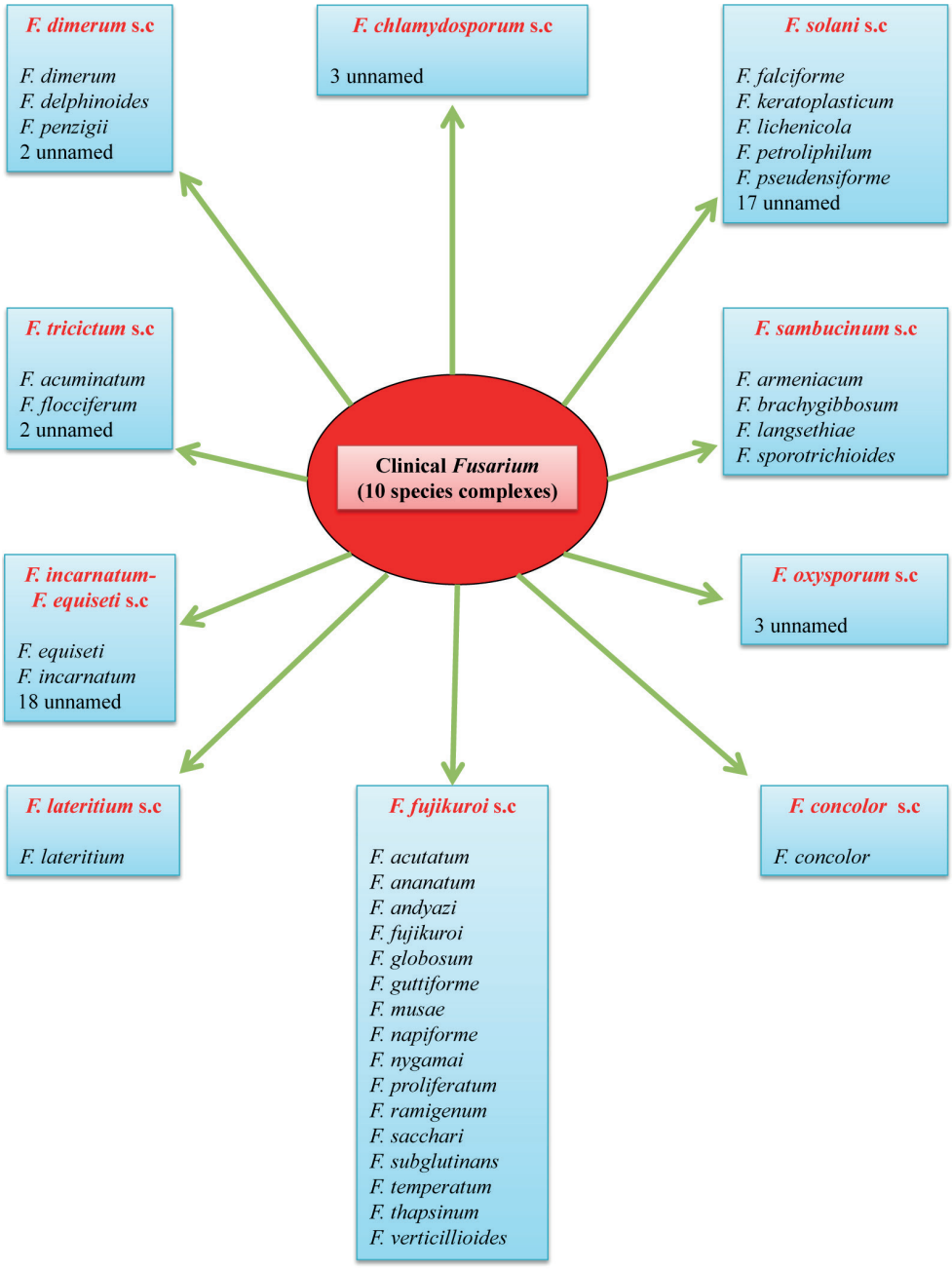

Fig 1. Diagram illustrating *Fusarium* species reported from clinical cases and belonging to ten species complexes.

The use of molecular genetic data appears to be essential to recognize monophyletic *Fusarium* species, the genus comprising at least 20 clades which are also referred to as species complexes (Fig. 2). Multilocus data have been applied to separate closely related taxa and provide support for species borderlines in *Fusarium*[48] as successfully used in other fungi. DNA sequencing of partial genes have been used to supplement morphological identification of *Fusarium* species. Today, nuclear, mitochondrial and protein-coding gene genealogies provide a robust and reliable means for phylogenetic species recognition,[49,50,15] although much undescribed diversity remains to be studied. Many molecular phylogenetic studies have used ribosomal RNA genes including nuclear and mitochondrial ribosomal rDNA (18S, 28S, ITS, IGS, mtSSU, mtLSU),[51] because these are conserved regions of the genome which have been well characterized and are easily amplified by PCR. Although analysis of ITS2 is confounded by the occurrence of non-orthologous copies, especially in the *F. fujikuroi* species complex (FFSC), ITS polymorphisms do exist in other *Fusarium* spp.[52] Stielow et al.[53] reported on newly identified potential barcodes, particularly 60S ribosomal subunit rDNA (60S), as one of the promising barcodes for *Fusarium*.

Protein-coding genes are also in use, such as RNA polymerase (*RPB1/2*), β-tubulin (*BT2*), elongation factor (*TEF1*),[54] and ATP citrate lyase (*ACL1*).[55] Geiser et al.[54] concluded that *TEF1* has excellent phylogenetic utility for the following reasons: (i) it is highly informative at the species level; (ii) non-orthologous copies of the gene have not been detected across the genus; and (iii) it is alignable across the genus *Fusarium*. Some single-copy protein-coding genes such as *RPB1* and *RPB2* are also promising for phylogeny and barcoding in *Fusarium*.[56] In general, protein-coding genes may be invariant in their amino-acid sequences but show high levels of divergence in third codon positions and introns, which make them appropriate for distinguishing closely related. In the present study, which investigates *Fusarium* using multi-locus sequencing, several DNA fragments were involved including two recently discovered DNA barcode loci, topoisomerase I (*TOP1*) and phosphoglycerate kinase (*PGK*), in combination with other routinely used markers such as *TEF1* and *RPB2*.[15]

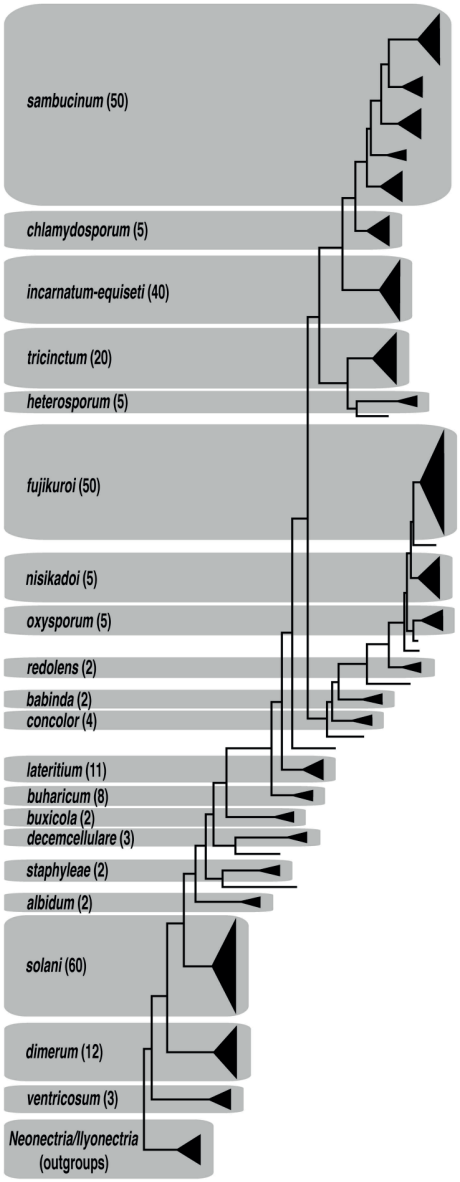

Fig 2. Diagrammatic representation of *Fusarium* phylogeny inferred from a combined *RPB1* + *RPB2* dataset (3383 bp) rooted on sequences of *Neonectria* and *Ilyonectria* (modified from Fig. 1 in O'Donnell et al. 2013), The approximate number of phylogenetically distinct species within each species complex based on more inclusive GCPSR-based analyses is indicated in parentheses. With permission from the publisher (Springer, License Number 3864851438385).

The emergence of *Fusarium* as a clinical entity

The genus *Fusarium* contains an extraordinary genetic diversity and is globally distributed in plants, soil, water, and man-made habitats. Plant-pathogenic members of the genus cause diseases in many agriculturally important crops, with billions of dollars of economical losses annually. Their presence as food contaminants is detrimental because of production of biologically active, highly toxic secondary metabolites.

Remarkably, human fusariosis does not have a long history. This is in contrast to comparable opportunistic pathogens like *Scedosporium* spp., which have been known since the 19th century. The first case of human *Fusarium* infection was reported only in 1958 and concerned an eye infection caused by a blow with a cow tail.[1] Today, *Fusarium* causes a very wide spectrum of disease, ranging from mildly superficial to fatally disseminated.[2] During the initial years, most reported infections were caused by traumatic inoculation. Keratitis is still the most common infection by *Fusarium* species, occurring especially in the warmer climates of India, China, and Brazil.[3] After 1960, the increasing use of antibiotics became a major predisposing condition.[4] Since 1970, prolonged neutropenia due to intensified cytotoxic treatment of hematologic malignancies was the leading risk factor for novel invasive diseases of fusariosis.[5] Since 1980, *Fusarium* infections have been seen in severely immunocompromised patients with a 100% mortality rate if the underlying disease is not reverted, e.g., in cases of cerebral involvement.[6] Today, complex surgery, organ transplantation, chronic steroid treatment, and aggressive cytotoxic therapy are the main risk factors of fusariosis.[7] *Fusarium* shows a dynamic response to opportunities provided by underlying immune disorders of the host.

Route of infection and risk factors

The most common route of human infection is by inoculation of conidia via thorns or plant debris, which is particularly observed in farmers and agricultural workers. A second route is inhalation of airborne conidia, and this is seen especially in the immunocompromised host. *Fusarium* poses a challenge for human disease management because infectious propagules may disperse over long distances in the atmosphere and new resources and susceptible hosts are quickly found.[17] Smith et al.[18] noted that *Fusarium* conidia are water-borne and become air-borne when dried. Schmale et al.[19] showed that large-scale atmospheric features known as lagrangian coherent structures (LCSs) enhance transport of *Fusarium* in the lower atmosphere. Lin et al.[20] demonstrated that atmospheric populations of fusaria are mixed and that conidial counts do not vary across consecutive sampling intervals, suggesting constant airborne transport.

One of the major risk factors for immunocompromised hosts is inhalation of contaminated air. Moretti et al.[21] established a link between *Fusarium* in the air and in blood of infected patients and suggested that unfiltered hospital air may be problematic for these patients. Short et al.[22] investigated hospital plumbing systems for the occurrence of fungi and found that these

are hidden reservoirs for *Fusarium*. Prevalence of *Fusarium* species in compromised patient populations is not proportional to their environmental abundance,[23] suggesting that infection is not merely a random process.

Epidemiology and genotyping

Fusarium species cause a broad spectrum of infections and fusariosis is, after aspergillosis, the second most common mould infection in humans.[23] Members of the genus *Fusarium* have been involved in infections ranging from localized (nail, skin, eye, or other location), mainly in immunocompetent hosts, to disseminated infections in immunocompromised patients.[16] Over the years, the number of case reports of fusariosis both in immunocompetent and compromised hosts has steadily increased. About 40 % of these reports were due to members of the *F. solani* species complex (FSSC), while the *F. oxysporum* species complex (FOSC) was involved in approximately 20 % of these reports.[57] Species diversity in the different types of fusariosis have to be derived from the few population studies done so far. Studies are available on onychomycoses and skin infections in Italy,[58] Switzerland,[59] Colombia,[60,61] Thailand,[62] Turkey,[63] Qatar,[64] and India.[65]

Another common infection in immunocompetent individuals is keratitis. Awareness of keratitis caused by *Fusarium* with correct recognition of the fungal nature of the infection is growing.[66,67] Traumatic *Fusarium* keratitis is observed especially among farmers and outdoor workers. However, the widespread use of contact lenses has dramatically increased the incidence of keratitis among urban populations.[68] The traumatic event may be minor, such as a small abrasion from a foreign body, or tear insufficiency. The spectrum of etiological *Fusarium* species is dependent on geographic and climatic factors and may also change with populations living in rural or urban areas, and in western *versus* developing countries.[69]

Most reported cases of fusariosis are superficial infections, such as keratitis and onychomycosis. In recent times disseminated infections have increased dramatically, mainly affecting patients with haematological malignancies but lso occasional reports have been published in immunosuppressed patients due to transplantation or solid tumors, and those with autoimmune disorders.[63] Less frequently infections are associated with burns, chronic wounds, peritonitis, pneumonia, catheter-associated fungemia, sinusitis, thrombophlebitis, osteomyelitis, septic arthritis and endophtalmitis.[70]

During the last three decades, the number of fusariosis cases increase globally and the role of genotyping is becoming increasingly important to recognize unsuspected infections, transmission routes, sources of infection, natural hosts, and geographical distributions. Typing studies are compulsory for understanding genetic and epidemiological relationships between clinical and environmental isolates. Understanding pathogen distribution and relatedness is essential for determining epidemiology of nosocomial infections, monitoring outbreaks, and

aiding in the design of pathogen control.

In the current study, we examine trends in global *Fusarium* infections, distribution, clinical presentation and prevalence during the period 1958–2015. Phylogeny and molecular epidemiology of geographically diverse isolates representing different *Fusarium* species was evaluated by using partial sequences of the *RPB2* and *TEF1* genes, and these were compared with amplified fragment length polymorphisms (AFLP) data. This combined approach improved discrimination of species and populations.

Diagnosis

Diagnosis of *Fusarium* infection remains a problem, particularly in the immunocompromised host, because clinical features and histopathology are similar to those of *Scedosporium*, *Aspergillus* and other relatively common hyaline hyphomycetes.[71,72] *Fusarium* infections are often clinically indistinguishable from other invasive mould infections and diagnosis relies on recovery of the etiological agent from clinical samples.[73] Diagnosis is based on clinical presentation, radiology and laboratory parameters. Microscopically, the hyphae of *Fusarium* in tissue resemble those of *Aspergillus*; the filaments are hyaline, septate and 3–8 μm in diameter. *Fusarium* is one of the few genera of molds that are able to sporulate *in vivo* and that are capable of growing in blood culture bottles, and positive bloodcultures is a hallmark of disseminated fusariosis.[74] *Fusarium* spp. grow easily and rapidly on most culture media, but species level identification requires molecular methods. Serological tests such the galactomannan test, which was developed for *Aspergillus* infections, may cross-react with *Fusarium*.[75] Galactomannan (GM) is not a good marker for fusariosis because cross-reactivity of *Fusarium* with *Aspergillus* is posible, although negative GM together with a positive 1,3-β-D-glucan test in a high-risk patient with mould infection is highly suggestive of fusariosis.[73]

Given that histopathological, radiological and culture morphology methods have limitations in correct species identification of agents of *Fusarium* infection, an increasing number of molecular techniques have been developed to achieve more accurate identification.[76] At present, molecular techniques are considered the new gold standard for *Fusarium* identification. The use of nucleic acid probes and amplification-based molecular approaches provide more rapid identification of *Fusarium* species compared with traditional phenotypic methods. Novel approaches include multiplex-PCR, real-time PCR (RT-PCR), restriction fragment length polymorphism (RFLP), isothermal methods, probe-based assays and DNA sequencing. Multi-locus sequence typing (MLST) is currently regarded as the best method for the identification of *Fusarium* isolates to species level.[37] However, sequencing of multiple genes is time-consuming, associated with high costs, and needs increased expertise.

Among the isothermal DNA amplification techniques, loop-mediated isothermal amplification (LAMP) and rolling circle amplification (RCA) assays have been applied to

several *Fusarium* plant pathogens.[77] RCA has been applied to specific groups of *Fusarium,* such as *F. oxyporum* and *F. incarnatum-equiseti* species complexes.[78] Furthermore, multiplex PCR platforms have been developed for the identification.[79]

Another promising approach for the quick identification of *Fusarium* is matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF).[76] MALDI-TOF MS uses species-specific patterns of peptides and protein masses to identify microorganisms. These peptides are converted into ions by either addition or loss of one or multiple protons.[80] Several studies have been conducted on *Fusarium* species with high success rates of 82–99%.[76, 81-84] During our research, this method was performed in the *F. fujikuroi* species complex. A database was built as a result of this research which can be used as a future reference tool.

***In vitro* antifungal susceptibility**

Antifungal susceptibility testing provides information to clinicians to guide antimicrobial therapy. Although up to now no breakpoints have been defined for *Fusarium* species, CLSI epidemiological cutoff values (ECVs) were established for the more common *Fusarium* complexes.[38] The typical antifungal susceptibility profile of *Fusarium* species is that of high resistance to most antifungal agents. However, *in vitro* susceptibility may vary between different species.[29] Efforts have been made to evaluate the *in vitro* susceptibility of the most common *Fusarium* species,[29, 85-92] to a wide range of antifungals, but no solid data are available for the less common species. For rare clinical *Fusarium* species a modified *in vitro* susceptibility protocol based on CLSI was developed.[29,42]

Antifungals are grouped into three main classes based on their site of action towards pathogenic fungi. The polyenes exert their activity via binding to ergosterol in the fungal cell membrane. Azoles comprise several active compoundss such as itraconazole, fluconazole, voriconazole, posaconazole, and isavuconazole. Azoles exert their activity by blocking the demethylation of lanosterol, therefore inhibiting ergosterol synthesis. The echinocandins include caspofungin, micafungin and anidulafungin. The mechanism of activity of echinocandins is attributed to inhibition of synthesis of 1,3-beta-D-glucan in the cell wall.[93]

Commercially available assays such as disk-diffusion and Etest, in addition to the reference broth microdilution methods of Clinical and Laboratory Standards Institute (CLSI) and the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) are available for testing *Fusarium*. In order to guide antifungal therapy, we first need to acquire more information on the *in vitro* antifungal susceptibility of *Fusarium* species against various antifungal agents. These are determined according to the CLSI reference method for broth dilution antifungal susceptibility testing of filamentous fungi.[94]

Combination therapy

Combination therapy might be an alternative chemotherapeutic approach for management of *Fusarium*, given the limited information on antifungal susceptibilities of the etiological agents, their limited *in vivo* efficacy, and the limited number of clinical trials assessing antifungal monotherapy.[41,42, 95] Liu et al.[96] noticed an increase in case reports describing the efficacy of combination therapy. Campo et al.[97] retrospectively reported 37 patients who received combination antifungal therapy consisting of a lipid formulation of AMB and a triazole. For 27 of these patients, 41 % achieved a complete or partial response after 90 days of therapy, and these results agreed with previous studies,[98, 99] where monotherapy was used. In this thesis, various *in vitro* drug interactions were investigated mainly for various *Fusarium* species causing keratitis.

***Fusarium* antifungal resistance**

Fusarium species are intrinsically resistant to azole antifungals. Five azole fungicides are widely used for plant protection: propiconazole, bromuconazole, epoxiconazole, difenoconazole, and tebuconazole. These azoles are generally inexpensive and have broad-spectrum activity and long stability. Azoles that are used in human medicine have derivative imidazole or triazole rings.[24] All azoles target the same active site, i.e., lanosterol-14α-demethylase.[25] Effects on *Fusarium* population dynamics in agricultural fields are likely due to decreased competition with susceptible *Fusarium* species.[26] This may be reinforced by antifungal prophylaxis in high-risk patients in clinical settings, enhancing selective pressure that favours multidrug-resistant fungi, including *Fusarium*.[27] Population dynamic effects have not been studied before because *Fusarium* was not considered to be a matter of concern.[28] The growing incidence of severe human *Fusarium* infections may change this situation.

Clinically relevant members of *Fusarium* are also resistant to echinocandins and polyenes. This poses a major challenge in medicine and agriculture, particularly with emerging and globally spreading fungi like *Fusarium*. There are only few options left for treating patients and crops. Intrinsic, primary resistance is found naturally among some *Fusarium* species without prior exposure to the drug.[29] Secondary resistance to azoles develops among previously susceptible strains after exposure to the antifungal agent, as seen, e.g., in *Aspergillus fumigatus*, and is usually dependent on altered expression of *CYP51*, the gene encoding sterol 14α-demethylase.[30] Recently, Fan et al. showed that *CYP51* in *Fusarium* has three paralogues (*CYP51*A, -B, and -C), with *CYP51*C being unique to the genus.[31] *CYP51*A deletion demonstrated decreased MICs to all azoles in fungi such as *A. fumigatus*,[25] whereas the opposite was found in *Fusarium*: *CYP51*A deletion increases the sensitivity of *Fusarium graminearum* to azoles and other fungicides (prochloraz, tebuconazole, and epoxiconazole) that are used in plant protection.[31] The exact resistance mechanisms in *Fusarium* are not entirely understood, but combinations of CYP51A amino acid alterations and/or *CYP51*A gene overexpression might be involved.

Recent findings indicate that a mutation occurring in the *FKS1* gene might contribute to the intrinsic echinocandin resistance in *Fusarium*. In support of this, evidence has been presented that hot spot 1 substitution P647A and F639Y in *FKS1* contribute to resistance of *Fusarium solani*.[32] Furthermore, *Fusarium* has an effective efflux mechanism to remove xenobiotics from its cells,[33] and this may also reduce azole sensitivity. Amphotericin B, second-generation broad spectrum triazoles (fluconazole, itraconazole, voriconazole, and posaconazole), antimetabolites (5-fluorocytosine), and echinocandins (caspofungin, anidulafungin, and micafungin) all have limited activity against *Fusarium* species. High-level cross-resistance to fluconazole and itraconazole was reported in almost all *Fusarium* species. Cross-resistance has been observed among the three echinocandins in *Fusarium* species.

Management of *Fusarium* infections

Treatment of fusariosis is a major challenge. For disseminated fusariosis in immunocompromised patients, the 12-week survival time has increased significantly in the last decade, judging from single center studies,[34] national studies,[35] and worldwide evaluations.[36] This better outcome of treatment is probably associated with the introduction of voriconazole in 2002. Therapy with amphotericin B deoxycholate gives poor survival results of 28 % when compared to lipid amphotericin B (53 %) or voriconazole (60 %).[36] Recent European guidelines[37] suggest treating disseminated fusariosis with voriconazole and lipid amphotericin B, although evidence is based on expert opinion and case series rather than on clinical trials. Of the azoles, only the new triazoles, voriconazole and posaconazole, show moderate activity, with mode minimal inhibitory concentrations (MICs) of 2–8 mg/L and 0.5–8 mg/L, respectively, depending on the species complex. The mode MIC of amphotericin B is 2 mg/L irrespective of the species complex (Fig. 3).[38] The newest class of antifungal drugs, the echinocandins, have activity against *Candida* and *Aspergillus* species,[30] but for *Fusarium* they appear to be inactive with high MICs of >16 mg/L (Fig. 3). Terbinafine is another option to treat some *Fusarium* species, but this compound is only registered to treat superficial infections.[39] Natamycin (5 %) and/or topical amphotericin B (0.5 %) are first-line treatment of fungal keratitis in some countries. Elsewhere, topical 1 % voriconazole and/or 5 % natamycin are used for this type of infection.[40]

Considering the poor outcome obtained with monotherapy, attempts have been made to determine whether combination of drugs lead to improved efficacy. Spader et al.[41] reported that synergistic interactions were observed for the combinations of amphotericin B with caspofungin (68.7 %), amphotericin B with rifampin (68.7 %), amphotericin B with 5-flucytosine (59.3 %), and amphotericin B with voriconazole (37.5 %). Al-Hatmi et al.[42] determined *in vitro* antifungal activity of natamycin alone and in combination with voriconazole for *Fusarium* isolates obtained from cases with keratitis, and found that MICs of these compounds alone were >4 and 4−8 mg/L, respectively, and that the combinations tested showed *in vitro* synergistic

effects against a significant number of isolates (70 %). MICs values were reduced to 0.02−0.5 mg/L and to 0.13−2 mg/L in combination, respectively.[42] Combinations of voriconazole, amphotericin B, and posaconazole showed poor efficacy in experimental murine infections by *F. verticillioides*, while the combination of liposomal amphotericin B and terbinafine showed good results.[23] However, clinical studies have not been performed, and the most efficacious combination remains to be explored. Further work on interactions in animal models or clinical trials with the aim to obtain higher cure rates of *Fusarium* infections is overdue.

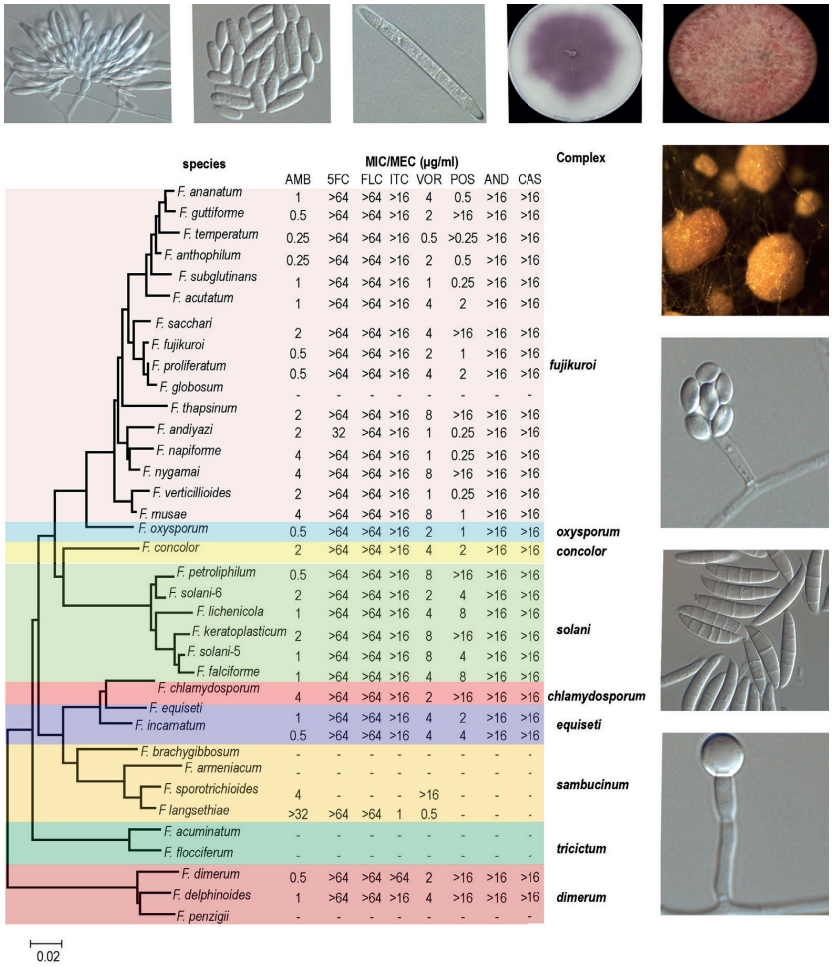

Fig 3. Sequence-based phylogeny of clinically related *Fusarium* species and associated antifungal susceptibilities with morphological features. Neighbor-Joining tree created by MEGA6 from *TEF1* sequences using 1,000 bootstrap replicates. The minimum inhibitory concentration (MIC) profiles of eight antifungals against each species have been incorporated into the figure. "-," no data available for these species. AMB = amphotericin B, FLC = fluconazole, ITC = itraconazole, VOR = voriconazole, POS = posaconazole, CAS = caspofungin, 5FC = 5-flucytosine, AND = anidulafungin.

Outline of thesis

The research presented in this thesis discusses the impact of *Fusarium* on human health and some of the challenges in treating infections. Plant pathogenic species of *Fusarium* are serious food contaminants due to the production of mycotoxins such as trichothecenes, zearalenone and fumonisins, but they also have the ability to cause opportunistic infections in humans. This thesis is focused on four main themes: (1) Taxonomy and barcoding of agents of fusariosis, (2) diagnosis, human infection and molecular epidemiology and (3) *in vitro* antifungal susceptibility testing and combinations. The new insights of the above mentioned themes are subsequently used to answer several questions in this thesis.

In **Chapter 1**, an introduction to the genus *Fusarium* is given and an overview on the history of taxonomy is provided, including the various techniques used for classification and species delimitation. Furthermore, details are provided on recent developments in diagnosis and typing, and an overview of antifungal testing for clinically relevant *Fusarium* species is given. In addition, options and challenges in treating *Fusarium* infections in human are briefly reviewed.

Part I: Taxonomy and barcoding of agents of fusariosis

In **Chapter 2,** phylogenetic taxonomy was employed as a fundamental model for classification and for selecting DNA barcoding markers for identification of opportunistic pathogens in *Fusarium*.

In **Chapter 3**, a description is given of *F. ficicrescens*, isolated from contaminated fig fruits in Iran, as a distinct species within the *F. fujikuroi* species complex, using multiphasic approaches including morphology, sequencing, AFLP and MALDI-TOF MS. The fungus proved to be able to grow well at 27 °C, and was still able to grow at 37 °C, but not at 40 °C. Potentially the species should be able to cause disease in humans, but human or animal infections have as yet not been observed.

Chapter 4 describes the occurrence and genotypic diversity of clinical *Fusarium* isolates from Qatar. Phylogenetic analysis of 44 strains revealed eleven species and the etiological agents belonged to four species complexes. Site of infections were blood, cornea, skin, wounds, burns and nails. This study indicates that *F. acutatum* is an endemic species in the Middle East. Mortality was 66.7% when fungemia with *F. petroliphilum* and *F. solani* was present. *Fusarium solani* species complex predominantly caused cornea, nail and bloodstream infections.

Part II: Diagnosis, human infection and molecular epidemiology

Conventional identification of *Fusarium* species by culture methods takes up to one week and routinely used molecular methods take up to 48 h, depending on the availability of the sequence data. By contrast, MALDI-TOF MS can be performed with minimal amounts from a young

colony and takes only a few minutes, with comparable levels of resolution. MALDI-TOF MS has the potential to become an important tool for the routine identification of pathogenic *Fusarium* species, provided that the availability of extended reference databases is guaranteed. During our research, this method was applied to the *F. fujikuroi* species complex. A database was built as a result of this research (**Chapter 5**) which can be used as a reference for future identification.

Chapter 6 describes the diagnostic concept of ecthyma gangrenosum (EG) due to the fungal agent *F. oxysporum*. Most cases of ecthyma are bacterial in etiology, mainly *Pseudomonas aeruginosa*. However, an EG-like lesion due to fungi was confirmed by different diagnostic aspects such as clinical diagnosis, culture, histopathological tests and molecular identification. A proposed definition of EG was discussed.

Chapter 7 reports the first case of *F. ramigenum* in a patient with common variable immunodeficiency (CVID). Histopathological examination confirmed hyaline hyphae in smears from lung tissue imprints, suggesting an invasive pulmonary fungal disease. BAL and biopsy culture were both positive for a *Fusarium* species. The cellular immune profile was further analyzed and an important qualitative cellular deficiency was found: a defective production of both gamma-interferon-γ (IFN-γ) and IL-17. Further molecular identification showed *F. ramigenum* which belongs to *F. fujikuroi* species complex. Moreover, this paper provides evidence indicating *F. ramigenum* as a potential human opportunist especially in immunocompromised patients.

Chapter 8 describes a novel opportunist: *F. temperatum,* an opportunistic species of *Fusarium* that caused severe keratitis in an immunocompetent Mexican farmer, as a result of the new taxonomy and classification. *F. temperatum* was previously misidentified as *F. subglutinans*.

Chapter 9 describes two human cases of mycetoma caused by two different *Fusarium* species; *F. keratoplasticum* and *F. pseudensiforme*. *F. pseudensiforme* is reported for the first time from human infections as a novel opportunist. Also in this chapter, a systematic literature review was performed to assess general features, identification, treatment and outcome of eumycetoma infections due to *Fusarium* species and previously reported *Fusarium* agents of mycetoma were molecularly verified.

Chapter 10 describes a case of an immunocompromised patient diagnosed with proximal subungual onychomycosis (PSO) caused by *F. falciforme* which successfully treated with posaconazole. This may provide an alternative treatment for patients presenting with onychomycosis.

Chapter 11 describes the first epidemiological data of fusariosis in a university hospital in Turkey during a 20-year period. The Turkish *Fusarium* population is investigated using multilocus sequence analysis (MLSA). A comparison between genotypic data, *in vitro*

antifungal susceptibility profiles and patient categories is given.

In **Chapter 12**, *Fusarium* keratitis is described which can lead to severe damage of the cornea. In this chapter, we review ten cases of keratitis due to *Fusarium* species from a single hospital in India during a two-year period and distinguished them from other corneal pathogens. Traumatic implantation was the portal of entry in most cases. Etiological agents belonged to the *F. solani* species complex (90%) and to the *F. sambucinum* species complex. All strains proved to be multi-resistant to azoles and caspofungin, with moderate susceptibility of some isolates to amphotericin-B.

In **Chapter 13,** the global molecular epidemiology and genetic diversity of the human opportunist *Fusarium* was investigated during the period 1958–2015, using AFLP as independent molecular epidemiological approach and two gene sequence typing, i.e. partial second largest subunit of RNA polymerase II (*RPB2*) and translation elongation factor 1-α (*TEF1*). Considerable species diversity is observed, with no differences between clinical and environmental isolates. This suggests that infections with *Fusarium* species are truly opportunistic.

Part III: *In vitro* antifungal susceptibility testing and combinations

Chapter 14 evaluates the *in vitro* activity of a new triazole antimycotic, isavuconazole, and seven comparators (amphotericin B, fluconazole, itraconazole, micafungin, natamycin, posaconazole and voriconazole) against 13 species of the *F. fujikuroi* species complex causing various human infections. Resistance patterns in the *F. fujikuroi* species complex are species-specific and therefore identification down to species level is important for the choice of antifungal treatment.

In **Chapter 15,** *in vitro* antifungal susceptibility and multilocus DNA sequencing was performed for sixty-five fusaria isolated from patients with keratitis in India. Susceptibilities of these fusaria to ten antifungals were determined *in vitro* by the broth microdilution method. An impressive phylogenetic diversity of fusaria was reflected in susceptibilities differing at species level. Typing results revealed that the isolates were distributed among species in the species complexes of *F. solani* (FSSC), *F. oxysporum* (FOSC), *F. fujikuroi* (FFSC) and *F. dimerum* (FDSC). Amphotericin B, voriconazole, and posaconazole proved to be the most effective drugs.

In **Chapter 16,** *in vitro* antifungal susceptibility using the EUCAST-AFST method against 39 clinical *Fusarium* strains isolated from local and invasive infections during the last 10 years from Qatar assessed for the *in vitro* activities of amphotericin B (AmB) and triazole antifungal agents. Results showed that species and strain specific differences in antifungal susceptibility exist within *Fusarium* and that susceptibility testing is important and may improve the prognosis of these infections.

In **Chapter 17,** the combined use of two antifungals with different mechanisms of action is discussed, which may improve the efficacy and shorten the course of therapy of fusariosis. Therefore, to determine if combination therapy can be used for fusariosis, the *in vitro*

interactions of natamycin with voriconazole, itraconazole and micafungin were tested against clinical *Fusarium* strains causing keratitis. The combination of natamycin with voriconazole was strongly synergistic at clinically achievable concentrations.

As *Fusarium* is notoriously refractory to therapy, detailed studies regarding *in vitro* susceptibility are necessary. **Chapter 18** describes a comparative evaluation of Etest and EUCAST methods with the CLSI broth microdilution method for amphotericin B, voriconazole and posaconazol against clinically relevant *Fusarium* species. Etest offers an attractive alternative for rapidly testing *Fusarium* isolates, provided it is further evaluated. Standardized susceptibility testing can aid the selection of the most relevant antifungal therapy for the management of *Fusarium* infections.

Chapter 19 contains a summary of the results and conclusions of this thesis. The impact of the present study in understanding the genetic diversity, phylogeny, taxonomy, barcoding, ecology, pathogenicity and treatment of species in the genus *Fusarium* is discussed. Furthermore, perspectives on future research are given.

Aim of the study

The aims of this thesis are i) to acquire understanding of the phylogeny and ecology of the genus *Fusarium* and establish a solid taxonomic platform for the causative agents of fusariosis, ii) to define a barcode for *Fusarium*, iii) to develop tools for identification and diagnosis of *Fusarium* species, iv) molecular genotyping, which may provide a better understanding of genetic and epidemiologic relationships between environmental and clinical isolates and thereby allow assessment of potential routes of transmission, and v) providing *in vitro* resistance data of a worldwide test set including strains of all recently described and clinically relevant *Fusarium* species. Furthermore, given the interest of combination therapy for treating invasive and superficial fusariosis, assessment of *in vitro* interaction of antifungal drugs for different *Fusarium* species is crucial.

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Part I

Taxonomy and barcoding of agents of fusariosis

- Evaluation of two novel barcodes for species recognition of opportunistic pathogens in *Fusarium*
- DNA barcoding, MALDI-TOF, and AFLP data support *Fusarium ficicrescens* as a distinct species within the *Fusarium fujikuroi* species complex
- Phylogenetic diversity of human pathogenic *Fusarium* and emergence of uncommon virulent species

CHAPTER 2

Chapter 2

Evaluation of two novel barcodes for species recognition of opportunistic pathogens in *Fusarium*

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Fungal Biology 2016; 120: 231-245.

Abstract

The genus *Fusarium* includes more than 200 species of which 73 have been isolated from human infections. *Fusarium* species are opportunistic human pathogens with variable aetiology. Species determination is best made with the combined phylogeny of protein coding genes such as elongation factor (*TEF1*), RNA polymerase (*RPB2*) and the partial b-tubulin (*BT2*) gene. The internal transcribed spacers 1, 2 and 5.8S rRNA gene (ITS) have also been used, however, ITS cannot discriminate several closely related species and has nonorthologous copies in *Fusarium*. Currently, morphological approaches and tree-building methods are in use to define species and to discover hitherto undescribed species. After a species is defined, DNA barcoding approaches can be used to identify species by the presence or absence of discrete nucleotide characters. We demonstrate the potential of two recently discovered DNA barcode loci, topoisomerase I (*TOP1*) and phosphoglycerate kinase (*PGK*), in combination with other routinely used markers such as *TEF1*, in an analysis of 144 *Fusarium* strains belonging to 52 species. Our barcoding study using *TOP1* and *PKG* provided concordance of molecular data with *TEF1*. The currently accepted *Fusarium* species sampled were well supported in phylogenetic trees of both new markers.

Key words

Character-based DNA barcoding, *Fusarium*, Intra- and inter-specific variation, Phosphoglycokinase (*PGK*), Topoisomerase I (*TOP1*)

Introduction

The genus *Fusarium* comprises more than 200 species, many occurring as pathogens on a wide range of important crop plants. They are also relevant to human health by causing opportunistic, superficial or disseminated infections, and by producing mycotoxins (Tortorano et al. 2014). The highly speciose genus is found in different climates zones, particularly in tropical and subtropical regions, and is associated with various habitats, such as sheltered forests, harsh deserts or soils (Leslie & Summerell 2006). Al-Hatmi et al. (2014) noted that 71 taxonomic species, verified by multilocus sequence typing (MLST), have been reported from human or animal infections. Recently two more species were added to this list: *Fusarium musae*, reported from blood samples and biopsies of immune-suppressed patients in Belgium and France (Triest et al. 2014), and *Fusarium langsethiae* from keratitis in India (Ruban et al. 2015).

Morphological identification of *Fusarium* is largely based on macro- and micromorphological characters including colony colour and appearance on specific culture media, and on size, shape, and development of sexual and asexual spores, as well as spore-forming structures (Moretti 2009). However, successful identification may be difficult because of similar, inconspicuous or degraded diagnostic characters in culture. Molecular methods have replaced traditional identification methods in most taxonomic research. DNA-based methods, particularly using geneological concordance analyses, frequently lead to the detection of novel sibling species (Scheel et al. 2013; Nucci et al. 2013). Standard diagnostic nuclear rDNA markers have limited resolution to distinguish currently recognized taxa in *Fusarium* (Geiser et al. 2004).

The concept of DNA barcoding (Herbert et al. 2003) had a large impact on standardizing identification of eukaryotes. Short, easily amplified regions of DNA, based on authoritatively identified vouchers, led to a more robust identification process (Riaz et al. 2011). Although tremendous progress has been made in terms of availability of molecular markers to study genetic diversity, there is a need to identify additional reliable and effective loci that can function at all taxonomic levels in *Fusarium*.

A large number of molecular loci have been used in *Fusarium* phylogenetics, including nuclear and mitochondrial ribosomal rDNA (18S, 28S, ITS, IGS, mtSSU, mtLSU) (O'Donnell et al. 1998), although analysis of the ITS is confounded by the occurrence of nonorthologous copies (O'Donnell & Cigelnik 1997). Protein-coding genes are also in use, such as RNA polymerase (*RPB2*), β-tubulin (*BT2*), elongation factor (*TEF1*) (O'Donnell et al. 2012) and ATP citrate lyase (*ACL1*) (Gráfenhan et al. 2011). Geiser et al. (2004) concluded that *TEF1* has high phylogenetic utility for the following reasons: (i) it is highly informative at the species level; (ii) nonorthologous copies of the gene have not been detected across the genus; and (iii) it is alignable across the genus *Fusarium*. Some single-copy protein-coding genes such as *RPB1* and *RPB2* are also promising for phylogeny and barcoding in *Fusarium* (O'Donnell et al. 2013).

In this study, two newly characterized genes were used as molecular markers for *Fusarium* species genotyping, i.e. topoisomerase I (*TOP1*) and phosphoglycerate kinase (*PGK*). They were discovered during genome scans guided by protein families (Lewis et al. 2011), and tested extensively for their utility for a broad range of fungi, but mostly Ascomycetes, by Stielow et al. (2015). The DNA topoisomerases enzymes (*Top1p* & *Top2p*) were discovered in the 1970s and their function is to relax supercoiling that accumulates in DNA following polymerization. Polymerase causes supercoiling because it is fixed and cannot rotate. Topoisomerases relieve supercoiling by either cutting one strand (*Top1p*) or both strands (T*op2p*). Partial nucleotide sequences of *TOP2* were used for identifying both eukaryotes and prokaryotes (Li et al. 2007). Topoisomerase I has not yet been used for phylogenetic or barcoding studies of *Fusarium*, although some of the *TOP1* and *PGK* data reported here was included and partially analysed in the large scale analysis of Stielow et al. (2015). Phosphoglycerate kinase (*Pgkp*) is a housekeeping enzyme involved in the Calvin cycle and glycolysis. It codes for the nuclear-encoded plastid enzyme (Huang et al. 2002) and, in common with *TOP1,* has not been used for phylogeny or identification of *Fusarium* species.

In our study, marker assessment and comparison were carried out using a tree-based DNA identification technique, as well as the distance-based technique for larger data sets, to assess the accuracy and robustness of DNA barcoding of *Fusarium* species using the sampled genes. Our hypothesis is that the genes topoisomerase I (*TOP1*) and phosphoglucokinase (*PGK*) may be reliable barcoding markers to overcome some of the identification problems for species in *Fusarium* inherent with the recognized barcode ITS (Schoch et al. 2012) and the informal but widely used secondary barcode *TEF1* (Geiser et al. 2004). The present research addresses further testing of specific primer sets based on *TOP1* and *PGK* presented by Stielow et al. (2015) to generate sequences that enhance discrimination of species of *Fusarium*.

Materials and methods

Strains

Strains of different *Fusarium* species with clinical relevance and their environmental counterparts were chosen for analysis, including type and authentic strains. Particular attention was paid to the *Fusarium fujikuroi* species complex (FFSC), which includes molecular siblings separated by few bp differences in *TEF1* (O'Donnell et al. 1998). The set comprised 144 clinical (n = 33) and environmental (n = 111) isolates (Table S1), of which 80 were taken from the reference collection of the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands, and 64 from the Department of Agriculture and Agri-Food (AAFC) culture collection, Ottawa, Canada (DAOMC). At CBS, stock cultures were maintained on slants of 2 % malt extract agar (MEA; Oxoid) and oatmeal agar (OA, home-made at CBS). Culture plates were incubated at 24 °C for one week. At AAFC, strains were grown on Difco Potato Dextrose agar (PDA) for 7-10 d prior to DNA extraction.

Specific primer design strategy

Topoisomerase I (*TOP1*) and phosphoglycerate kinase (*PGK*) were identified as potentially useful barcodes from a Pfam analysis of available fungal genomes by Lewis et al. (2011). As described in detail by Stielow et al. (2015), intensive universal primer testing and redesign for all fungi was undertaken for a 1 kb fragment of *PGK* and a 700 bp fragment of *TOP1*, with M13 primers added and tested for sequencing as an additional option to increase DNA sequencing success after PCR and facilitate high throughput when sequencing multiple markers.

DNA extraction

At CBS, DNA was extracted following the Quick CTAB protocol. 1-10 mm^3 material was transferred to two mL screw-capped tubes filled with 490 µL CTAB buffer 2x and 6-10 acid washed glass beads. 10 µL Proteinase K were added and mixed thoroughly on a MoBio vortex for 10 min. 500 µL Chloroform: isoamylalcohol (24:1) were added and shaken for 2 min after incubation for 60 min at 60 °C. Tubes were centrifuged for 10 min at 14 000 r.p.m. The supernatant was collected in a new Eppendorf tube. To ~400 µL DNA sample 2/3 vol (~270 µL) of ice-cold iso-propanol was added and centrifuged again at 14 000 r.p.m. for 10 min and the upper layer was dissolved in 1 mL ice-cold 70 % ethanol. Tubes were centrifuged again at 14 000 r.p.m. for 2 min, air-dried and re-suspended in 50 µL TE-buffer. Quality of genomic DNA was verified by running 2-3 mL on a 0.8 % agarose gel. DNA was quantified with NanoDrop 2000 spectrophotometer (Thermo Fisher, Wilmington, U.S.A.). Samples were stored at 20 °C until use. The methods used at AAFC for DNA isolation was performed with the OmniPrep™ Genomic DNA Extraction Kit (G-Biosciences, St. Louis, Missouri, U.S.A.) following the procedure outlined in Stielow et al. (2015).

DNA amplification and sequencing

Four gene regions were amplified directly from the genomic DNA: translation elongation factor-1a (*TEF1*), internal transcribed spacer regions of the nrDNA (ITS), topoisomerase I (*TOP1*) gene and the phosphoglycerate kinase (*PGK*) gene. Primers used for amplification and sequencing were PGK_480-F/R, PGK_533-F/R (only used for a few strains at AAFC) and TOP1_501-F/R from Stielow et al. (2015), EF1 and EF2 from O'Donnell et al. (1998), ITS1 and ITS4 from and White et al. (1990).

Methods for amplification and sequencing of *TEF1*, *PGK* and *TOP1* at AAFC are detailed in Stielow et al. (2015). At CBS, PCR reaction mixture (12.5 µL final vol) contained 10x PCR buffer 1.25 µL, water 7.5 µL, dNTP mix (2.5 mM) 0.5 µL, 0.25 µL of each primer (10 pmol), Taq polymerase (5 U mL^{-1}) 0.05 mL, DMSO 0.7 µL, and template DNA (100 ng mL^{-1}) 1 µL. PCR reactions were performed in a Hybaid Touchdown PCR machine (Hybaid, Middlesex, U.K.). *TEF1* and ITS were amplified and sequenced following the methods reported

previously by Al-Hatmi et al. (2015). For *TOP1* and *PGK*, a touchdown PCR protocol consisted of two phases: Phase 1 included an initial step of 95 °C for 5 min, followed by ten cycles of denaturation at 95 °C for 45 s, annealing at variable temperatures for 1 min, and extension at 72 °C for 1 min. In the first cycle, the annealing temperature was set to 68 °C and, at each of the ten subsequent cycles, the annealing temperature was decreased by 1 °C (i.e., it varied from 68 to 58 °C at 1 °C decrements along the ten cycles). Phase 2 consisted of 30 cycles of 95 °C for 45 s, 58 °C for 45 s, 60 °C for 45 s and 72 °C for 1 min and post elongation step of 5 min at 72 °C. After the last PCR cycle, the samples were cooled to 10 °C, and a 6-µL aliquot of the amplification products was electrophoresed on a 1 % agarose gel (1x TAE) and visualized under ultraviolet (UV) light. Amplicons were purified using exoSAP. Both strands of the PCR fragments were sequenced with M13 primers. Sequencing PCR was performed as follows: 1 min at 95 °C, followed by 30 cycles consisting of 10 s at 95 °C, 5 s at 50 °C and 2 min 60 °C. Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, U.S.A.) with ABI PRISM BigDye terminator cycle sequencing kit (Applied Biosystems). The ABI PrismH BigDye Terminator v. 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) was used for sequencing PCR. Sequences were determined with an ABI PRISM 3100 Genetic Analyzer.

Barcoding resolution

Barcoding gaps were evaluated by (i) analyses of pairwise distances, and (ii) tree based analysis of barcodes of significant clades after tree reconstruction, for which parallel analyses were carried out on the four alignments

Analyses of pairwise distances

Aligned sequences were analysed in R (R Core Team 2014), APE to generate pairwise distances (Paradis et al. 2004), and using GGPLOT2 for generating graphics (Wickham 2009). The raw pairwise distances were calculated for each pair of strains and categorized as being intra- or inter-specific.

Tree based assessment

Sequences were edited and assembled SeqMan of the Lasergene package (DNAStar, Madison, WI, U.S.A.) and manually corrected. Sequences were aligned using the MAFFT server (www.ebi.ac.uk/Tools/msa/mafft/) under default parameters and whenever indicated, manually improved in BioEdit v. 7.0.5.2 (Hall 1999). The best-fit model of evolution was determined by ModelTest v. 2.3 (Nylander 2004). Neighbour-Joining (NJ) trees were generated based on the Kimura2-parameter model for each DNA marker to provide visual displays of genetic variation

within and between species. Additional trees were generated with Maximum Likelihood (ML) and Bayesian Inference (BI) with MrBayes v. 3.1.2 on the Cipres portal (http:// www.phylo.org/sub_sections/portal). Two parallel runs of four chains were run for 10000000 generations and trees were sampled every 1000 generations. TRACER version 1.5 was used to verify that the mean likelihood value, effective sample size (ESS) and other parameters reached a plateau. For each run, 10 % of the trees were discarded as they were obtained during the burning phase. Maximum likelihood (ML) analysis was done with RAxML-VI-HPC v. 7.0.3 with nonparametric bootstrapping using 1000 replicates, a bootstrap percentage value as good support was regarded above 70 %, and the substitution model was Tamura-Nei and uniform rates among sites. To check the congruency of the data sets, a 70 % Neighbour-Joining (NJ) reciprocal bootstrap method with maximum likelihood distance was performed on each individual locus (Mason-Gamer & Kellogg 1996). Trees were viewed and edited with TreeView v. 1.6.6, FIGTREE v. 1.1.2 and MEGA6.2.

Results

Strain designations and corresponding GenBank accession numbers of 144 *Fusarium* cultures (111 environmental and 33 clinical isolates; 80 sequenced at CBS and 64 at AAFC) representing the 52 species used in this study. The ITS, *TEF1*, *TOP1* and *PGK* genes were selected as candidate markers to compare their suitability for the identification of the 52 *Fusarium* species.

PCR amplification success rates evaluated at CBS for the four loci were 100 % for ITS, 96 % for *TEF1*, 95 % for *TOP1* and 93 % for *PGK*. Properties of the sequences and alignments of the tested loci are summarized in Table 1. No multiple bands were detected and sequencing success rates were 100 % of the isolates for ITS, 98 % for *TEF1*, 96 % for *TOP1* and 95 % for *PGK*. ITS had the average shortest length of 469 bp followed by *TEF1*, which had an average total alignment length of 589 bp, *TOP1* with 708 bp, and *PGK* at 828 bp. *TEF1* had the highest number of variable sites (314/589 = 53.3 %), followed by *TOP1* (359/708 = 50.70 %), P*GK* (300/828 = 36.2 %), and ITS (137/469 = 29.2 %). *TOP1* had the most phylogenetically informative sites (327/700 = 46.1 %), followed by *TEF1* (256/ 589 = 43.4.2 %), *PGK* (240.828 = 28.99 %) and ITS (105/469 = 22.5 %). The lengths and guanine-cytosine (G + C %) contents of the markers varied significantly and was highest at 57.8 % for *PGK*, followed by *TEF1* at 52.5 %, ITS with 50.7 % and 48.5 % with *PGK*.

Table 1. Amplification success, phylogenetic data and the substitution models used in the phylogenetic analyses, per locus.

	Gene data sets			
Markers	ITS	*TEF1*	*TOP1*	*PGK*
Best model used	K2+G+I	K2+G+I	TN93+G+I	TN93+G
Number of aligned sites	469	589	708	828
Amplification success (%)	100%	98%	96%	95%
Conserved sites	319	258	342	514
%	68.01	43.80	48.3	62.07
Variable sites	137	314	359	300
%	29.2	53.3	50.70	36.2
Parsimony informative sites	105	256	327	240
%	22.3	43.4	46.1	28.9
Singleton sites	32	49	31	60
%	6.82	8.31	4.37	7.24
G+C (average) %	50.7	52.5	48.5	57.8

Analyses of pairwise distances

The distributions of intra- and interspecific distances were compared for ITS, *TEF1*, *TOP1* and *PGK* in order to better visualize the 'barcode gap' (Fig 1).

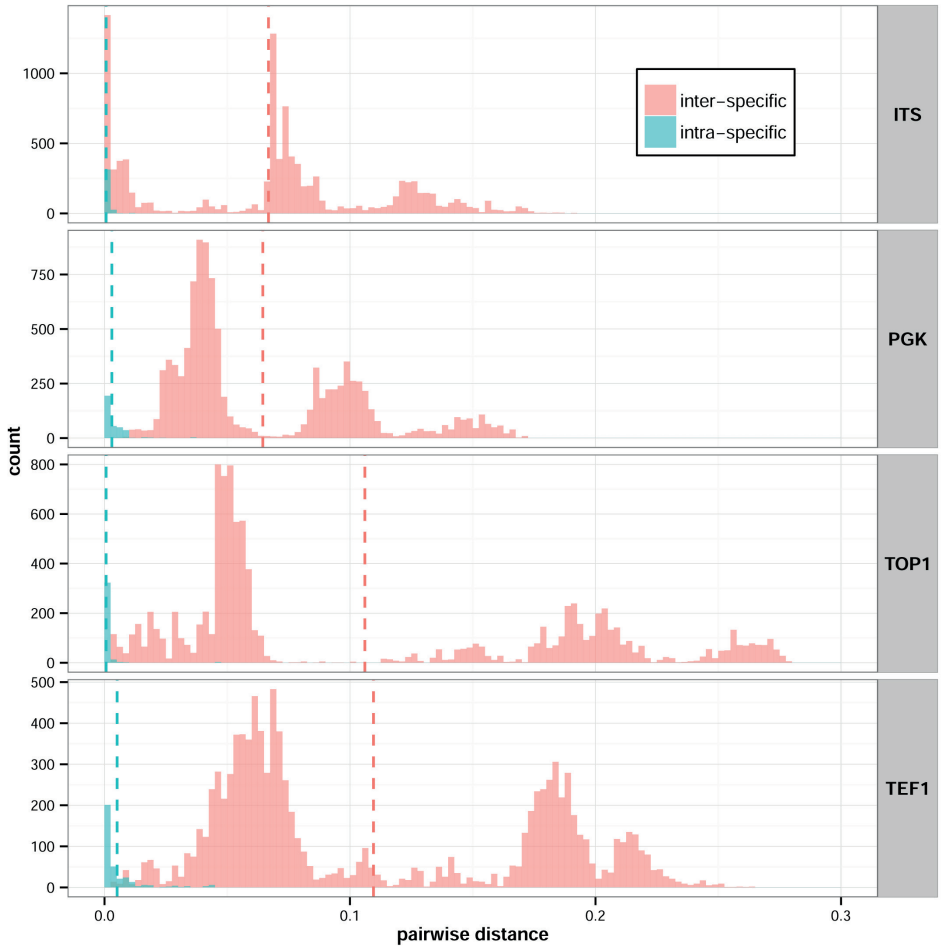


Fig 1. Distribution of intra- and inter-specific pairwise distances for all strains, for each marker. The average distance is represented by the dotted lines.

Not surprisingly, ITS showed the greatest overlap between intra- and inter-specific distance. The barcode gap appeared to be similar for *PGK* and *TEF1*, however, the interspecific variation was much smaller in *PGK*. *TOP1* and *TEF1* had similar interspecific variation but TOP1 had less intraspecific variation. The interspecific variation was compared for each pair of markers (Fig 2). The most important part of this graph is close to the origin on the axes. One can see that there are many instances where the ITS pairwise distances are zero but are above zero for the other markers. *PGK* and *TEF1* appear to be similar close to the origin but *TEF1* always showed a higher pairwise distance for more distant species.

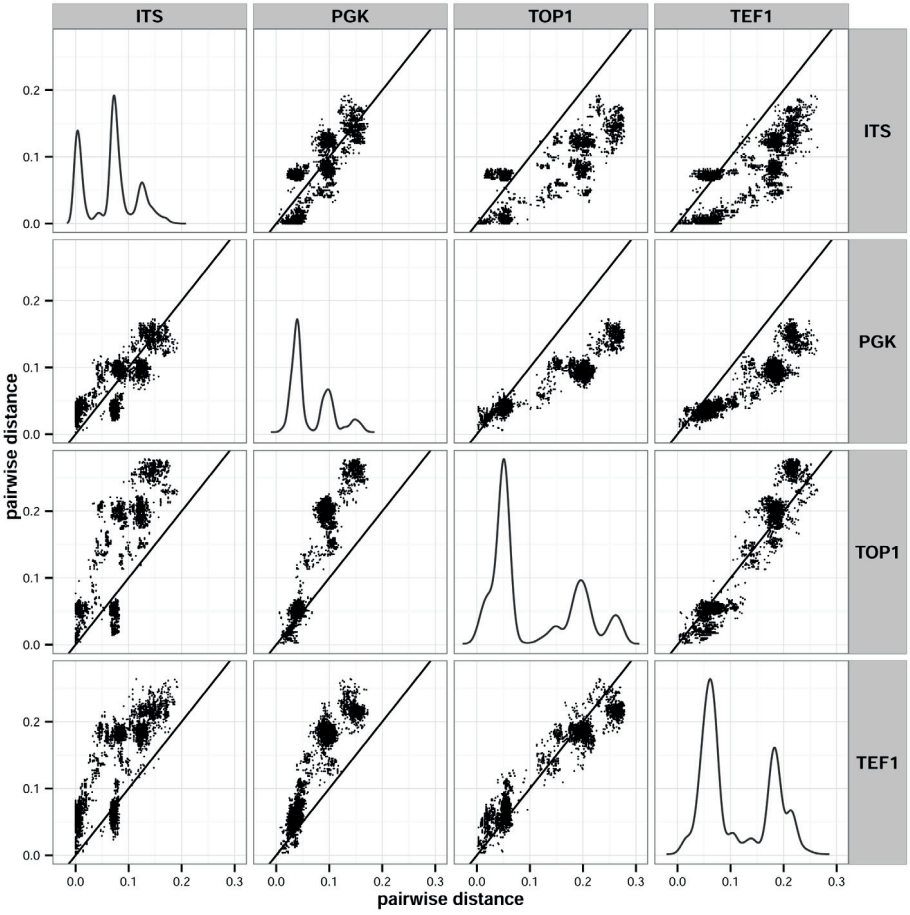


Fig 2. Comparison of interspecific pairwise distances between each marker pair. The points along the zero line for one axis represents the species pairs that cannot be differentiated by this primer. For each point, the coordinate with the higher number is the superior marker for species differentiation. The points that are along the line of x = y represent the species pairs where the distance is the same for both markers.

ITS yielded smallest barcoding gaps in *Fusarium* between sibling at a minimum of 0.1% for the species pairs *F. succisae* / *F. anthophilum*, *F. andiyazi* / *F. anthophilum*, *F. circinatum* / *F. anthophilum*, *F. culmorum* / *F. crookwellense*, *F. proliferatum* / *F. mangiferae*, and *F. proliferatum* / *F. fujikuroi*. The most divergent species in the ITS data set were *F. petroliphilum* and *F. armeniacum*.

Intra-specific variability

The most useful barcoding locus should have no overlap between inter- and intra-specific K2P distances. Twenty-three of the 52 *Fusarium* species had more than one strain in the data set and were selected for analysis (Fig 3).

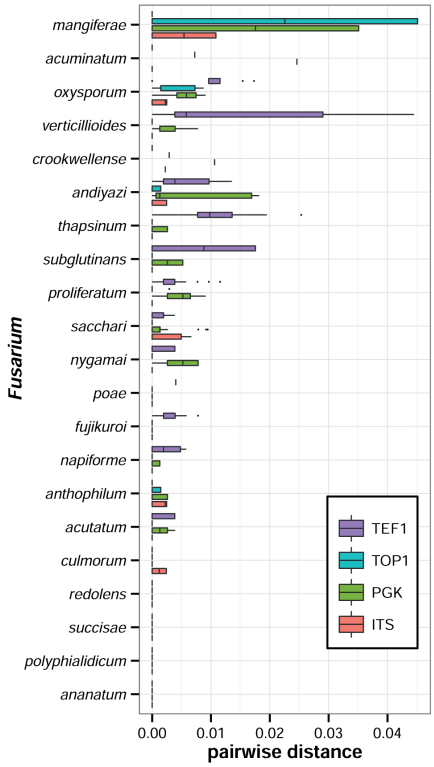
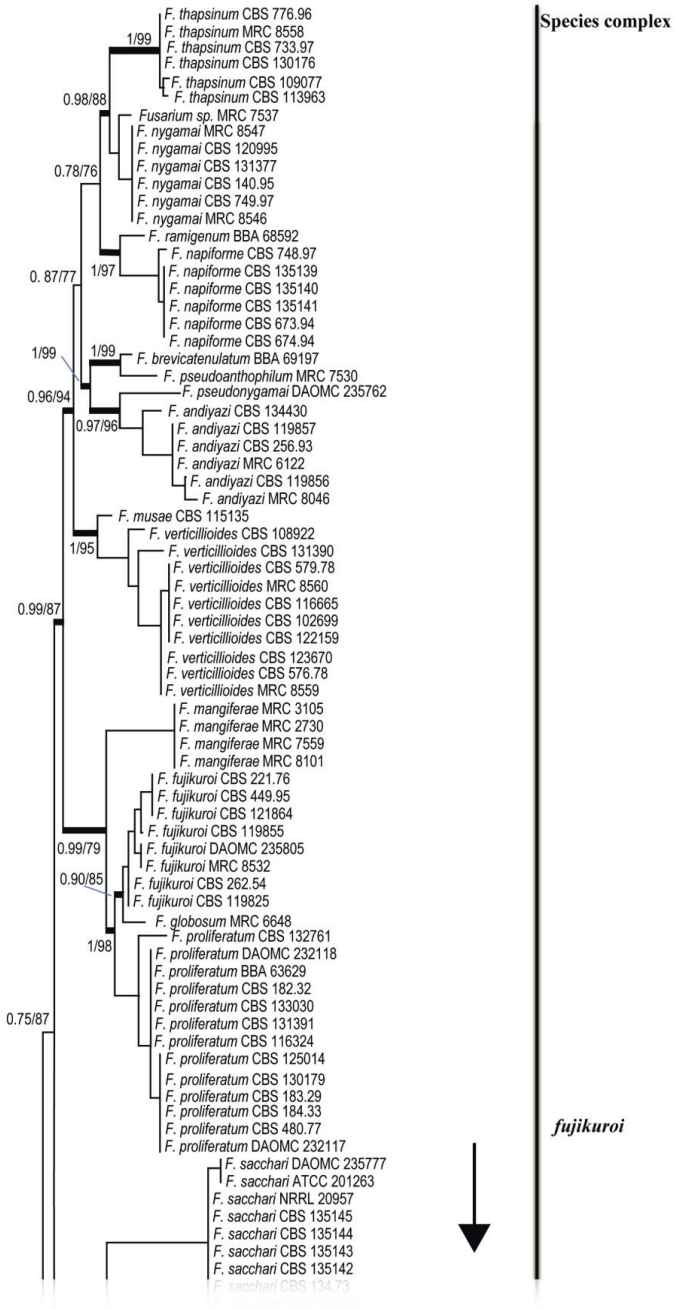


Fig 3. Intraspecific variation among all strains of each species pairs for the four markers. All strains had sequence data for the four markers. The line in the middle of the box is the median. When all markers of a species do not have a box, there was only a pair of strains for this species. When there is a mixture of lines and boxes for a species, the lines represent no variation in the intraspecific pairwise distance. The bottom and the top of a box represent the 25th and 75th percentiles and the whiskers are 1.5 times the interquartile range above and below the box limits. The dots are outliers, i.e. beyond ±2.7 standard deviations.

Phylogenetic analysis and congruence among loci

Bootstrap analyses were performed on each data set, with 70 % as cut-off. We used the program ModelTest v. 2.3. within MEGA6.2 (Tamura et al. 2013) to determine the nucleotide substitution model that best fit our data. K2 þ G + I was selected as the best model for the ITS and *TEF1* data sets, TN93 + G + I for *TOP1*, and TN93 + G for *PGK*. The nodes, e.g. nodal distance from the root node to the branches on a phylogenetic tree obtained from the single gene data sets using ML and Bayesian analyses showed significant discordance with the ITS tree (not shown) and the other three markers, but revealed no conflicts between *TEF1*, *TOP1*, and *PGK*. Tree-based DNA identification was assessed using BI and ML without considering an outgroup. ML and BI analyses showed that the data sets for all terminal clades representing species were identical for *TEF1*, *TOP1*, and *PGK*.

Based on the data generated here (Figs 4-6), we could delineate the *Fusarium* species using any one of the three protein coding genes, i.e. *TEF1*, *TOP1*, and *PGK*, individually. The currently accepted species were all well-supported in the trees of the three markers. Most species represented by several strains formed monophyletic groups with posterior probabilities higher than 1 and bootstrap values higher than 70%.


Species complex
F. thapsinum CBS 776.96
F. thapsinum MRC 8558
F. thapsinum CBS 733.97
F. thapsinum CBS 130176
F. thapsinum CBS 109077
F. thapsinum CBS 113963
Fusarium sp. MRC 7537
F. nygamai MRC 8547
F. nygamai CBS 120995
F. nygamai CBS 131377
F. nygamai CBS 140.95
F. nygamai CBS 749.97
F. nygamai MRC 8546
F. ramigenum BBA 68592
F. napiforme CBS 748.97
F. napiforme CBS 135139
F. napiforme CBS 135140
F. napiforme CBS 135141
F. napiforme CBS 673.94
F. napiforme CBS 674.94
F. brevicatenulatum BBA 69197
F. pseudoanthophilum MRC 7530
F. pseudonygamai DAOMC 235762
F. andiyazi CBS 134430
F. andiyazi CBS 119857
F. andiyazi CBS 256.93
F. andiyazi MRC 6122
F. andiyazi CBS 119856
F. andiyazi MRC 8046
F. musae CBS 115135
F. verticillioides CBS 108922
F. verticillioides CBS 131390
F. verticillioides CBS 579.78
F. verticillioides MRC 8560
F. verticillioides CBS 116665
F. verticillioides CBS 102699
F. verticillioides CBS 122159
F. verticillioides CBS 123670
F. verticillioides CBS 576.78
F. verticillioides MRC 8559
F. mangiferae MRC 3105
F. mangiferae MRC 2730
F. mangiferae MRC 7559
F. mangiferae MRC 8101
F. fujikuroi CBS 221.76
F. fujikuroi CBS 449.95
F. fujikuroi CBS 121864
F. fujikuroi CBS 119855
F. fujikuroi DAOMC 235805
F. fujikuroi MRC 8532
F. fujikuroi CBS 262.54
F. fujikuroi CBS 119825
F. globosum MRC 6648
F. proliferatum CBS 132761
F. proliferatum DAOMC 232118
F. proliferatum BBA 63629
F. proliferatum CBS 182.32
F. proliferatum CBS 133030
F. proliferatum CBS 131391
F. proliferatum CBS 116324
F. proliferatum CBS 125014
F. proliferatum CBS 130179
F. proliferatum CBS 183.29
F. proliferatum CBS 184.33
F. proliferatum CBS 480.77
F. proliferatum DAOMC 232117
F. sacchari DAOMC 235777
F. sacchari ATCC 201263
F. sacchari NRRL 20957
F. sacchari CBS 135145
F. sacchari CBS 135144
F. sacchari CBS 135143
F. sacchari CBS 135142
fujikuroi
1/99
0.98/88
0.78/76
0. 87/77
1/97
1/99
1/99
0.96/94
0.97/96
1/95
0.99/87
0.99/79
0.90/85
1/98
0.75/87

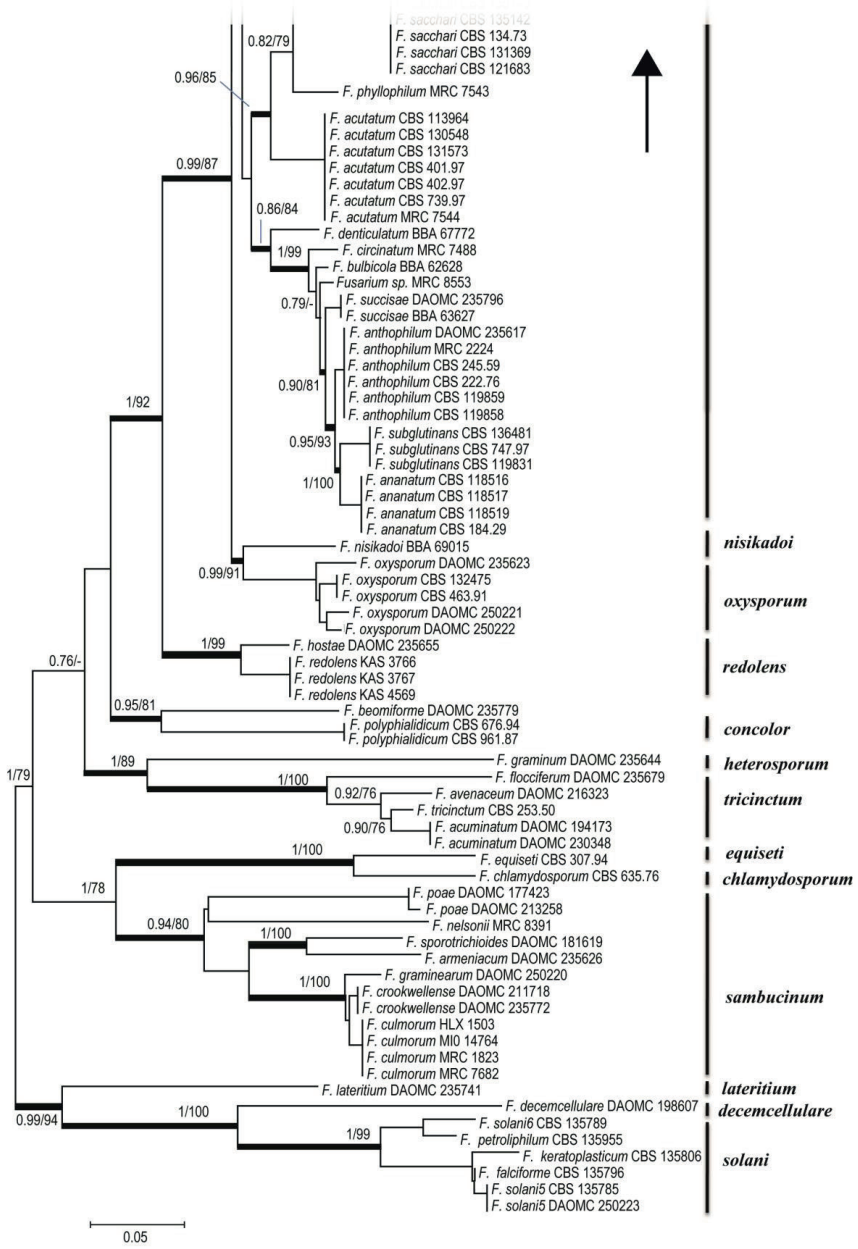
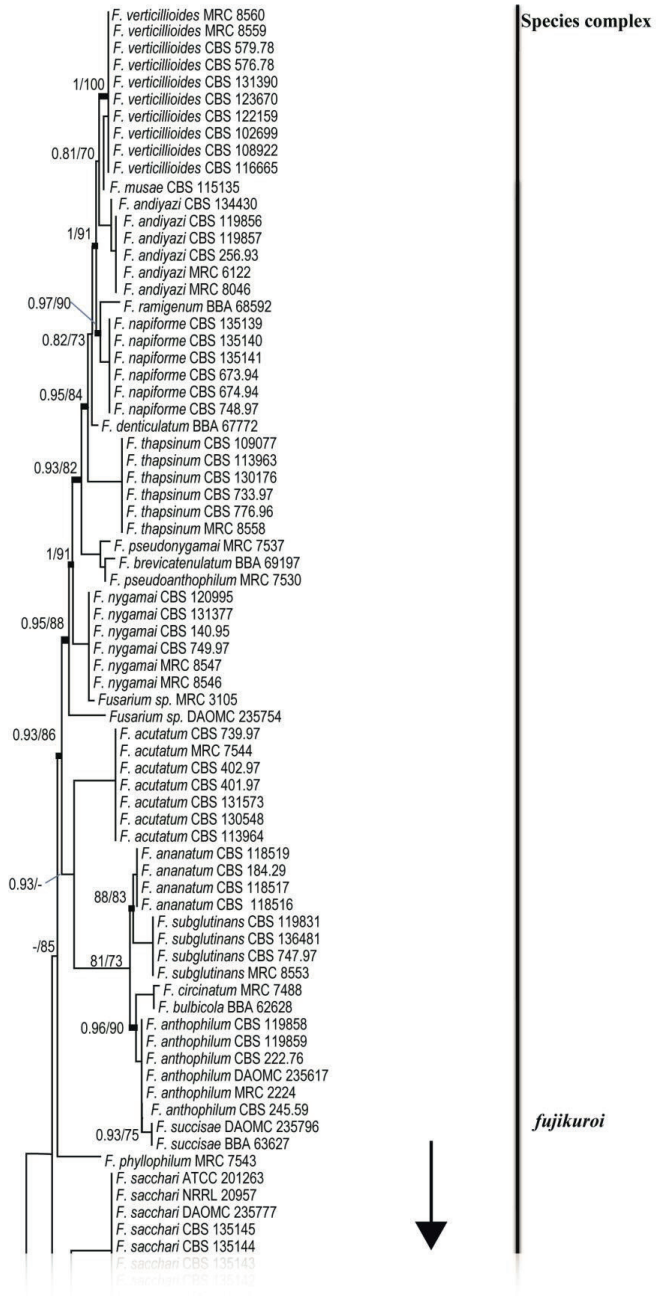


Fig 4. Phylogenetic tree generated by Bayesian inference (BI) and maximum likelihood (ML) trees from 144 -*TEF1* sequences, 589 characters, 10 000 000 generations, 4 mcmc runs. Numbers on the branches are Bayesian posterior probabilities (PP) and percentages of 1000 bootstrap-replications of MEGA6-maximum likelihood (PP/ML). The tree was rooted with the *F. solani* complex.


F. verticillioides MRC 8560
F. verticillioides MRC 8559
F. verticillioides CBS 579.78
F. verticillioides CBS 576.78
1/100
F. verticillioides CBS 131390
F. verticillioides CBS 123670
F. verticillioides CBS 122159
F. verticillioides CBS 102699
0.81/70
F. verticillioides CBS 108922
F. verticillioides CBS 116665
F. musae CBS 115135
F. andiyazi CBS 134430
1/91
F. andiyazi CBS 119856
F. andiyazi CBS 119857
F. andiyazi CBS 256.93
F. andiyazi MRC 6122
F. andiyazi MRC 8046
0.97/90
F. ramigenum BBA 68592
0.82/73
F. napiforme CBS 135139
F. napiforme CBS 135140
F. napiforme CBS 135141
F. napiforme CBS 673.94
0.95/84
F. napiforme CBS 674.94
F. napiforme CBS 748.97
F. denticulatum BBA 67772
F. thapsinum CBS 109077
0.93/82
F. thapsinum CBS 113963
F. thapsinum CBS 130176
F. thapsinum CBS 733.97
F. thapsinum CBS 776.96
F. thapsinum MRC 8558
1/91
F. pseudonygamai MRC 7537
F. brevicatenulatum BBA 69197
F. pseudoanthophilum MRC 7530
F. nygamai CBS 120995
F. nygamai CBS 131377
0.95/88
F. nygamai CBS 140.95
F. nygamai CBS 749.97
F. nygamai MRC 8547
F. nygamai MRC 8546
Fusarium sp. MRC 3105
Fusarium sp. DAOMC 235754
0.93/86
F. acutatum CBS 739.97
F. acutatum MRC 7544
F. acutatum CBS 402.97
F. acutatum CBS 401.97
F. acutatum CBS 131573
F. acutatum CBS 130548
F. acutatum CBS 113964
F. ananatum CBS 118519
F. ananatum CBS 184.29
0.93/-
F. ananatum CBS 118517
88/83
F. ananatum CBS 118516
F. subglutinans CBS 119831
F. subglutinans CBS 136481
-/85
F. subglutinans CBS 747.97
81/73
F. subglutinans MRC 8553
F. circinatum MRC 7488
F. bulbicola BBA 62628
0.96/90
F. anthophilum CBS 119858
F. anthophilum CBS 119859
F. anthophilum CBS 222.76
F. anthophilum DAOMC 235617
F. anthophilum MRC 2224
F. anthophilum CBS 245.59
F. succisae DAOMC 235796
0.93/75
F. succisae BBA 63627
F. phyllophilum MRC 7543
F. sacchari ATCC 201263
F. sacchari NRRL 20957
F. sacchari DAOMC 235777
F. sacchari CBS 135145
F. sacchari CBS 135144
Species complex
fujikuroi

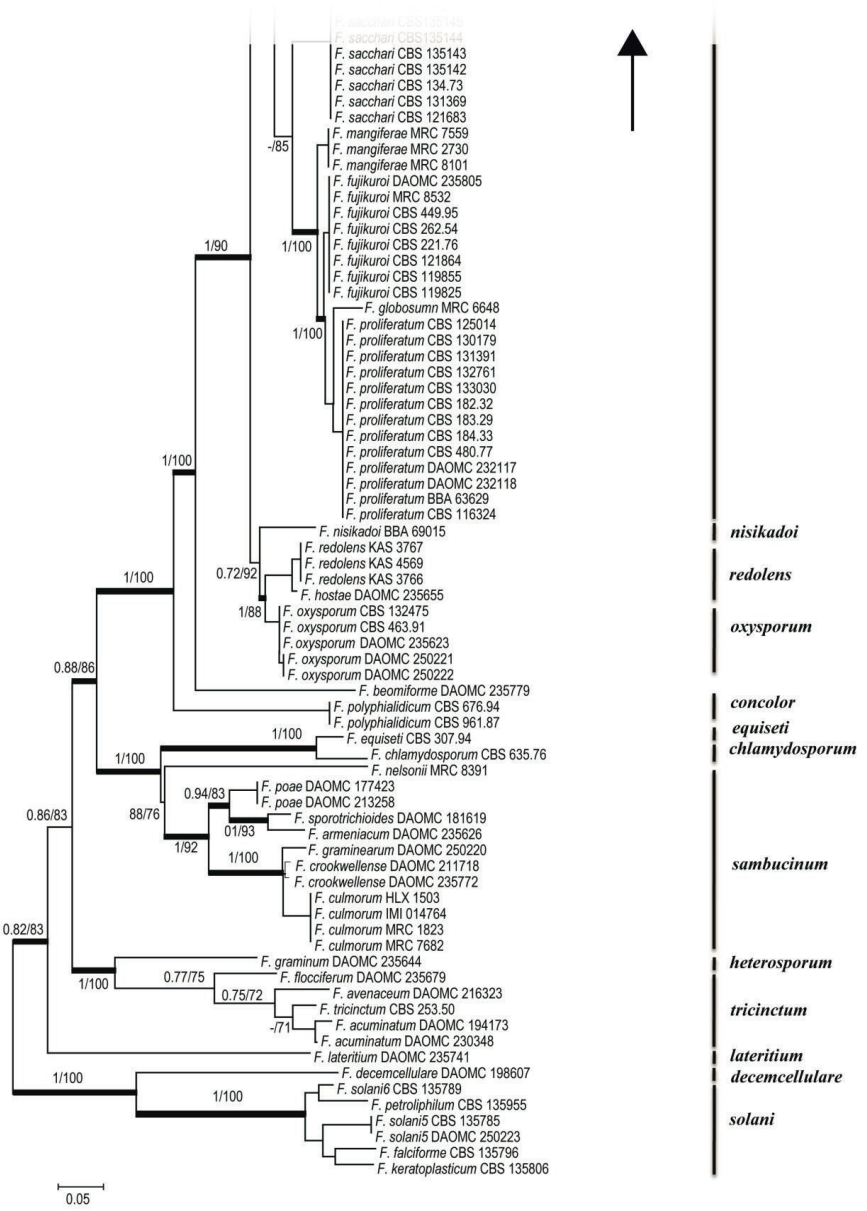
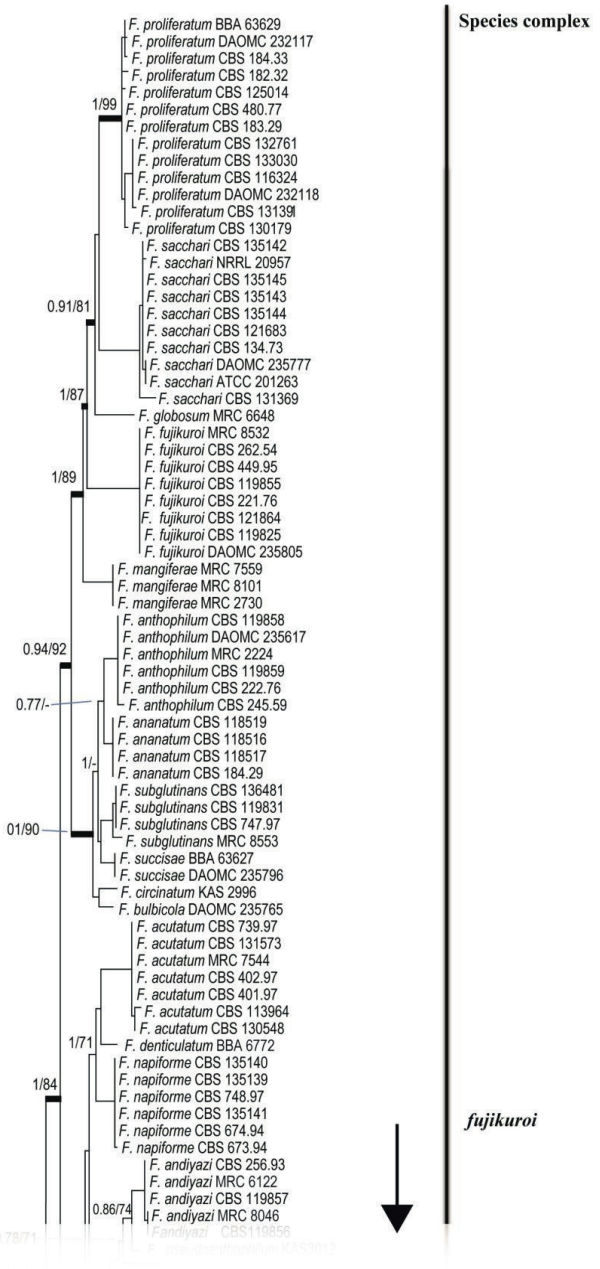


Fig 5. Phylogenetic tree generated by Bayesian inference (BI) and maximum likelihood (ML) trees from 144 - *TOP1* sequences, 708 characters, 10 000 000 generations, 4 mcmc runs. Numbers on the branches are Bayesian posterior probabilities (PP) and percentages of 1000 bootstrap-replications of MEGA6-maximum likelihood (PP/ML). The tree was rooted with the *F. solani* complex.


Species complex
F. proliferatum BBA 63629
F. proliferatum DAOMC 232117
F. proliferatum CBS 184.33
F. proliferatum CBS 182.32
F. proliferatum CBS 125014
1/99
F. proliferatum CBS 480.77
F. proliferatum CBS 183.29
F. proliferatum CBS 132761
F. proliferatum CBS 133030
F. proliferatum CBS 116324
F. proliferatum DAOMC 232118
F. proliferatum CBS 131391
F. proliferatum CBS 130179
F. sacchari CBS 135142
F. sacchari NRRL 20957
F. sacchari CBS 135145
F. sacchari CBS 135143
0.91/81
F. sacchari CBS 135144
F. sacchari CBS 121683
F. sacchari CBS 134.73
F. sacchari DAOMC 235777
F. sacchari ATCC 201263
1/87
F. sacchari CBS 131369
F. globosum MRC 6648
F. fujikuroi MRC 8532
F. fujikuroi CBS 262.54
F. fujikuroi CBS 449.95
1/89
F. fujikuroi CBS 119855
F. fujikuroi CBS 221.76
F. fujikuroi CBS 121864
F. fujikuroi CBS 119825
F. fujikuroi DAOMC 235805
F. mangiferae MRC 7559
F. mangiferae MRC 8101
F. mangiferae MRC 2730
F. anthophilum CBS 119858
F. anthophilum DAOMC 235617
0.94/92
F. anthophilum MRC 2224
F. anthophilum CBS 119859
F. anthophilum CBS 222.76
0.77/-
F. anthophilum CBS 245.59
F. ananatum CBS 118519
F. ananatum CBS 118516
1/-
F. ananatum CBS 118517
F. ananatum CBS 184.29
F. subglutinans CBS 136481
F. subglutinans CBS 119831
01/90
F. subglutinans CBS 747.97
F. subglutinans MRC 8553
F. succisae BBA 63627
F. succisae DAOMC 235796
F. circinatum KAS 2996
F. bulbicola DAOMC 235765
F. acutatum CBS 739.97
F. acutatum CBS 131573
F. acutatum MRC 7544
F. acutatum CBS 402.97
F. acutatum CBS 401.97
F. acutatum CBS 113964
F. acutatum CBS 130548
1/71
F. denticulatum BBA 6772
F. napiforme CBS 135140
1/84
F. napiforme CBS 135139
F. napiforme CBS 748.97
F. napiforme CBS 135141
F. napiforme CBS 674.94
F. napiforme CBS 673.94
fujikuroi
F. andiyazi CBS 256.93
F. andiyazi MRC 6122
F. andiyazi CBS 119857
0.86/74
F. andiyazi MRC 8046

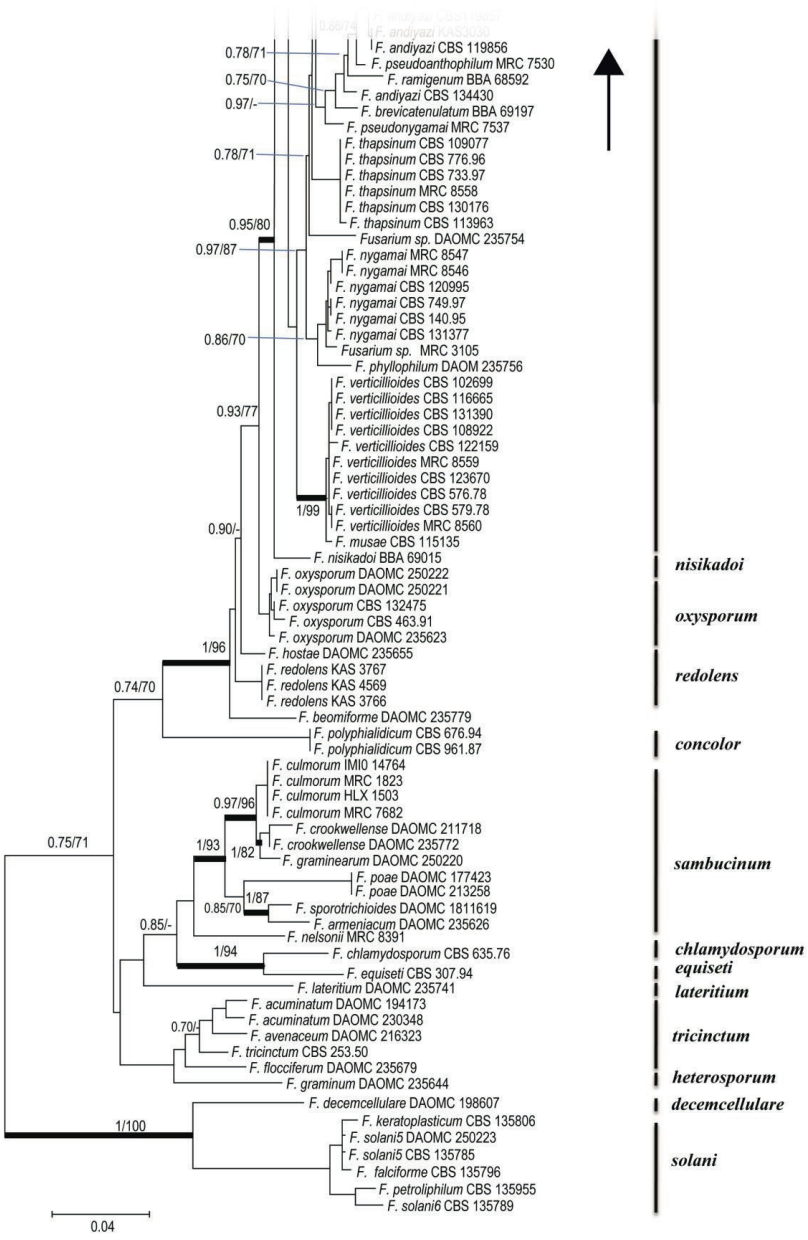


Fig 6. Phylogenetic tree generated by Bayesian inference (BI) and maximum likelihood (ML) trees from 144 *PGK* sequences, 828 characters, 10 000 000 generations, 4 mcmc runs. Numbers on the branches are Bayesian posterior probabilities (PP) and percentages of 1000 bootstrap-replications of MEGA6-maximum likelihood (PP/ML).The tree was rooted with the *F. solani* complex.

Discussion

The need for rapid identification of *Fusarium* species has always been apparent because of the frequency of diseases and mycotoxin production in plants. The genus is increasingly significant for human health, particularly because mortality rates from disseminated fusariosis are rising in severely immunocompromised patients. Molecular taxonomic studies have focused on resolving evolutionary relationships within species complexes or clades of agriculturally and medically important *Fusarium* (O'Donnell et al. 2012), where particularly *RPB2* and *RPB1* have been regarded significant (O'Donnell et al. 2013). The aim of the present study was to evaluate the efficacy of DNA barcoding using alternative markers in addition to ITS, i.e. the widely used translation elongation factor 1-a (*TEF1*, Geiser et al. 2004), and the newly identified potential barcodes topoisomerase I (*TOP1*) and phosphoglycokinase (*PGK*, Stielow et al. 2015).

We obtained sequences from representatives of 52 species in 13 species complexes of *Fusarium*, demonstrating the ability of the primers for all four markers to amplify a wide range of species within the genus. High-quality DNA was obtained from all strains and all amplicons were easily sequenced. ITS rDNA as the recommended barcode for fungi has many advantages, in particular the large number of reference sequences available in GenBank (Schoch et al. 2012), but for most species complexes in *Fusarium* this gene is insufficiently variable to discriminate molecular siblings (Summerbell et al. 2005; Balajee et al. 2009).

We analysed potential barcode alternatives according to criteria listed by Letourneau et al. (2010) that the barcode should be short (500-800 bp), and easily amplifiable, with a low intraspecific variation and a higher interspecific than intraspecific variation. PCR amplification success rates were 98 % for *TEF1*, 96 % for *TOP1* and 95 % for *PGK*. Generally, specific primer binding resulted in single bands and only a few strains evidenced a complete lack of amplification. For both *TOP1* and *PGK*, we used M13 primers for sequencing in our first trial and a 100 % success rate was obtained (Table 1). The identity of all strains with *TEF1* sequences was confirmed using BLAST, *Fusarium* MLST database (http://www.cbs.knaw.nl/fusarium) (O'Donnell et al. 2010) and the FUSARIUM-ID database (Geiser et al. 2004). The identities of some unknown strains originally sequenced only for *TOP1* or *PGK* sequences were inferred by correlation with similar sequences obtained for other strains identified using *TEF1* sequences. Although amplification and sequencing success rates of ITS were high, close to 100 % in our study, the gene could not discriminate between *Fusarium* species in this analysis. This could be because ITS sequences are identical in many *Fusarium* complexes and they do not tend to evolve at a rate correlated with speciation. In contrast, protein-coding genes usually have higher information content and tend to evolve at higher rate than the more commonly used ITS (Liu et al. 1999). In our data sets, *TEF1* had the highest number of variable sites (53.3 %), followed by *TOP1* (50.70 %), *PGK* (36.2 %), and ITS (29.2 %). In terms of phylogenetically informative sites among four markers, *TOP1* had (46.1 %), followed by *TEF1* (43.4.2 %), *PGK* (28.99 %)

and ITS (22.5 %). Thus, the higher variability in intron-rich portions of protein coding genes generally allow these genes to do much better (Geiser et al. 2004), and could therefore represent better candidates for *Fusarium* DNA barcoding. These results demonstrate sufficient variability for species identification in *Fusarium* for all three protein coding genes. According to concepts outlined by Jeffroy et al. (2006), this could reflect either degeneracy of the genetic code, which means that even when a protein is completely conserved, the DNA sequence encoding that protein can vary, or invariant protein-coding regions combined with highly variable introns or intergenic spacer regions provide adequate barcode signals. Stielow et al. (2015) developed novel protein based primers and recommended a far broader application of *TEF1* for barcoding in fungi. *TEF1* is already in use as barcode in Hypocreales, e.g. *Fusarium*, *Tricholoma*, *Hypocrea* and various genera in the *Nectriaceae* (Pino-Bodas et al. 2013).

Lengths and guanine-cytosine contents (G + C %) of gene regions amplified varied among markers. The *PGK* data set had the highest G + C % content (57.8 % on average), followed by *TEF1* (52.5 %) and *TOP1* (48.5 %). Amplicons of *TEF1*, *TOP1* and *PGK* ranged from 500 to 800 bp. Amplicon lengths and G + C % contents were generally species-specific, but similar G + C % contents do occur in different species. If G + C % contents are very different, however, the strains cannot be considered members of the same species (Baron 1996). There are no absolute rules for the appropriate barcode gap distance between species, but it is axiomatic that genes yielding wider inter-specific gaps are more reliable for delimiting species within a genus. There were no clear gaps between the minimum inter-specific variations for the four studied barcodes (Fig 1). However, we have only limited data for the intra-specific variation because many species were represented by single strain. In studies where intra-specific variability was included in more detail, it was noted that discrete barcode gaps are not always present. For example, in *Ochroconis* and related genera (Samerpitak et al. 2016), a remarkably high degree of divergence was noted in all markers, so that ITS and even LSU were useful for species distinction. This led to large inter-specific differences, but because intraspecific heterogeneity was also high, most markers did not demonstrate an ambiguous barcoding gap. In contrast, *Scedosporium* species outside the *S. apiospermum* complex (Chen et al. 2016) form compact clusters, where ITS has insufficient resolution to separate the entities. Within the *S. apiospermum* complex no absolute barcoding gaps were present either, while other, unambiguously delimited species at larger phylogenetic distances did exist. In *Fusarium*, additional studies on intraspecific variability are necessary to confirm the performance of these loci, and whether or not unambiguous barcoding gaps are present among phylogenetic siblings. Considering our high success rates for amplification and sequencing, both *TOP1* and *PGK* are promising supplementary barcodes for identifying *Fusarium* species.

Our analyses of *Fusarium* show that *TOP1* and *PGK* help to resolve large-scale genus level as well as detailed species level phylogenetic relationships. Sequences were alignable

across the genus and had sufficient variability to resolve within genus relationships. Phylogenetic results provided strong Bayesian and Maximum Likelihood support rates for a monophyletic 'terminal *Fusarium* clade' (TFC) (Figs 4-6). TFC was described previously in *Fusarium* by Gráfenhan et al. (2011), O'Donnell et al. (2013) and Lombard et al. (2015). *TEF1*, *TOP1* and *PGK* genes produced congruent gene trees and we predict that *TOP1* and *PGK* will have a higher potential to resolve phylogenetic relationships among *Fusarium* species when analysed in combination with other routinely used markers such as *TEF1*, *RPB1* and *RPB2*. All loci resolved most of the terminal clades in their respective gene-trees. The loci can be combined to reach taxonomically sound conclusions about the relationships among species.

We conclude that any of the three protein-coding loci tested in this study (*TEF1*, *TOP1* and *PGK*) reliably identify all of the species studied. As noted in previous studies (e.g. O'Donnell et al. 2010), ITS is often insufficiently informative at the species level, but can be useful to identify species complexes. The ITS gene tree proved to be incongruent with those from other loci. However, this does not preclude the use of this gene as a barcoding locus for species identification within the fungal kingdom and it is a good starting point to rapidly identify genera level within Ascomycota (Schoch et al. 2012). It is critical that ITS sequences continue to be generated for newly described *Fusarium* species, and that the species sharing identical ITS sequences be explicitly identified, so that information on *Fusarium* can be extracted from amplicon-based environmental sequencing at the maximum degree of resolution possible within the limitations of the marker. *TEF1*, *TOP1* and *PGK* can more effectively be used as barcoding markers for precise identification of cultures known to represent *Fusarium* species.

Acknowledgements

This study was partially funded by the Ministry of Health, Oman. Formal Agreement no. 28/2014. Abdullah. M. S. Al Hatmi received a PhD scholarship from the Ministry of Health, Oman. Genome mining at CBS and AAFC, and primer development and testing at AAFC, were supported by grants from the A.P. Sloan Foundation Programme on the Microbiology of the Built Environment**.**

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CHAPTER 3

Chapter 3

DNA barcoding, MALDI-TOF, and AFLP data support *Fusarium ficicrescens* as a distinct species within the *Fusarium fujikuroi* species complex

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Fungal Biology 2016; 120: 265-278.

Abstract

The *Fusarium fujikuroi* species complex (FFSC) is one of the most common groups of fusaria associated with plant diseases, mycotoxin production and traumatic and disseminated human infections. Here we present the description and taxonomy of a new taxon, *Fusarium ficicrescens* sp. nov., collected from contaminated fig fruits in Iran. Initially this species was identified as *Fusarium andiyazi* by morphology. In the present study the species was studied by multi-locus sequence analysis, amplified fragment length polymorphism (AFLP), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and phenotypic characters. Multilocus analyses were based on translation elongation factor 1α (*TEF1*), RNA polymerase subunit (*RPB2*) and beta-tubulin (*BT2*) and proved *F. ficicrescens* as a member of the FFSC. Phylogenetic analysis showed that the fungus is closely related to *Fusarium lactis*, *Fusarium ramigenum*, and *Fusarium napiforme*; known plant pathogens, mycotoxin producers, and occasionally occurring multidrug resistant opportunists. The new species differed by being able to grow at 37 °C and by the absence of mycotoxin production. *TEF1* was confirmed as an essential barcode for identifying *Fusarium* species. In addition to *TEF1*, we evaluated *BT2* and *RPB2* in order to provide sufficient genetic and species boundaries information for recognition of the novel species.

Key words: β-tubulin, Ecology, Fumonisin, Phylogeny, Taxonomy, Translation elongation factor 1 α

Introduction

Species of the genus *Fusarium* show close interactions with plant hosts as saprobes on plant debris and endophytic in living hosts, as well as plant pathogens. They also occur as potential opportunists of immunocompetent humans, with a traumatic portal of entry, and occasionally cause disseminated infections in severely debilitated patients. Their distribution is in temperate and tropical climate zones (Schmale & Munkvold 2009). Since individual species can be found in divergent habitats including agricultural and aquatic systems, natural forests, grassland and deserts, as well as in man-made environments, the natural ecology of *Fusarium* remains enigmatic (Al-Hatmi et al. 2014). Smith et al. (2007) noted that *Fusarium* conidia are water-borne and become air-borne when dried, while the chlamydospores are typically soil-borne. Sexual spores, when produced, are distributed by air. Some species of *Fusarium* have a restricted host-range, while others either cause diseases of many plant species (Short et al. 2013) or are able to infect humans relatively effectively (Nucci et al. 2014).

During the past two decades 15 species have been described in the *F. fujikuroi* species complex (FFSC) based on the biological species concept, but by using molecular data the existence of at least 50 distinct phylogenetic species have been surmised (Kvas et al. 2009). Of these, 34 species have been formally described with morphological characters.

Mycotoxin contamination of food products due to growth of *Fusarium* is a widely recognized problem. Figs are an economically important crop in the Mediterranean area, with Algeria, Egypt, and Turkey being the main producing countries worldwide (Heperkan et al. 2012). *Fusarium verticillioides* and *F. proliferatum* are commonly isolated from figs and are known to produce fumonisins (Kosoglu et al. 2011). Moretti et al. (2010) reported the presence of *F. ramigenum*, a species able to produce fumonisin B1 (FUM), on dried figs in Turkey and on fresh figs in Italy. In addition, *F. proliferatum*, *F. ramigenum*, and *F. solani* were isolated from fig samples having endosepsis-like symptoms in the Apulia region in Italy (Moretti et al. 2010). *Fusarium verticillioides* and *F. solani* were reported as causative agents of endosepsis in cultivated and wild Capri figs collected in California and in figs produced in Turkey (Kosoglu et al. 2011). According to Heperkan et al. (2012) the identification of *Fusarium* etiologic agents in both studies was based on morphological characters only, and thus strains may have been misidentified according to modern standards. Californian fungal fig strains identified by Subbarao & Michailides (1993) as *F. verticillioides* were sequenced by O'Donnell et al. (1998) and these authors identified them as belonging to the closely related species *F. ramigenum* or *F. lactis*. These two species were considered as fig specific. However, in a survey on dried figs in Apulia, Italy only *F. ramigenum* was found, while *F. lactis* was not encountered (Moretti et al. 2010). In their phylogenetic analysis using *TEF1* gene, a phylogenetic cluster was noted composed of three *Fusarium* strains (ITEM 2913, ITEM 2921, and ITEM 2922) which were not assigned to any described species yet.

In the present study, an apparently undescribed species with *Fusarium*-like morphology was isolated from fig fruit samples from Estahban areas of Fars province in Iran, during the month June of two consecutive years (2007-2008). A sample set of seven isolates morphologically identified as *F. cf. andiyazi* (n = 3) and *F. cf. proliferatum* (n = 4) were sent to the CBS-KNAW Fungal Biodiversity Centre in Utrecht, the Netherlands, for molecular characterization. The three strains previously identified as *F. andiyazi* appeared to be located in a separate clade, suggesting an as yet undescribed species whereas the four remaining strains molecularly were confirmed to be *F. proliferatum*. Therefore, we assume that the same fungus isolated from different figs fruits in the Mediterranean area requires formal description.

The new species is described below as a member of the *F. fujikuroi* species complex. The objectives of the present paper were: (1) to describe a novel species of *Fusarium* by using multilocus sequencing, AFLP, MALDI-TOF MS, and morphological characteristics, (2) assess whether these isolates are able to produce fumonisin B1 (FUM), (3) evaluate the antifungal susceptibility profile of the species, and (4) define the optimal barcoding gene for its routine identification.

Materials and methods

Strains and culture conditions

Our re-identification of *Fusarium* strains in the CBS-KNAW collection was based on BLAST analysis in GenBank using sequences of the following genes: internal transcribed spacer region (ITS; White et al. 1990), partial nuclear large subunit (LSU; Hopple & Vilgalys 1999), elongation factor 1α (*TEF1*; O'Donnell et al. 1998), the second largest subunit of RNA polymerase (*RPB2*; Reeb et al. 2004), and β-tubulin (*BT2*; Glass & Donaldson 1995; O'Donnell & Cigelnik 1997) (Table 1). A total of 100 *Fusarium* strains were isolated during June of two consecutive years (2007-2008), *Fusarium* species associated with fig fruit samples of two non-edible fig cultivars [Danesefid and Pouzdonbali of Capri fig (*Ficus carica* cv. Capri)] were isolated from Estahban and EiJ areas of Fars province. Edible fig samples were collected from Calimyrna trees (*Ficus carica* cv. Calimyrna) of Estahban during harvest time, before processing, during processing stages and after packaging in October and November of 2008 and 2009. Fig fruits were cultured on potato dextrose agar (PDA, Merck, White house Station, NJ, U.S.A.) and incubated at 25 °C for 5-7 d. Preliminary identification was done using morphological characteristics observed on carnation leaf agar (CLA) medium. Based on the morphological identification, representatives of seven strains were sent to the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, for further characterization.

Table 1. PCR primers used for amplification and sequencing in this study				
Locus	**Primers**	**primer sequence (5′-3′)**	**size (bp)**	**Reference**
BT2	BT-2a	GGTAACCAAATCGGTGCTGCTT	500	(Glass & Donaldson 1990)
	BT-2b	TTACGTCCCTGCCCTTTGTA		(O'Donnel & Cigelnik 1997)
ITS	ITS1	TCCGTAGGTGAACCTGCGG	500	(White et al. 1990)
	ITS4	TCCTCCGCTTATTGATATGC		
LSU	LROR	ACCCGCTGAACTTAAGC	600	(Vilgalys & Hester 1990)
	LR5	TCCTGAGGGAAACTTCG		
RPB2	7cr	CCCATRGCTTGYTTRCCCAT	800	(Reeb et al. 2004)
	5f2	GGGGWGAYCAGAAGAAGGC		
TEF1	EF1	ATGGGTAAGGARGACAAGAC	600	(O'Donnell & Sutton 2010)
	EF2	GG ARGTACCAGTSATCATGTT		
MAT1	GFmat1a	GTTCATCAAAGGGCAAGCG	200	(Steenkamp et al. 2000)
	GFmat1b	TAAGCGCCCTCTTAACGCCTTC	200	

Morphology and physiology

Colony characteristics and growth morphology were studied by inoculating the isolates onto plates of malt extract agar (MEA; Oxoid, Basingstoke, U.K.), oatmeal agar (OA; homemade at CBS-KNAW), potato dextrose agar (PDA; Oxoid), synthetic nutrient agar (SNA; home-made at CBS-KNAW) and carnation leaf agar (CLA; home-made at CBS-KNAW; Leslie & Summerell 2006). Culture plates were incubated at 25 °C under alternating 12 h cycles of light/dark using 325 nm UV light. Microscopic mounts in lactic acid with cotton blue were made from cultures grown on a CLA plate and slide cultures were observed after 5 d of incubation at 25 °C. Slides were examined and measured with a light microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan), and pictures were taken using a camera attached to the microscope (digital-sight DS-5 M; Nikon). A minimum of ten measurements per structure were taken after processing in Adobe Photoshop CS3 (Adobe Systems, San José, CA, U.S.A.), and the average was calculated. Cardinal growth temperatures were determined by pre-incubating strain (CBS 125178) and (CBS 119856, type strain for *F. andiyazi*) on CLA plates with subsequent transfer to the centers of 8.5 cm MEA plates, measuring growth at 25, 27, 30, 33, 35, 36, 37, and 40 °C after 72 h. Average growth rates per species were calculated and expressed as diametric growth per 24 h.

Mycotoxins

To assess whether the strains produce Fumonisin B1 (FUM), isolates were grown on rice grains and evaluation was performed by using HPLC-IAC as described by Mirabolfaty & Karami-

Osboo (2006). Briefly, FUM was measured by extraction with methanol-water (80:20, v/v) solvent, followed by purification with immunoaffinity columns (IACs). Fumonisin was converted to fluorescent derivatives by OPA (ortho-phetaldehide). Fumonisin B1 was quantified through high-performance liquid chromatography (HPLC, MERCK, Germany) with fluorescence detector (Em = 335 nm, Exi = 440 nm). Standard curve was drawn between 0.3125 and 40 µg ml $^{-1}$ by using six Fumonisin working standards. Validation of the method was estimated by Certified Reference Material (CRM). The mean of recovery obtained through this method was 90.7 %.

MALDI-TOF MS

For MALDI-TOF analysis conventional formic acid protein extraction was performed on all isolates as described by Ranque et al. (2014). Mass spectra acquisition and analysis was performed on a Bruker Microflex platform using MALDI Biotyper v2.0 software.

Mating

Crosses of the three strains CBS 125177, CBS 125178, and CBS 125181 were conducted as described by Klittich & Leslie (1988). Crosses were set up in duplicate on at least two separate occasions. Strains were inoculated by using the 'barrage zone' method on carrot agar (CA; home-made at CBS-KNAW) and incubated at 25 ± 2 °C with alternating 12 h light/dark cycle with both fluorescent and near-UV light. Eventual production of ascocarps was monitored for 6 weeks following inoculation.

DNA extraction and multi-locus sequencing

Strains were transferred to fresh MEA plates and incubated at 25 °C for 3–7d. Approximately 1 cm2 mycelium was transferred to a 2 mL Eppendorf tube containing 490 µL CTAB (cetyltrimethylammonium bromide) buffer [(Tris-HCl 100 mM, EDTA 20 mM, NaCl 1.4 M, CTAB 2% (w/v), β-mercaptoethanol 0.2% (v/v), pH 8.0)] and 6–10 acid-washed glass beads (Sigma G9143; Sigma-Aldrich, St. Louis, MO, U.S.A.). Ten µL proteinase K (50 mg/mL) was added and the sample was vortexed for 10 min, followed by incubation of the mixture at 60 °C for 2h. Subsequently, 500 µL chloroform:isoamylalcohol (24:1, v/v) was added to the solution and shortly mixed by inversion for 2 min and centrifuged for 10 min at 14,000 rpm. The supernatant was transferred to a new tube with 0.55 volume ice cold isopropanol, mixed carefully by flipping, kept at -20 °C overnight and centrifuged for 10 min at 14,000 rpm. The pellet was washed with ice cold 70% ethanol and after drying at room temperature, re-suspended in 50 µL TE buffer (Tris 0.12% w/v, Na-EDTA 0.04%, w/v). DNA quality was verified with a NanoDrop® ND-1000 Spectrophotometer using ND-1000 v3.3.0 software (Coleman Technologies, Wilmington, DE, U.S.A.). Alternatively, 1% agarose gel (1× TE) was

used to evaluate the DNA by visualizing under ultraviolet (UV) light. DNA extracts were stored at -20 °C prior to use.

PCR reaction were performed in a volume of 12.5 µL containing 1.25 µL 10× PCR buffer, 7.5 µL water, 0.5 µL dNTP mix (2.5 mM), 0.25 µL of each primer (10 pmol), 0.05 µL *Taq* polymerase (5 U/µL), 0.7 µL DMSO, and 1 µL template DNA (100 ng/µL). Amplification was performed in an ABI Prism 2720 (Applied Biosystems, Foster City, U.S.A.). Cycling conditions included 95 °C for 4 min, followed by 35 cycles consisting of 95 °C for 45 sec, 52 °C for 30 sec and 72 °C for 2 min, and a delay at 72 °C for 7 min for ITS and LSU. One cycle of 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 48 °C and 2 min at 72 °C, followed by one cycle of 7 min at 72 °C, at 10 °C for BT2 (BT-2a, BT-2b). One cycle of 5 min at 94 °C, ten cycles of 45 sec at 94 °C, 45 sec at 55 °C and 1.5 min at 72 °C, 30 cycles of 45 sec at 94 °C, 45 sec at 52 °C and 1.30 min at 72 °C and post elongation step of 6 min at 72 °C for *TEF1* (Fus1, Fus2) and Pre-denaturation for 3 minutes at 95 °C, five cycles of 45 sec at 95 °C, 45 sec at 58 °C and 2 min at 72 °C, five cycles of 45 sec at 95 °C, 45 sec at 56 °C and 2 min at 72 °C, 30 cycles at 45 sec at 95 °C, 45 sec at 52 °C and 2 min at 72 °C and post elongation step of 8 min at 72 °C for *RPB2* (5F2, 7cr). PCR products were visualized by electrophoresis on 1% (w/v) agarose gels. Sequencing PCR was performed as follows: 1 min at 95 °C, followed by 30 cycles consisting of 10 sec at 95 °C, 5 sec at 50 °C and 2 min 60 °C. Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequencing was done on an ABI 3730xL automatic sequencer (Applied Biosystems) with BigDye™ v3.1 terminator cycle sequencing kit (Applied Biosystems).

Phylogenetic inference

To confirm the identity of our three presumed new *Fusarium* species, we evaluated their position in Bayesian phylogenetic trees of the following individual gene markers (ITS, LSU, *BT2*, *TEF1*, and *RPB2*). In these analyses, our sequences, together with sequences retrieved from GenBank were analysed together (Table 2). Consensus sequences were computed with SeqMan from the Lasergene package (DNAStar, Madison, WI, U.S.A.). Sequences were aligned with the program MAFFT (www.ebi.ac.uk/Tools/msa/mafft/), followed by manual adjustments with MEGA v6.2 and BioEdit v7.0.5.2. A single alignment was constructed for *TEF1* and *BT2* and *RPB2* barcodes, including 39 sequences representing 37 species for *BT2* and *TEF1* and 23 sequences representing 21 species within FFSC for *RPB2*. The best-fit model of evolution was determined by ModelTest v0.1.1. Also ITS and LSU sequences were generated and analysed but these were not able to distinguish the strains sufficiently up to species level and were not analysed further. A tree was constructed by the outgroup method and edited in MEGA v6.2. Sequences included in this study were retrieved from GenBank *F. inflexum* was used as outgroup (Table 2). Genetic relationships were investigated by phylogenetic analysis using Bayesian

inference (BI) and maximum likelihood (ML) methods. Bayesian analysis was performed with MrBayes v3.1.2. Four MCMC (Markov chain Monte Carlo) which is the most popular class of numerical approximation algorithms in Bayesian inference. Chains were run simultaneously for 1 x 10^7 generations (Ronquist & Huelsenbeck 2003). The first 25 % of sampled trees were treated as burn in and discarded. A consensus tree with posterior probability was assessed from the remaining trees. Tracer 1.5 (Rambaut & Drummond 2007) was used to check convergence of the runs and effective sample sizes for the different parameters.

Table 2. GenBank accession numbers of *Fusarium* spp. of the *F. fujikuroi* species complex used in phylogenetic analysis of *F. ficicrescens*

Species	Collection	β-tubulin	*TEF1*	Reference
F. acutatum	NRRL 13308	U34431	AF160276	Scauflaire *et al.* 2011
F. andiyazi	CBS119857	KP662894	KP662901	This study
F. anthophilum	NRRL 13602	U61541	AF160292	Scauflaire *et al.* 2011
F. bactridioides	NRRL20476	U34434	AF160290	Scauflaire *et al.* 2011
F. begoniae	NRRL25300	U61543	AF160293	Scauflaire *et al.* 2011
F. brevicatenulatum	NRRL25446	U61623.1	AF160265	Scauflaire *et al.* 2011
F. bulbicola	NRRL 13618	U61546	AF160294	Scauflaire *et al.* 2011
F. circinatum	NRRL 25331	U61547	AF160295	Scauflaire *et al.* 2011
F. concentricum	NRRL25181	U61548	AF160282	Scauflaire *et al.* 2011
F. denticulatum	NRRL25302	U34453.1	AF160271	Scauflaire *et al.* 2011
F. dlaminii	NRRL13164	U34430	AF160277	Scauflaire *et al.* 2011
F. ficicrescens	CBS125177	KP662895	KP662898	This study
F. ficicrescens	CBS125178	KP662896	KP662899	This study
F. ficicrescens	CBS125181	KP662897	KP662900	This study
F. fractiflexum	NRRL28852	AF160315	AF160288	Scauflaire *et al.* 2011
F. fujikuroi	NRRL13566	U34415	AF160279	Scauflaire *et al.* 2011
F. globosum	NRRL26131	U61557	AF160285	Scauflaire *et al.* 2011
F. guttiforme	NRRL22945	U34420	AF160297	Scauflaire *et al.* 2011
F. inflexum	NRRL20433	U334435	AF8479	Scauflaire *et al.* 2011
F. konzum	MRC 8544	EU220234	EU220235	Scauflaire *et al.* 2011
F. lactis	NRRL25200	U61629	AF160272	Scauflaire *et al.* 2011
F. mangiferae	NRRL25226	U61561	AF160281	Scauflaire *et al.* 2011
F. musae	NRRL28893	FN545374	FN552092	Van Hove *et al.* 2011
F. napiforme	NRRL 13604	U34428	AF160266	Scauflaire *et al.* 2011
F. nygamai	NRRL13448	U34426	AF160273	Scauflaire *et al.* 2011
F. oxysporum	NRRL 22902	U34424	AF160312	Scauflaire *et al.* 2011
F. phyllophilum	NRRL 13617	U34432	AF160274	Scauflaire *et al.* 2011
F. proliferatum	NRRL22944	U34416	AF160280	Scauflaire *et al.* 2011
F. pseudoanthophilum	NRRL2520	U61631	AF160264	Scauflaire *et al.* 2011
F. pseudocircinatum	NRRL22946	U34453	AF160271	Scauflaire *et al.* 2011
F. pseudonygamai	NRRL13592	U34421	AF160263	Scauflaire *et al.* 2011
F. ramigenum	NRRL25208	U61632	AF160267	Scauflaire *et al.* 2011
F. sacchari	NRRL13999	U34414	AF160278	Scauflaire *et al.* 2011
F. subglutinans	NRRL 22016	U34417	AF160289	Scauflaire *et al.* 2011
F. succisae	NRRL 13613	U34419	AF160291	Scauflaire *et al.* 2011
F. sterilihyphosum	CML 283	DQ445780	DQ452858	Scauflaire *et al.* 2011
F. temperatum	MUCL 52436	HM067692	HM067684	Scauflaire *et al.* 2011
F. thapsinum	NRRL22045	U34444	AF160270	Scauflaire *et al.* 2011
F. udum	NRRL22949	U34433	AF160275	Scauflaire *et al.* 2011
F. verticillioides	NRRL 22172	U34413	AF160262	Scauflaire *et al.* 2011

Amplified fragment length polymorphism genotyping

Twenty eight strains belonging to eight closely related species within FFSC and the novel *Fusarium* species were subjected to amplified fragment length polymorphism (AFLP) genotyping by using a previously described method (Lackner et al. 2014). However, for the amplification of the DNA fragments the selective residues of the HpyCH4IV and MseI primers were replaced (5'-Flu-GTAGACTGCGTACCCGTAC 3' and 5'-GATGAGTCCTGACTAATGAG-3', respectively). Amplicons were 20x diluted using ddH2O; 1 µL of the diluted amplicon was then added to a mixture of 8.9 µL ddH2O and 0.1 µL LIZ600 (Applied Biosystems) followed by a heating step for 1 min at 100 °C followed by cooling down to 4 °C. Fragment analysis was carried out using an ABI3730xL Genetic Analyser (Applied Biosystems) according to the manufacturer's instructions. Raw data were then inspected visually after importation into BioNumerics v6.6 (Applied Maths, St. Martens-Latem, Belgium) and analysed by UPGMA clustering using the Pearson correlation coefficient.

Antifungal susceptibility

Antifungal susceptibility testing (AFST) of CBS 125177, CBS 125178, and CBS 125181 was performed by the CLSI broth microdilution as described in the CLSI document M38-A2 (Clinical and Laboratory Standards Institute 2008) as described by (Al-Hatmi et al. 2015a). The following drugs were used, amphotericin B (Sigma-Aldrich), fluconazole (Pfizer, Groton, CT, U.S.A.), itraconazole (Janssen Pharmaceutica, Tilburg, The Netherlands), voriconazole (Pfizer), posaconazole (Merck), isavuconazole (Basilea Pharmaceutica, Basel, Switzerland), micafungin (Astellas, Ibaraki, Japan), and natamycin (DSM, Delft, The Netherlands). Three reference strains (*Paecilomyces variotii* ATCC 22319, *Candida krusei* ATCC 6258, and *Candida parapsilosis* ATCC 22019) were included as quality controls.

Results

Phylogenetic inference

BLAST results of the *TEF1* sequence in GenBank revealed that CBS 125178 matched *F. lactis* with 97 % similarity. Further comparison using the FUSARIUM ID database (http://isolate.fusariumdb.orgl/) (Geiser et al. 2004) revealed *Gibberella fujikuroi* species complex (GFSC) with 96.7 % and in the *Fusarium* MLST database (http://www.cbs.knaw.nl/fusarium) (O'Donnell et al. 2010) *Fusarium* sp. (*G. fujikuroi* species complex). The different indications of species complexes are due to the use of either the name of the sexual of the asexual morph, respectively; at present the name *Fusarium* is preferred over *Gibberella* and hence the same species complex is now known as FFSC. The best hits for *BT2* yielded *Fusarium* sp. and the best hit for *RPB2* matched *F. lactis* with 97 % similarity. For further understanding of relations between species, a phylogenetic tree was constructed for each locus separately, i.e. *TEF1*,

BT2, and *RPB2*. Sequences were retrieved for *Fusarium* species within the *F. fujikuroi* species complex from GenBank (Table 2).

PCR amplification of the studied isolates was positive for all five markers analysed. Sequences of the *TEF1* region of isolates were compared with all known *Fusarium* species belonging to the *F. fujikuroi* species complex and validated by inclusion of ex-type materials. *TEF1* partial sequences proved to be highly variable for clinical and environmental *Fusarium* species. The alignment including the outgroup sequence had a length of 570 nucleotides of which 372 were conserved, 186 variable ones and 94 were parsimony-informative. Based on these *TEF1* partial sequences, a Bayesian phylogenetic tree was constructed in which all presently recognized species could be recognized with 60-100 % support in their posterior probability. *TEF1* phylogenetic analyses showed that strains CBS 125177, CBS 125178, and CBS 125181 were nested within the *F. fujikuroi* complex, close to *F. lactis*, *F. ramigenum*, and *F. napiforme* but did not match with *F. andiyazi*. All species formed well-supported monophyletic branches suggesting that further phylogeny is necessary for species delimitation for the above-mentioned isolates.

Based on the given topology in Fig 1, a clade of the novel species could be discerned and the partial β-tubulin gene was chosen in addition to *TEF1* to resolve species-level, because of the availability of *BT2* sequences in GenBank for clinical and environmental *Fusarium* species and also because *BT2* is one of the recommended secondary barcodes in the genus *Fusarium* (O'Donnell et al. 2012). The *BT2* dataset comprising 39 sequences consisted of 36 taxa with 474 characters, from which 186 were variable, 54 parsimony-informative and 52 were singletons. The tree inferred from *BT2* alignment showed that our three isolates CBS 125177, CBS 125178, and CBS 125181 are members of *F. fujikuroi* complex and form a monophyletic clade (Fig 2). Most of the basal branches were supported by Bayesian posterior probabilities (PP) and bootstrap, also some deeper internal branches of the CBS 125177, CBS 125178, and CBS 125181 received strong support (100%). The novel species originating from fig fruits was phylogenetically distinct from *F. andiyazi*, and a sister taxon of *F. lactis*, *F. ramigenum*, and *F. napiforme*. The latter is a causative agent of systemic fusariosis and is multidrug resistant.

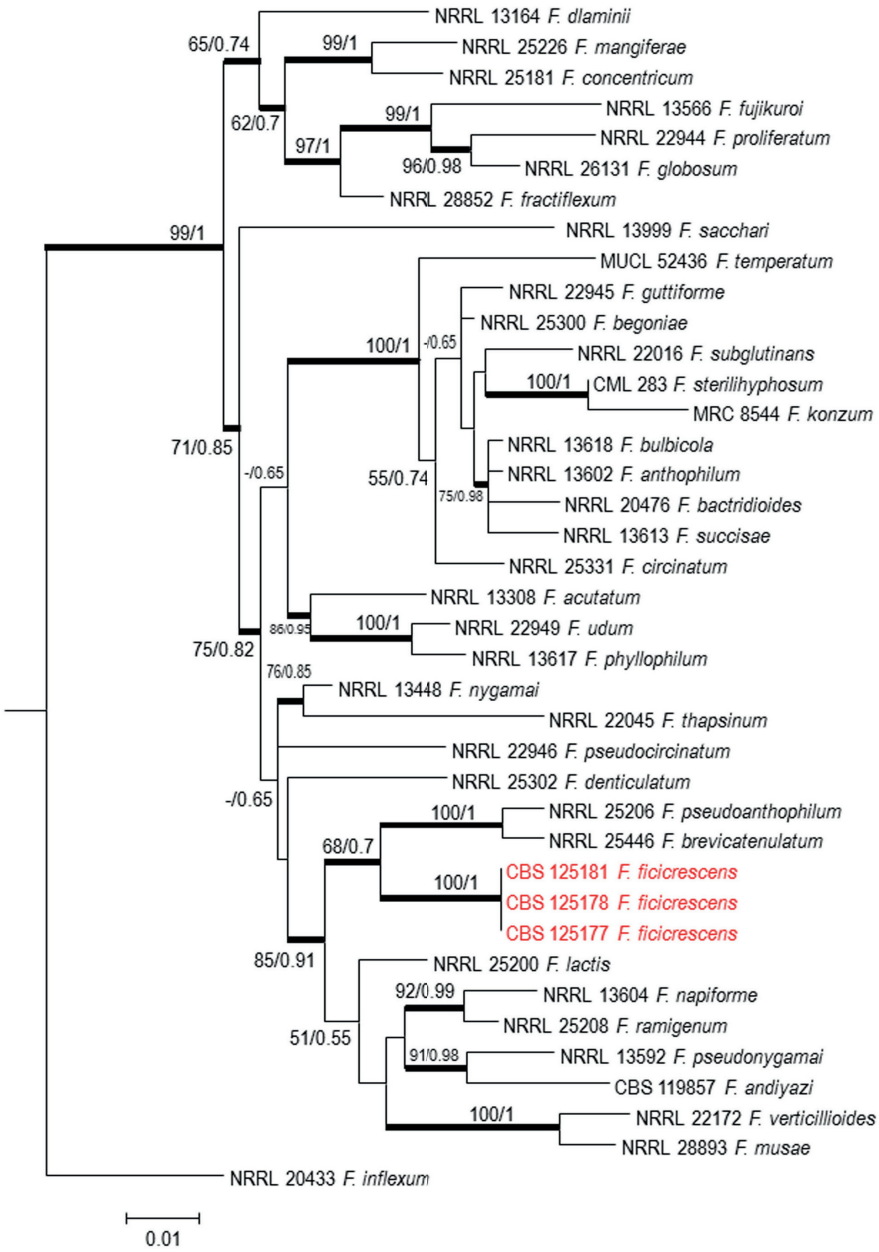

Fig 1. Phylogenetic tree generated by Bayesian inference (BI) and maximum likelihood (ML) trees from 39-*TEF1* sequences, 570 characters, 10 000 000 generations, 4 mcmc runs. Numbers on the branches are Bayesian posterior probabilities (PP), percentages of 1000 bootstrap-replications of MEGA6-maximum likelihood (PP/ML). The tree was rooted with the two strains *F. inflexum* NRRL 20433.

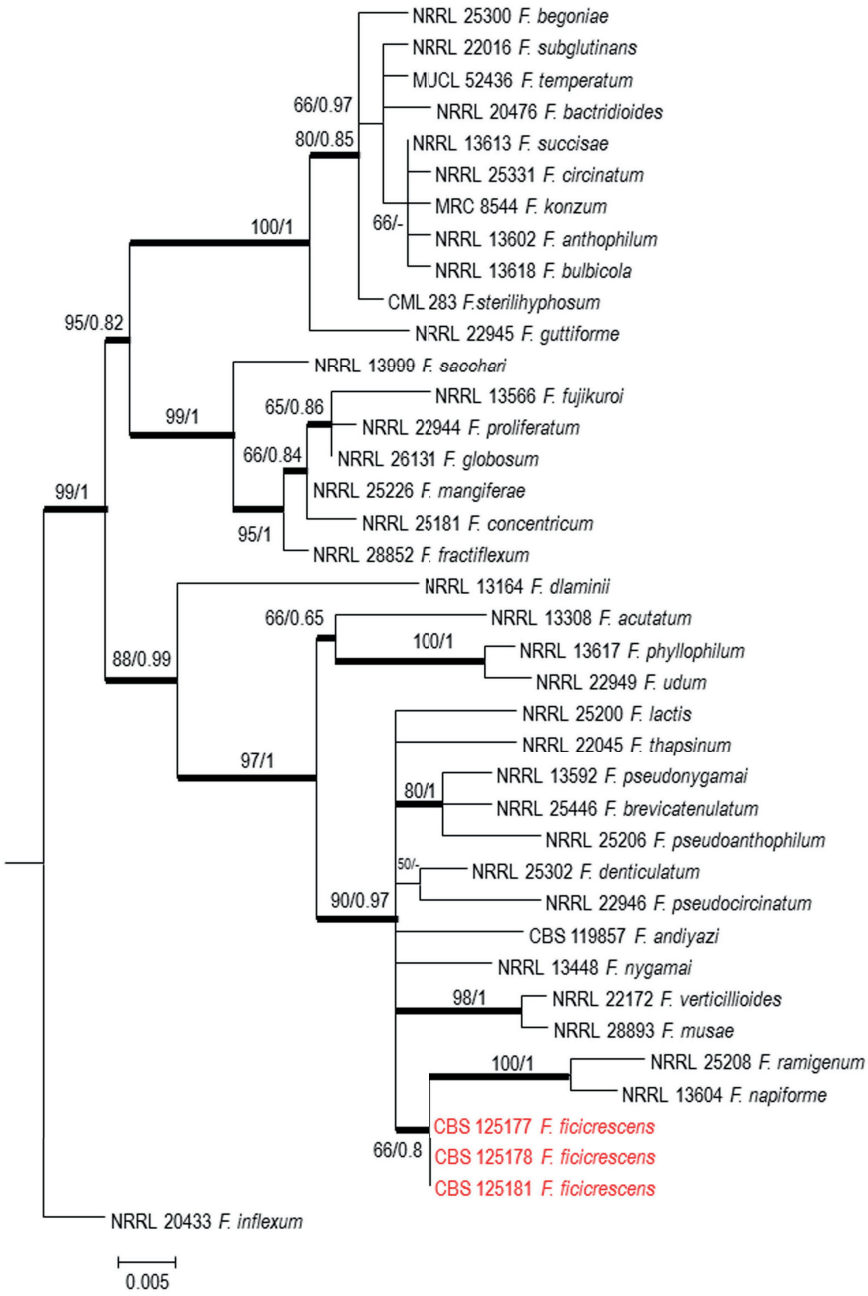

Fig 2. Phylogenetic tree generated by Bayesian inference (BI) and maximum likelihood (ML) trees from 39-*BT2* sequences, 474 characters, 10 000 000 generations, 4 mcmc runs. Numbers on the branches are Bayesian posterior probabilities (PP), percentages of 1000 bootstrap-replications of MEGA6-maximum likelihood (PP/ML). The tree was rooted with the two strains *F. inflexum* NRRL 20433.

Ribosomal polymerase B2 (*RPB2*) is one of the most informative gene fragments and resolves taxonomy at or near the species-level in *Fusarium*, but its drawback is that less sequences are available in GenBank. In our study, we were not able to cover all the taxa used in earlier analyses using *RPB2* sequences. The alignment of *RPB2* sequences had a length of 899 nucleotides when the outgroup was included; 721 nucleotides were conserved, while 98 were parsimony-informative. A Bayesian phylogenetic tree constructed with *RPB2* sequences of available strains appeared well-resolved without polytomies (Fig 3); a clade containing isolates CBS 125177, CBS 125178, and CBS 125181 was found within FFSC. All clades had a statistical support between 70 and 100 % and all species were well separated.

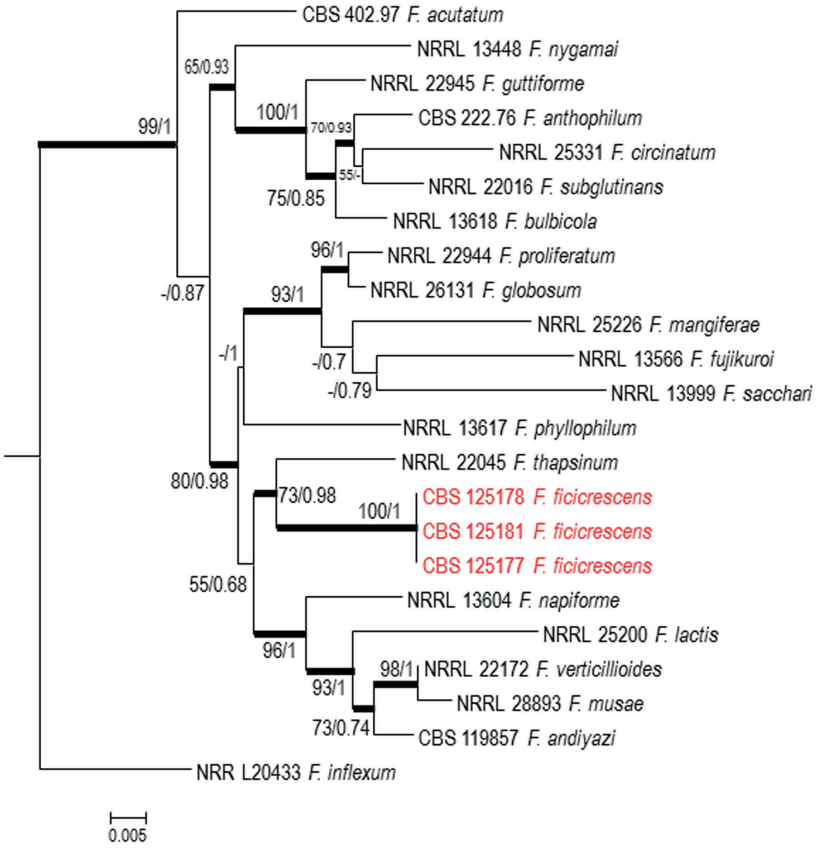

Fig 3. Phylogenetic tree generated by Bayesian inference (BI) and maximum likelihood (ML) trees from 23-*RPB2* sequences, 899 characters, 10 000 000 generations, 4 mcmc runs. Numbers on the branches are Bayesian posterior probabilities (PP), percentages of 1000 bootstrap-replications of MEGA6-maximum likelihood (PP/ML). The tree was rooted with the two strains *F. inflexum* NRRL 20433.

AFLP

The isolates CBS 125177, CBS 125178, and CBS 125181 formed a well-defined cluster in an UPGMA dendrogram based on AFLP data (Fig 4). The data indicate that the three isolates are separated from previously described species within the FFSC based on all molecular approaches used. AFLP analysis revealed clear polymorphisms both within and between species of the FFSC. The three strains under study that were grouped by sequence analysis as a novel species formed an AFLP cluster that was clearly differentiated from remaining species of the *F. fujikuroi* species complex. The three strains showed slight genetic variability: CBS 125177 and CBS 125178 were identical, while CBS 125181 showed a slightly different pattern.

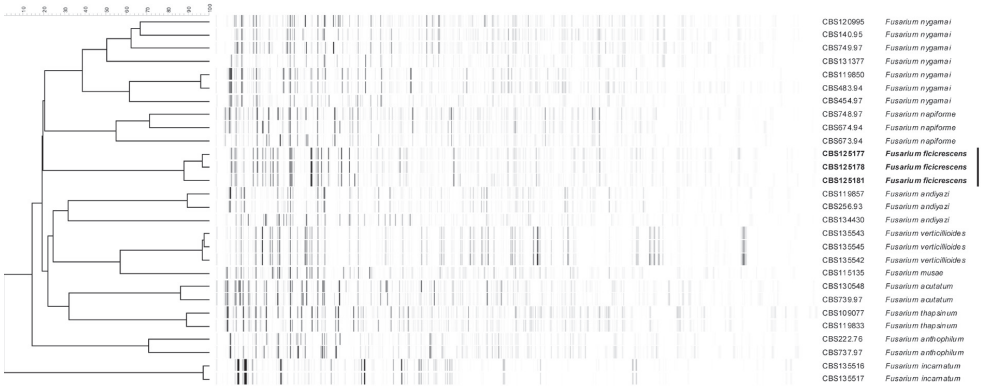

Fig 4. AFLP profile of *F. ficicrescens* (CBS 125177), (CBS 125178) and (CBS 125181) in relation to that other closely related species within *F. fujikuroi* species complex (including the type strains) and *F. incarnatum* (CBS 135516 and CBS 135517).

MALDI-TOF MS

The three isolates under study were identified by MALDI-TOF MS as '*Fusarium* species' by an in-house database for *Fusarium* species with confidence log scores 2.193, 2.20, and 2.226, respectively indicating correct generic and probable specific identification. The strains CBS 125177, CBS 125178, and CBS 125181 had initially been identified morphologically as *F. andiyazi*. Two separate clusters were visualized by using MALDI-TOF MS, one comprising three strains including the type strain of *F. andiyazi*, and another with strains CBS 125177, CBS 125178, and CBS 125181 (Fig 5). This indicates that MALDI-TOF MS has a potential to distinguish closely related and morphologically identical *Fusarium* species. Here, the MALDI-TOF MS data are used as an additional parameter in our polyphasic classification of this cryptic species.

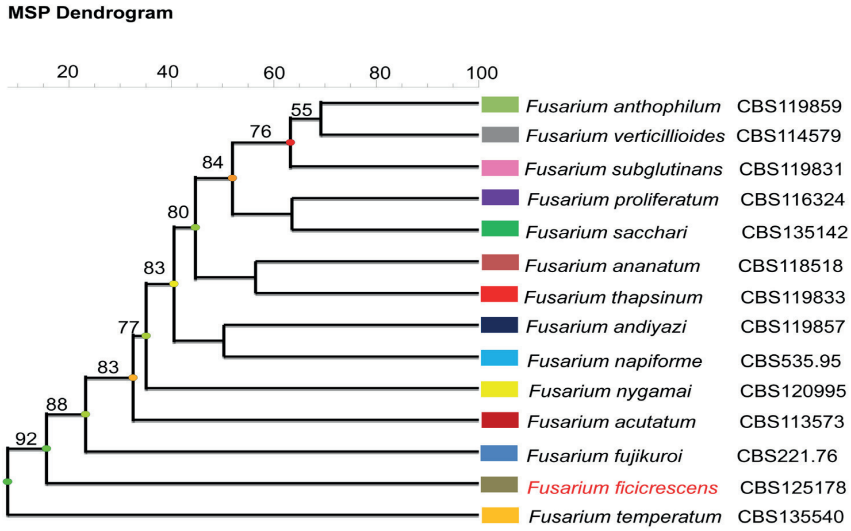

Fig 5. Dendrogram generated from hierarchical cluster analysis of MALDI-TOF MS spectra of *F. ficicrescens* and 13 isolates of other *F. fujikuroi* species complex species. Distances are displayed in relative units.

Mating experiments and mycotoxins

The *in vitro* mating tests crossing strains CBS 125177, CBS 125178, and CBS 125181 in all possible combinations remained without result; no sexual stage was generated under the used laboratory conditions. Physiological features included rapid growth and sporulation on MEA, OA, PDA, SNA, and CLA media from 24 to 37 °C, and inability to grow at 40 °C (Fig 6). Plates with individual strains all eventually produced ascocarp-like structures (Fig 7 D-F). Analysis of the presence of mating type genes using primers for the MAT1-1 and MAT1-2 idiomorphs showed that all three strains contain both mating idiomorphs and therefore can be considered a homothallic species in an otherwise mostly heterothallic species complex. None of the isolates were found to produce fumonisins B1 (FUM), common to other species in the FFSC.

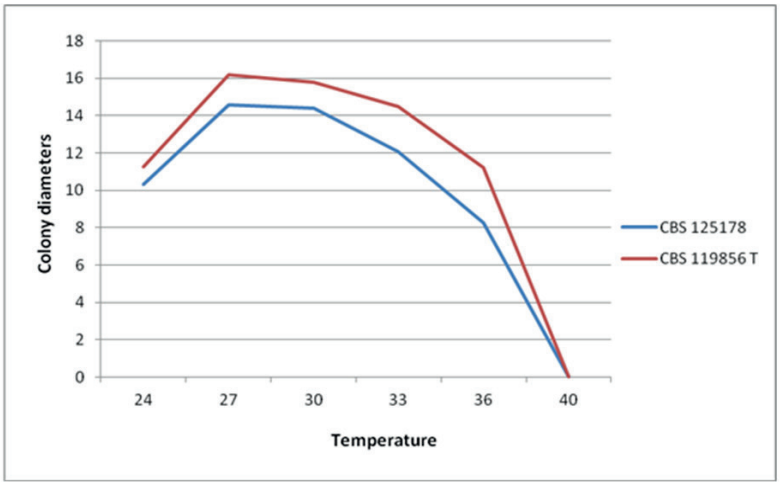

Fig 6. Colony diameters (mm) at various temperatures ranging from 24 °C to 40 °C, measured after one week of incubation on 2 %.

Antifungal susceptibility

Table 3 summarizes the MIC values of eight antifungal drugs against strains CBS 125177, CBS 125178, and CBS 125181. There was a uniform pattern of low MICs of amphotericin B, voriconazole, and posaconazole. The highest MICs were for fluconazole followed by itraconazole and micafungin. Natamycin and isavuconazole showed moderate activity against these isolates.

Table 3. MIC values of the isolates CBS 125177, CBS 125178 and CBS 125181

	MIC values (µg/ml)							
No/Drug	**AMB**	**FLC**	**ITC**	**VOR**	**POS**	**NATA**	**ISA**	**MICA**
CBS 125177	0.5	>64	>16	1	0.25	4	4	>8
CBS 125178	1	>64	>16	0.5	0.5	4	4	>8
CBS 125181	0.5	>64	>16	1	0.25	4	8	>8

AMB: Amphotericin B, FLC: Fluconazole, ITC: Itraconazole, VOR: Voriconazole, POS: Posaconazole, ISA: Isavuconazole, NATA: Natamycin, MICA: Micafungin

Taxonomy

Fusarium ficicrescens A. M. S. Al-Hatmi, M. Mirabolfathy, B. Stielow & G.S. de Hoog, sp. nov. (Fig 7), MB811380.

Etymology: 'ficicrescens' refers to the fungus growing on figs, which may be colonization or pathogenicity. The only known host plant for this species is *Ficus carica*.

Holotype: CBS 125178, living and dried, herbarium specimen CBS H-21815, isolated from fig fruits, Estahban, Iran, 2009.

Description based on CBS 125178 on PDA, SNA, and CLA growing in the dark at 25 ^{o}C after 7 d. Colonies growing rapidly, attaining 45=58 mm (mean = 52) diam. Aerial mycelium cottony, initially pinkish white and later becoming light purple in the centre. Reverse slightly darker purple (Fig 7B and C). Sterile fruiting bodies produced on SNA with filter paper (Fig 7D-F). Sporodochia pale orange, formed within 10 d on carnation leaf pieces on CLA. Conidiophores consisting of monophialides up to 11 x 1.8 µm wide; longer and more slender monophialides up to 28 x 1.5 µm occasionally present (Fig 7R). Polyphialides up to 22 x 3.2 µm (Fig 7Q). Mesoconidia spindle-shaped lacking foot-cell, 0-1- septate 15.9-21.2 (mean = 17.6) µm x 1.9-2.4 (mean = 2 µm) (Fig 7H and I). Microconidia abundant, ovoidal, 4.6-6.5 (mean =5.6) µm x 1.5-2.8 (mean =1.8) µm (Fig 7G), borne in long chains (>10 microconidia) (Fig 7N) or in false heads clumping at the end of the phialides (Fig 7O). Coiled hyphae usually present (Fig 7P). Chlamydospores produced after 4 weeks on SNA, single, or with several in chains, consisting of enlarged, thick-walled vegetative cells within hyphae, 9-12 µm diam (Fig 6K-M). Sclerotia absent.

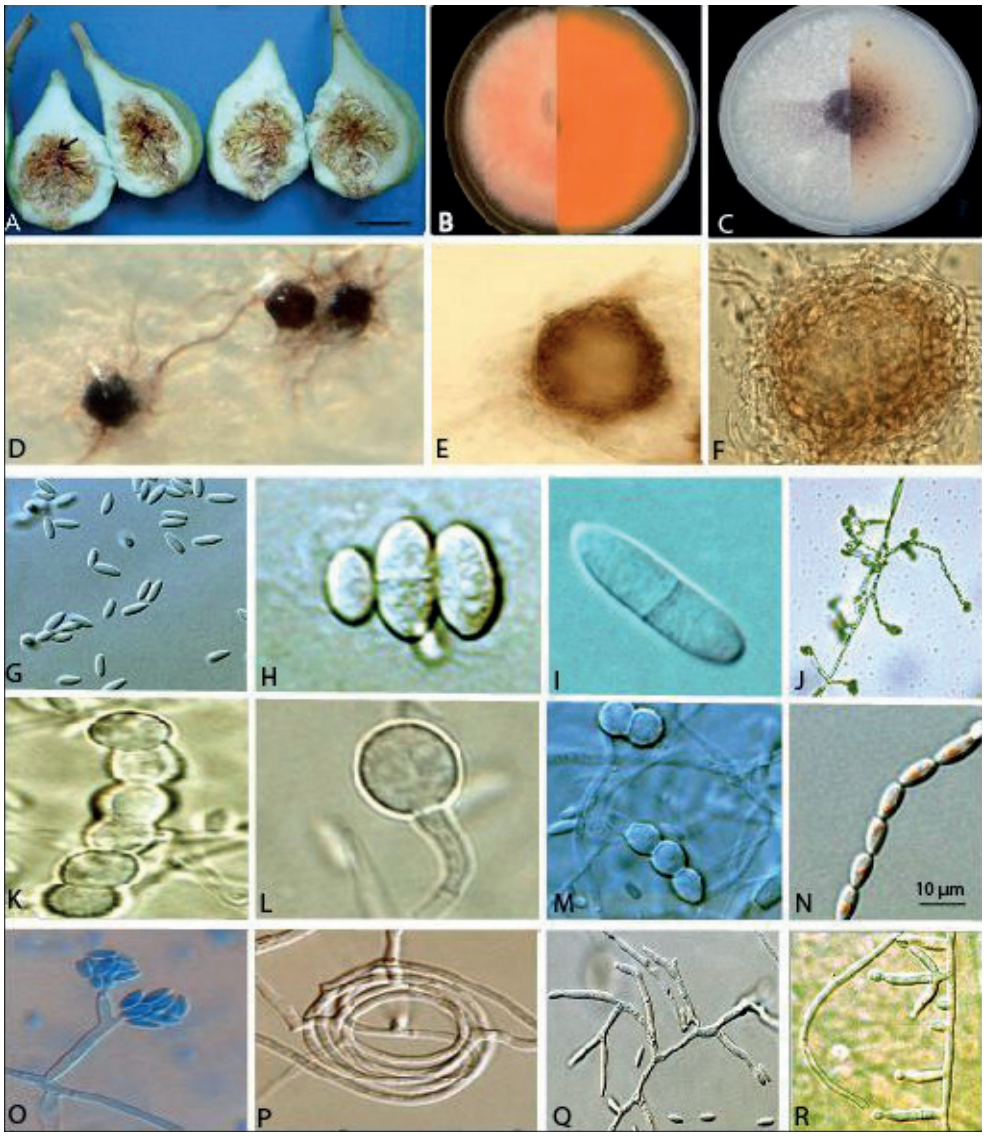

Fig 7. Morphology of *F. ficicrescens* CBS 125178. (A) Infected figs. (B) Growth of the isolate *F. ficicrescens* on MEA agar, front pinkish white, reverse orange. (C) Growth of the isolate *F. ficicrescens* on PDA agar, front pinkish white and the reverse is light purple after 14 d. (D-F) Fruiting bodies on synthetic nutrient agar (SNA) with filter paper. (G) Microconidia. (H-I) Mesoconidia from 0 to 1 septata. (J) Microconidia in situ on CLA. (K) Chain of verrucose chlamydospore of *F. ficicrescens*. (L) Single, verrucose chlamydospore on the tip of hyphae. (M) Two to three chlamydospores in the middle of the hyphae. (N) Microconidia in chains. (O) Small false head with few microconidia. (P) Coiling hyphae. (Q) Branching polyphialides. (R) Short monophialides and polyphialides. Scale bar 10 µm.

Discussion

Many taxonomic changes have taken place in *Fusarium* especially in the *F. fujikuroi* species complex (Nirenberg & O'Donnell 1998; O'Donnell et al. 2000). Recognition of species in *Fusarium* is sometimes problematic due to the limited number of morphological characteristics for distinction (Marasas et al. 2001). Our phylogenetic analysis based on a three gene dataset (*TEF1*, *BT2*, and *RPB2*) shows that our isolates CBS 125177, CBS 125178, and CBS 125181 belong to *F. fujikuroi* species complex and that no name for this group is available. The new species *F. ficicrescens* is morphologically similar to other *Fusarium* species of FFSC that occur on human patients (de Hoog et al. 2011). FFSC is one of the most important groups of agents causing superficial and disseminated infections, the latter occurring in immunocompetent individuals. *F. ficicrescens* has phenotypic characters prevalent in FFSC, particularly in the presence of monophialides and polyphialides and the production of ovoidal microconidia. Presence of chlamydospores and microconidia in chains was also described by Phan et al. (2004) in *F. gaditjirrii*, a further member of FFSC. *F. ficicrescens* has mesoconidia in addition to chlamydospores, as well as microconidia and coiled hyphae. Another member of the FFSC, *F. subglutinans*, has mesoconidia, as in *F. ficicrescens*. *Fusarium andyazi* usually has pseudochlamydospores as swellings in the hyphae, while real chlamydospores are absent. Consequently, *F. ficicrescens* is a molecular sibling that differs by AFLP, MLST, and MALDI-TOFMS, but phenotypically may not be reliably differentiated from other *Fusarium* species. For diagnostics, mesoconidia, and coiled hypha are recommended. Although these particular characters are present in different species within the genus *Fusarium* it could be a diagnostic character in this complex to reduce the number of options.

Leslie (1995) reported that some *Fusarium* species within FFSC have an unknown sexual stage and suggested that additional biological species remained to be found which might be associated with such taxonomic entities. In *F. ficicrescens* mating experiments were performed by crossing the available isolates, but no perithecia were obtained. However, after prolonged incubation, ascocarp-like structures were observed in two out of three *F. ficicrescens* strains. No ascospores could be observed, but PCR analysis with primer pairs specific for the two mating type idiomorphs revealed presence of both mating types in all three strains analysed, suggesting homothallic mating.

Delimitation of the new species *F. ficicrescens* is based on a multigene approach to recognize species boundaries and also others molecular approaches including MALDI-TOF MS and AFLP. MLST of five gene regions (ITS, LSU, *BT2*, *TEF1*, and *RPB2*) were evaluated to recognize *F. ficicrescens*. *TEF1*, *BT2*, and *RPB2* genes showed strict concordance, indicating holds true for many *Fusarium* species (Geiser et al. 2004). Using partial coding genes *BT2*, *TEF1*, and *RPB2*, *F. ficicrescens* was easily distinguished from all described *Fusarium* species, clustering in a well-supported clade (PP = 1; bootstrap [B] = 100) close to *F. lactis*, *F. ramigenum*,

and *F. napiforme* (Figs 1-3). The *F. ficicrescens* clade is present in three of the five single-locus phylogeny analyses (data not shown). Thus our multilocus results strongly support the recognition of the *F. ficicrescens* isolates as a novel species within the FFSC; the differences observed for all phylogenetic markers are considered sufficient to propose *F. ficicrescens* as a new species in *fujikuroi* complex.

Supporting data were acquired from AFLP fingerprinting and MALDI-TOF MS. In the AFLP analysis the *F. ficicrescens* strains clustered separately with intra-group similarities of >90 % and having less than 10 % in common with eight closely related neighbouring species (Fig 4). MALDI-TOF MS has successfully been used for rapid and reliable identification of *Fusarium* species (Marinach-Patrice et al. 2009; Triest et al. 2015a; Al-Hatmi et al. 2015b). Data generated in the present study using the MALDI Biotyper v3.1 software package with our in-house database for the *F. fujikuroi* species complex (data not shown), demonstrated that strains CBS 125177, CBS 125178, and CBS 125181 matched with each other with log score values between 2.2 and 2.74, indicating that the strains are conspecific. When comparing the dendrogram representing proteomic differences to the phylogenetic tree based on *TEF1*, *RPB2*, and *BT2* gene analysis of *F. ficicrescens*, high concordance was found at the species level (Fig 5).

The phylogenetic position of the new species *F. ficicrescens* within the complex *fujikuroi* is shown in Fig 1. To date, this complex contains fifteen agents causing human fusariosis, namely *F. acutatum*, *F. ananatum*, *F. andiyazi*, *F. anthophilum*, *F. fujikuroi*, *F. guttiforme*, *F. musae*, *F. napiforme*, *F. nygamai*, *F. proliferatum*, *F. verticillioides*, *F. sacchari*, *F. thapsinum*, *F. subglutinans*, and *F. temperatum* (de Hoog et al. 2011). In *BT2*, *TEF1*, and *RPB2*, voucher strains CBS 125177, CBS 125178, and CBS 125181 of *F. ficicrescens* cluster as a sister clade to the *F. napiforme*; however, human infections by *F. ficicrescens* have not been reported. *Fusarium ficicrescens* probably is not a pathogen on *Ficus carica*, but rather an osmotolerant colonizer which takes advantage of increasing sugar concentrations during the maturation process of edible figs. *Fusarium ficicrescens* did not produce fumonisin B1 (FUM) when grown on rice medium. None of the examined edible fig fruits yielding *F. ficicrescens* was contaminated with fumonisins.

The genus *Fusarium* is remarkable by its ability to behave as an opportunist on numerous plant species and also being able to cause diseases in animals including humans (Sharon & Shlezinger 2013). It is also one of the more intensively investigated groups of fungi which has led to the description of many new species from a diversity of environments and host substrates (Zhang et al. 2006). The relation between opportunism on plants and on humans has not been elucidated. Some *Fusarium* species are more prone to human infection, whereas others seem to be host-specific plant pathogens (Hyde et al. 2010). Al-Hatmi et al. (2015c) noted that with the fifteen human-opportunistic species in the FFSC, this complex harbours the largest mammal-

infective ability within *Fusarium*. Possibly the euryoecious nature of FFSC, i.e. the ability to live under a broad range of conditions (Cantrell et al. 2011) might explain human opportunism. This conception was challenged by Al-Hatmi et al. (2014) who reported the maize pathogen *F. temperatum* to cause human keratitis. The precise ecological niche of *F. ficicrescens* is still unknown. All isolates originated from Iranian fig fruits, having been recovered repeatedly from fig fruit samples of two non-edible cultivars of Capri fig (*F. carica* cv. Capri) from Estahban areas of Fars province. Repeated isolation from a single host plant thus does not necessarily mean host-specificity, but rather suggests epidemic elevation of temporarily successful clones. All strains of *F. ficicrescens* had an optimum growth around 27 °C, and were still able to grow at 37 °C, but not at 40 °C (Fig 6). They thus potentially should be able to cause superficial infections in humans, but occurrence on humans or animals has as yet not been observed.

Acknowledgment

The work of Abdullah Mohammed Said Al-Hatmi was financially supported by Ministry of Health, Oman (Formal Agreement no. 28/2014).

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CHAPTER 4

Chapter 4

Phylogenetic diversity of human pathogenic *Fusarium* and emergence of uncommon virulent species

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Journal of Infection 2015; 71: 658-666.

Abstract

Objectives: *Fusarium* species cause a broad spectrum of infections. However, little is known about the etiological agents to the species level. We identified *Fusarium* species isolated from clinical specimens including those of high risk patients to better understand the species involved in the pathogenesis. **Methods**: A set of 44 *Fusarium* isolates were identified by two-locus sequence typing using partial sequences of the second largest subunit of RNA polymerase (*RPB2*) and translation elongation factor 1 alpha (*TEF1*). **Results**: The identified species belonged to four species complexes (SC); the most common SC was *Fusarium solani* (FSSC) (75%), followed by *Fusarium oxysporum* (FOSC) (4.5%), *Fusarium fujikuroi* (FFSC) (13.6%), and *Fusarium dimerum* (FDSC) (6.8%). Sites of infections were nails (n = 19, 43.2%), skin (n =7, 15.9%), cornea (n= 6, 13.6%), blood (n = 3, 9%), wound (n = 4, 6.8%), burn (n = 2, 4.5%), tissue (n = 2, 4.5%), and urine (n = 1, 2.27%). *Fusarium acutatum* was rare and seem restricted to the Middle East. Comorbidities associated with invasive infections were hematological malignancy and autoimmune disorders. **Conclusions**: Members of the FSSC predominantly caused cornea, nail and bloodstream infections. Less frequently encountered were the FOSC, FFSC and FDSC. More accurate molecular identification of *Fusarium* species is important to predict therapeutic outcome and the emergence of these species.

Key words: *Fusarium*, Emerging fungal infections, Two-locus sequence typing, Local infections, Invasive infections

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Introduction

Fusarium is a hyaline hyphomycete fungus that is commonly found in the environment, where it is isolated from soil, plants and water systems.[1-3] Some *Fusarium* species cause a broad spectrum of opportunistic infections in human, while invasive and disseminated infections occur predominantly in severely immunocompromised patients[3,4] and generally manifest as fever not responding to antimicrobial therapy.[5] Risk factors for the development of invasive fusariosis are neutropenia, hematologic malignancies, hematopoietic cell transplantation, and impaired cellular immunity.[4-7] More recently an invasive infection was also reported to occur in immunocompetent patients.[8] Other infections, such as keratitis or endophtalmitis and onychomycosis, are frequently manifested in immunocompetent persons and are often associated with previous trauma.[9-13] *Fusarium* species may occasionally cause infections in animals.[14]

Fusariosis is the leading mycosis affecting immunocompromised patients,[3] and represents the second most common cause of filamentous fungi infections after aspergillosis.[15,16] Nucci et al.[17] recently reported on 233 cases of invasive fusariosis from centers in 11 different countries. Another study reported 97 global cases, which were published in the medical literature between January 2000 and January 2010, and updated with an additional 26 cases reported from USA.[4] Invasive infections with *Fusarium* species are characterized with poor prognosis due to neutropenia of the infected patients[18] and the high resistance to antifungal agents and therapy.[19-22]

Often clinically important, morphologically similar *Fusarium* species are identified to species complex level only, and further genotypic characterization to species level is usually not done routinely in clinical laboratories.In the present study, we describe the molecular characterization of *Fusarium* species isolated from 43 patients with invasive and cutaneous infections, and the clinical manifestation of the disease in patients infected with fusariosis observed over 10 years at Hamad Medical Corporation (HMC), Qatar.

Materials and methods

Patients and specimens

A set of 44 *Fusarium* strains were isolated from 43 (38 immunocompetent and 5 immunocompromised) patients that were seen at Hamad Hospital, Doha, Qatar from July 2003 to June 2014. The demographic data, clinical specimens, and fungal etiology are reported in Table 1. The isolates were obtained from a variety of clinical specimens. Nineteen strains were isolated from nails, 7 from skin, 6 from cornea, 3 from blood, 4 from wounds, 2 from burn wounds, and 2 from skin tissue, and 1 from urine. One isolate was obtained from each patient except for two isolates that were taken from blood and skin respectively, from the same patient. The HMC research and ethics committee approved this study (number RC/104044/2015).

Isolation and morphological identification

Fusarium species were isolated and identified by morphology according to standard laboratory procedures. The clinical specimens were generally cultured on either Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, MI) plus chloramphenicol (SDA), SDA without antibiotics, or brain heart infusion plus 40 U/ml streptomycin and 20 U/ml penicillin. Blood cultures were performed using the Bactec automated culturing system (BD Diagnostic Systems). For culturing from urine, cysteine lactose electrolytedeficient agar (CLED) (Mast Diagnostics, UK) was used for the isolation of the organism.

Fusarium species isolated from nails were considered proven infections after two consecutive isolations from the same patient, direct microscopy showing compatible fungal cells, and the absence of dermatophytes in culture, according to the diagnostic criteria of Gupta et al.[23] Organisms isolated from specimens that did not meet such criteria were excluded from the study. Culture plates were incubated at 26°C and 37°C and were observed daily for growth up to 10 days.

The isolates were sent to CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands for molecular identification, where they were subcultured on oatmeal agar (OA; home-made at CBS) and incubated for 5 days at 28°C. Strains were deposited under accession numbers in the reference collection of the CBS-KNAW Fungal Biodiversity Centre (Table 1).

DNA extraction

Hyphae were harvested in 2 ml screw capped tubes with sterile sand, 750 µl of Lysis buffer and 750 µl of phenolchloroform (1:1; pH 8.0) were added and the mixtures were bead beaten at 2500 rpm for 3 min. The crude extract was spun at 17,000 g for 15 min at 4°C; about 700 µl of the supernatant was transferred to a fresh 1.5 ml tube. An equal amount of ice-cold 96% ethanol and 100 µl of 3.0 M ice-cold sodium acetate were added and mixed gently, then stored at -20°C for 30-60 min. The mixture was then spun down by centrifugation at 17,000 g for 10 minutes at 4°C. Supernatant was poured off in one flowing move, and the remaining ethanol was removed by pipetting. The DNA pellet was air-dried; and re-suspended in 100 µl of TE buffer. The solution was incubated successively at 37°C and 65°C both for 10 min, and stored at -20°C. The DNA quality was checked by 1.5% agarose gel electrophoresis.

Table 1. Clinical *Fusarium* (n=44) isolates, accession numbers and patients demographic data

Species	No[1]	Source	Year	Origin	Age/ Sex	CBS No	GenBank Numbers	
							RPB2	*TEF1*
FSSC [2]								
F. keratoplasticum (n=14)	1457	Skin	2013	Qatar	73/M	138573	KR673974	KR673951
	2630	Skin	2014	Yemen	29/M	139331	KR674007	KR673952
	1493	Nail	2013	Qatar	73/M	137549	KR673973	KR673950
	1465	Nail	2013	UK	48/F	137548	KR673980	KR673949
	1321	Nail	2013	Sri lanka	41/M	137547	KR673975	KR673948
	1139	Nail	2013	India	46/M	137640	KR673981	KR673947
	0601	Skin	2008	Sudan	53/M	137637	KR673979	KR673946
	0345	Nail	2007	Nepal	25/M	137638	KR673971	KR673945
	0309	Nail	2006	Pakistan	29/M	137538	KR673977	KR673944
	0093	Nail	2007	Egypt	62/F	137760	KR673978	KR673943
	1092	Nail	2009	Egypt	57/F	138571	KR673976	KR673942
	6687	Skin	2011	India	58/M	137542	KR673972	KR673941
	0842	Skin	2012	UK	47/F	139328	KR674006	KR673953
	0066	Wound	2008	India	52/M	137543	KR673970	KR673940
F. falciforme (n= 9)	7423	Cornea	2013	Nepal	34/M	137644	KR673990	KR673933
	0796	Nail	2007	Qatar	45/F	138569	KR673988	KR673934
	1541	Tissue	2013	Pakistan	51/M	137550	KR673986	KR673931
	7661	Cornea	2011	Pakistan	42/M	139330	KR674008	KR673932
	4467	Cornea	2013	Pakistan	40/M	137642	KR673987	KR673930
	4263	Cornea	2007	India	51/M	138357	KR673984	KR673935
	8098	Cornea	2012	India	35/M	138159	KR673983	KR673936
	0666	Skin	2013	India	69/F	137761	KR673985	KR673937
	0700	Nail	2007	India	57/M	137641	KR673989	KR673938
F. solani sensu stricto (n=3)	1068	Nail	2010	Sudan	58/F	137633	KR673994	KR673957
	0852	Cornea	2007	Bangladesh	48/M	139947	KR673992	KR673956
	0142	Nail	2007	Qatar	52/M	137539	KR673993	KR673955
F. lichenicola (n=2)	2532	Wound	2011	India	43/M	138750	KR674003	KR673967
	0678	Nail	2013	India	33/F	137551	KR674004	KR673966
F. petroliphilum (n=3)	9512	Blood	2003	Bahrain	80/F	137639	KR673996	KR673959
	4714	Blood	2011	Syria	37/M	137762	KR673998	KR673960
	0716	Nail	2012	Jordan	46/F	139333	KR674005	KR673962
F. solani sensu lato (n=2)	0158	Skin	2010	Sudan	24/F	137541	KR674001	KR673964
	3523	Blood	2010	Sudan	24/F	137540	KR674000	KR673965
FOSC [3]								
F. oxysporum (n=2)	1223	Wound	2014	Qatar	65/M	137650	KR674018	KR673928
	0435	Nail	2007	USA	47/M	137635	KR674017	KR673927
FFSC [4]								
F. proliferatum (n=2)	4792	Wound	2012	Pakistan	2/F	139334	KR674019	KR673925
	1592	Tissue	2005	Pakistan	18/M	137537	KR674015	KR673924
F. acutatum (n=4)	0896	Nail	2008	Qatar	45/F	137545	KR674013	KR673921
	0159	Nail	2007	Pakistan	62/M	137634	KR674010	KR673919
	0277	Nail	2014	India	58/F	137649	KR674011	KR673920
	1447	Nail	2013	India	33/F	138572	KR674012	KR673922
FDSC [5]								
F. dimerum (n=1)	3436	Urine	2012	Palestine	75/M	139948	KR674021	KR673913
F. delphinoides (n=1)	4158	Burn	2008	Sri lanka	24/M	137544	KR674022	KR673914
F. cf. dimerum (n=1)	0286	Burn	2012	Syria	16/F	139329	KR674025	KR673917

[1]Qatar Lab Reference number, [2]*F. solani*, [3]*F. oxysporum*, [4]*F. fujikuroi* and [5]*F. dimerum* species complexes.

DNA amplification and sequencing

Two-loci, namely the second largest subunit of the RNA polymerase gene (*RPB2*) and the translation elongation factor 1 alpha (*TEF1*) were amplified directly from the genomic DNA. For the partial sequence of *TEF1*, primers EF1 and EF2[24] were used with modification of the PCR program according to Davari et al.[25] For *RPB2* the primers used were 7cr and 5F2.[26] The PCR reaction mixture (12.5 µl final volume) contained 10X PCR buffer 1.25 µl, water 7.5 µl, dNTP mix (2.5 mM) 0.5 µl, 0.25 µl of each primer (10 pmol), Taq polymerase (5 U/ml) 0.05 ml, DMSO 0.7 µl, and template DNA (100 ng/µl) 1 ml. Amplification was performed in an ABI PRISM 2720 (Applied Biosystems, Foster City, U.S.A.) thermocycler. The PCR program used was as follows: Pre-denaturation for 3 min at 95 °C. Five cycles of 45 s at 95 °C, 45 s at 58 °C and 2 min at 72 °C, five cycles of 45 s at 95 °C, 45 s at 56 °C and 2 min at 72 °C, 30 cycles at 45 s at 95 °C, 45 s at 52 °C and 2 min at 72 °C and a post elongation step of 8 min at 72 °C. PCR products were visualized by electrophoresis on a 1% (w/v) agarose gel. Amplicons were purified using exoSAP. Both strands of the PCR fragments were sequenced with the above-mentioned primers.

The ABI PrismH Big Dye™ Terminator v. 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) was used for sequencing PCR. Sequences were determined with an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems). Sequencing PCR was performed as follows: 1 min at 95 °C, followed by 30 cycles consisting of 10 s at 95 °C, 5 s at 50 °C and 2 min 60 °C.Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems) with the ABI PRISM BigDye™ terminator cycle sequencing kit.

Phylogenetic analyses

A consensus sequence for each gene was computed for each clinical isolate from the forward and reverse sequences with SeqMan from the Laser Gene package (DNAstar, Madison, WI). Sequences of eleven strains (2 type and 9 reference strains) of *Fusarium* species were included and obtained from GenBank. The used sequences of the reference *Fusarium* species from this study are listed in Table 2. All sequences were aligned using MAFFT v. 7.127 (http://mafft.cbrc.jp), followed by manual adjustments with MEGA v. 6. A combined alignment was constructed for both *TEF1* and *RPB2* markers, representing 12 phylogenetically distinct *Fusarium* species. Both *RPB2* and *TEF1* partial genes were used for identification of the clinical isolates. In order to have a complete overview of molecular species occurring in this study, we used some reference sequences from the CBS database, as well as some sequences from GenBank (Table 2). All the clinical strains were analyzed by using standard primers, but sequencing efficiency proved to differ slightly between *Fusarium* species. *RPB2* and *TEF1* sequences could be aligned confidently over the entire genus *Fusarium*. The best-fit model of evolution was determined by

Model Test v. 0.1.1. A phylogenetic tree was constructed by using MrBayes version 3.2.2 and RAxML (7.6.6) available online. The tree was mid-rooted by using the outgroup method and edited in MEGA v. 6. The two-locus tree (Fig. 1) including all the isolates in this study was based on 56 strains including our 44 clinical isolates and 12 representative type and reference strains of different *Fusarium* species.

Nucleotide sequence accession numbers

The *TEF1* and *RPB2* nucleotide sequences for all the isolates were deposited in GenBank under the accession numbers listed in Table 1.

Table 2. Type and reference strains from CBS and GenBank accession numbers used in this study for phylogenetic analysis of *Fusarium* species.

Species	Collection	*RPB2*	*TEF1*
F. keratoplasticum	FCRCS2477	JN235897	JN235712
F. falciforme	NRRL 32542	EU329590	DQ247008
F. cf. solani	NRRL 31169	U34426	DQ246923
F. petrophilum	NRRL 54988	KC808350	KC808210
F. solani sensu stricto	NRRL 28679	EU329556	DQ246912
F. lichenicola	NRRL 28030	EF470146	DQ246877
F. delphinoides	NRRL 36160	HM347219	HM347134
F. dimerum	CBS108944	KR260882	KP985063
F. oxysporum	NRRL 62545	KC808368	KC808222
F. proliferatum	NRRL 22944	JX171617	AF160280
F. acutatum	CBS402.97	KT154005	KR071754

Results

Case series

Forty-four isolates of *Fusarium* species originating from 43 patients were obtained. Information pertaining to the source of isolation and the demographic data of the patients yielding these isolates is provided in Table 1. Nail and skin were the most common sites of infections (nails n=19, and skin n=7) and all these were proven cases. The diagnosis of *Fusarium* nail and skin infections was established with repetitive cultures growing *Fusarium* species and by direct microscopy. The third frequent class of infections were infections of the cornea (n = 6) and all these were considered probable infections. Also the infection classes, wounds (n = 4), burn wound (n = 2), tissue (n = 2) and urine (n = 1), were all probable infections. Invasive *Fusarium* infection was defined as infection in each case with at least one positive blood culture or the isolation of the same strain from two or more sites. The invasive infections from blood specimens

(n = 3) were all proven infections with isolation of the fungal etiology in culture in the context of a compatible infectious disease process in immunocompromised patients with underlying diseases, i.e. autoimmune disease, T cell lymphoma, and acute myeloid leukemia. All three patients had neutropenia. The first patient received fluconazole and died, the second was treated with a combination treatment of amphotericin B and voriconazole + granulocyte macrophage colony stimulating factor (GM-CSF) and died, and the third was treated with amphotericin B + GM-CSF and healed with the relief of neutropenia. Overall, 2 of 3 patients who received antifungal therapy died from the infection.

Sequences based on species determination were performed using two-loci for the 44 isolates. Twelve phylogenetic species were identified, belonging to four species complexes: *F. solani* (FSSC), *F. oxysporum* (FOSC), *F. fujikuroi* (FFSC) and *F. dimerum* (FDSC). The most common infecting species involved in the infections in this study was *F. keratoplasticum* (n = 14, FSSC, 31.8%) all originating from skin and nail infections, followed by *F. falciforme* (n = 9, FSSC, 20.5%) with a majority isolated from cornea, and *F. acutatum* (n = 4, FFSC, 9%) isolated from cutaneous specimens. Less common species were *Fusarium solani sensu stricto* (n = 3, FSSC, 6.8%), *F. petroliphilum* (n = 3,FSSC, 6.8%), *Fusarium cf. solani* (n = 2, FSSC, 4.5%), *F. lichenicola* (n = 2, FSSC, 4.5%), *F. proliferatum* (n = 2, FFSC, 4.5%), *F. oxysporum* (n = 2, FOSC, 4.5%), *F. dimerum* (n = 1, FDSC, 2.27%), *Fusarium cf. dimerum* (n = 1, FDSC, 2.27%), and *F. delphinoides* (n = 1, FDSC, 2.27%). *Fusarium delphinoides* and one *Fusarium cf. dimerum* were isolated from patients in the burn unit, while the other *F. dimerum* came from a urine specimen.

Comorbidities associated with invasive infections were hematological malignancy and autoimmune disorders. Blood infection was associated with FSSC mainly *F. petroliphilum* and *F. solani sensu lato*. We identified 43 patients with proven or probable fusariosis: there were 27 male patients and 16 female patients.

Phylogeny

Both genes possessed enough polymorphism, and therefore, were excellent markers with 99-100% accuracy for the identification of *Fusarium* species from GenBank, the FUSARIUM ID database database (http://isolate.fusariumdb.orgl/; and the *Fusarium* MLST database (http://www.cbs.knaw.nl/fusarium). Maximum likelihood (ML) analysis used 1000 bootstraps and the Bayesian inferences (BI) used a Metropolis-coupled Markov chain Monte Carlo (MCMC) analysis of four chains started from random tree topology and lasting 10^7 generation. Trees were saved each 1000 generation resulting in 2000 trees. The burn-in period was set at 25%. All trees were constructed by the outgroup method and edited in MEGA v. 6. Topology of trees obtained from these individuals was concordant. The loci *TEF1* and *RPB2* were therefore combined in order to investigate species delimitation using Phylogenetic Species Recognition (PSR). The

resulting data set included 56 taxa and 1419 characters. Fig. 1 presents the phylogenetic tree with Bayesian inference. The generated tree was separated into four clades. Clade 1 included all members of FSSC, clade 2 represented FOSC, clade 3 FFSC, and clade 4 FDSC.

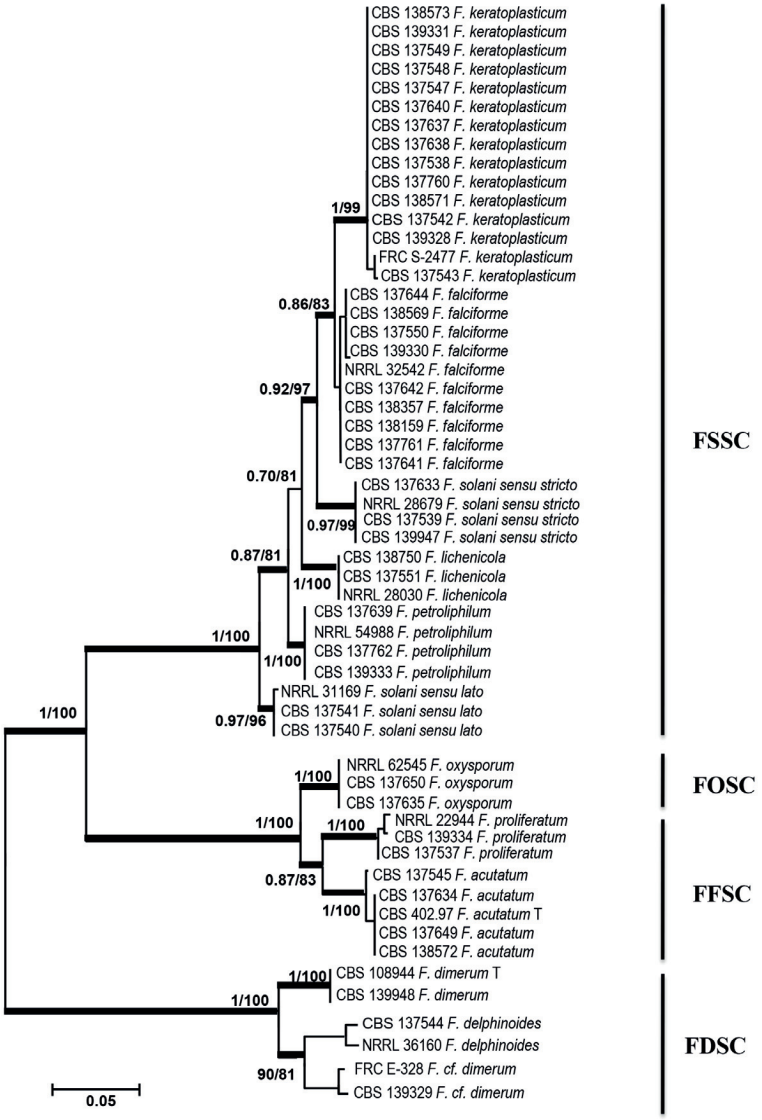

Fig 1. Phylogenetic tree resulting from Bayesian and RAxML analysis for the *TEF1* and *RPB2* genes (values of 0.7 for Bayesian inferences (BIs) and 80% for maximum likelihood (ML) bootstrap are shown. A tree of 56 *Fusarium* species including 12 type (T) and reference strains was inferred from a concatenated partitioned alignment of two gene fragments. Values at the nodes are concordance factors. The branches of the phyla represent at least 12 *Fusarium* species. The *F. dimerum* species complex was used as the outgroup.

Discussion

We presented the etiological agents of local and invasive *Fusarium* infections reported from 43 patients at Hamad Hospital, Qatar. Skin and nails were the predominant sites of infection, which coincides with other global studies on fusariosis reported in the literature.[4,19] Few discernible differences were observed in the microscopic morphologies of the strains. Conventional morphological identification of filamentous fungi to species level is a cumbersome and time-consuming method and the necessary expertise is only available in reference laboratories. In order to accomplish an easier and more accurate identification of clinical isolates molecular methods were developed for the identification of *Fusarium* species,[27] which may result in more adequate treatment of patients providing the availability of antifungal susceptibility profiles of the species, involved.

We performed *TEF1* and *RPB2* analysis and a BLAST search of *TEF1* and *RPB2* sequences against the FUSARIUM-ID[28] and GenBank databases (www.ncbi.nlm.nih.gov) was used as an initial step to identify isolates to species and/or species complex. Bayesian Inference (BI) and Maximum Likelihood (ML) analysis of 56 taxa (including outgroup) of *F. dimerum* species complex inferred from combined *TEF1* and *RPB2* (1419-bp alignment) sequences offered a better resolution and identified the strains of most of the four *Fusarium* species complexes to species level. The results of these two-loci turned out to be highly reproducible and effective for the identification of these isolates to FSSC, FOSC, FFSC and FDSC.

The most frequently identified species in our patients were members of the FSSC (75%), which proved to be more common in Qatar (49%) than reported in the literature.[4] Our results in the phylogenetic tree confirmed the separation of species within the FSSC that frequently cause human infections. [29] These species were *F. petroliphilum, F. keratoplasticum* and *F. falciforme*, but also *F. lichenicola, F. solani sensu stricto*, and one belong to FFSC as yet undescribed species was observed.[30]

Within the FSSC the two predominant clusters represented *F. keratoplasticum* and *F. falciforme*. The recently described *F. keratoplasticum*[30] was mainly involved in onychomycosis. These results are consistent with the findings of others[31] that members of FSSC appear to be a prevalent cause of onychomycosis and skin infections in Asia. Members of FSSC are widely distributed in the environment as important plant diseases and, also, as the main agent of fungal keratitis,[10] infections with severe neurological sequelae,[8] and usually fatal disseminated infections.[5,6,32] FSSC members are characterized by their resistance to most available antifungal agents.[4] Two members of the FSSC, *F. petroliphilum* and *F. solani sensu stricto* were the fusaria associated with bloodstream infections in this study.

It is not surprising that two other members of FSSC, *F. falciforme* and *Fusarium cf. solani* are the main fusaria responsible for cornea infections in this study. *F. falciforme* formerly known *as Acremonium falciforme* is a well known soil fungus in Asia,[33] and most of

our patients were immigrant workers from southeast Asia (India, Pakistan, and Nepal), who probably acquired the infection by inoculation of the fungus through cornea injury. Recently, *F. falciforme* was reported to cause vertebral osteomyelitis,[34] suggesting that this species is emerging as one of the most virulent fusaria. These results are consistent with other reports that FSSC are a predominant cause of ocular mycosis worldwide.[10,35,36]

The remaining members of the FSSC, *F. solani sensu stricto*, *F. lichenicola* and *Fusarium cf. solani* also formed a distinct group. *F. lichenicola* was firstly described by Summerbell and Schroers[37] and is considered an uncommon pathogen as the first human disseminated infection case was reported by Rodriguez-Villalobos in 2003,[38] and shortly thereafter it was reported to cause keratitis.[39] The phylogenetic tree also presented the monophyly of an undescribed species of *F. solani sensu stricto* with 3 strains, and *F. cf. solani* with 2 strains belonged to FSSC as yet undescribed species. The phylogenetic tree showed a well-supported relationship (100% bootstrap) between *F. keratoplasticum* and *F. falciforme*. Furthermore, the support value was above 80% between the other species within the complex. The present study confirms the high genetic diversity within the FSSC among clinical strains as described before in a number of studies.[30,40,41]

In addition to the FSSC strains included in our phylogenetic analysis, strains were found to be nested within known phylogenetic species in FOSC, FFSC and FDSC. O'Donnell and colleagues,[42] have studied the species delimitation intensively in FOSC. Both our FOSC isolates belong to the clade marked as sequence type [33], the most common clade containing human infecting strains.[42] A relatively low prevalence of *F. oxysporum* was detected in this study. The two cases were reported from infections of nail and wound, and this fungus was reported to be more common in Europe.[31] Thirteen species of the FFSC complex have been reported as opportunistic human pathogens[20] while we found only two in our study: This is the first study reporting four nail infections due to *F. acutatum*. Only one human infection has been attributed to this species rarely in a diabetic foot.[43] These results emphasize that *F. acutatum* is an emerging human pathogen that so far was only detected in Asia. The other FSSC species, *F. proliferatum* is one of the most common human opportunists of this species complex.

Three recognized species *F. dimerum*, *F. penzigii* and *F. delphinoides* and two unnamed lineages among 12 described species within FDSC were involved in human infection.[32] In the present study we reported one isolate of *F. dimerum* and *F. delphinoides* in addition to one undescribed species. Burn wound infections is a major health problem and globally the most devastating form of trauma.[44] Fungi contribute to 20-25% of these infections and are associated with high mortality.[45] In this study, two members of the FDSC, *F. delphinoides* and *Fusarium cf. dimerum* (Table 1) were involved in burn infections. Such infections probably develope due to antibacterial and burn wound care.[46,47] Therefore, we added burns as a significant risk factor for infection with *Fusarium*. *F. dimerum* is an uncommon agent of cutaneous and disseminated

infections in leukemia patients.[48,49]

The distribution of *Fusarium* species reported in the current analysis is different from that reported in the literature.[31,50,51] Invasive infection by *F. verticillioides* is predominant in Italy.[50] In our series the cases of invasive infections were caused by the predominance of FSSC members mainly *F. petroliphilum* and *F. solani sensu lato*. Our findings agree with the suggested variable local distributions of opportunistic *Fusarium* species.[31]

We concluded that phylogenetic diverse members of the FSSC, predominantly cause cornea, nail and bloodstream infections. Less frequently encountered are members of the FOSC, FFSC and FDSC. Molecular identification to identify a number of *Fusarium* species is important to predict therapeutic outcome and the emergence of these species.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgments

Supported by Grant NPRP 5-298-3-086 from the Qatar National Research Fund (a member of Qatar Foundation) to Saad J. Taj-Aldeen, Muna Almaslamani, Husam Salah and Teun Boekhout. The statements herein are solely the responsibility of the authors. Abdullah Mohammed Said Al- Hatmi was financially supported by Ministry of Health (28/2014), Muscat, Oman.

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Part II

Diagnosis, human infection and molecular epidemiology

- Rapid identification of clinical members of *Fusarium fujikuroi* complex using MALDITOF MS
- The concept of ecthyma gangrenosum illustrated by a *Fusarium oxysporum* infection in an immunocompetent individual
- *Fusarium ramigenum*, a novel human opportunist in a patient with common variable immunodeficiency and cellular immune defects: case report
- Keratitis by *Fusarium temperatum*, a novel opportunist
- *Fusarium* species causing eumycetoma: Report of two cases and comprehensive review of the literature
- Proximal subungual onychomycosis caused by *Fusarium falciforme* successfully cured with posaconazole
- Emergence of fusariosis in a university hospital in Turkey during a 20-year period
- Multidrug-resistant *Fusarium* keratitis: a clinico-mycological study of keratitis infections in Chennai, India
- Global molecular epidemiology and genetic diversity of *Fusarium*, a significant emerging group of human opportunists, 1958-2015

CHAPTER 5

Chapter 5

Rapid identification of clinical members of *Fusarium fujikuroi* complex using MALDITOF MS

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Future Microbiology 2015; 10: 1939–1952.

Abstract

Aim: To develop the matrix-assisted laser desorption ionization mass spectrometry (MALDITOF MS) method for identification of *Fusarium* species within *Fusarium fujikuroi* complex for use in clinical microbiology laboratories. **Materials & methods**: A total of 24 reference and 60 clinical and environmental isolates belonging to the *F. fujikuroi* complex were subjected to MALDI-TOF MS identification. Protein extracts of *Fusarium* isolates were obtained using formic acid extraction. Multilocus sequence analysis was used as a gold standard. **Results**: The MALDI-TOF MS Biotyper correctly identified 93.6% of the strains down to the species level, while the remaining isolates (6.4%) were identified at the genus level when using scores of ≥ 2.0 as cut-off values. Correct identification was obtained despite large intraspecific heterogeneities in MALDI-TOF spectra. **Conclusion**: MALDI-TOF MS is a rapid identification tool for the recognition of species within *F. fujikuroi* complex, provided a database is available.

Keywords: database, fingerprint, *Fusarium fujikuroi* complex, MALDI-TOF MS, multilocus, sequence analysis, spectral analysis

Introduction

Fusariosis is an emerging cause of mortality in severely immunocompromised patients worldwide. An important virulence factor in molds of the genus *Fusarium* is their ability to disseminate in the bloodstream [1]. Members of the *F. fujikuroi* complex are significantly opportunistic pathogens [2], and their identification is essential to guide therapy, detect treatment failures and predict patient outcomes. Thus, rapid diagnostic tools are of high importance to improve survival of susceptible patients [3]. Current identification of *Fusarium* species mostly involves conventional histopathology, culturing and microscopic techniques on a case-by-case basis [4]. Phenotypic analyses have been demonstrated to be inadequate for reliable identification of *Fusarium* at a species level. Consequently, the identification of 33–50% of *Fusarium* isolates is erroneous or missed [5]. Furthermore, histopathology and culturing methods are laborious, time consuming and require considerable experience [6].

As an alternative to morphological identification, DNA sequencing of specific regions has been developed and enables more accurate identification [7]. Multilocus sequencing and phylogenetic assessments have also led to the discovery of cryptic species within accepted morphological entities [8]. Today, strategies based on molecular phylogeny are considered to be the new gold standard for *Fusarium* identification [9]. However, DNA extraction and sequencing are time consuming, associated with higher costs, and increased expertise is needed for assay development, performance and analysis of uncommon species isolated in diagnostic laboratories [10].

The drawbacks of phenotypic and genotypic techniques have led to an increased interest in an emerging technique, MALDI-TOF MS, for rapid and reliable identification of clinically important fungi [11–14]. Identification of unknown isolates by this technology is based on the acquisition of unique protein profiles from isolated colonies and comparison of these data to a library of reference spectra derived from well-characterized isolates [15]. Compared with traditional methods, sample preparation for MALDI-TOF MS analysis, including protein extraction, requires inexpensive reagents that cost approximately $0.50 per identification, although the initial cost of a MALDI-TOF MS instrument can be expensive ($180,000–$250,000) [16]. In addition, commercially supplied spectral libraries can be supplemented with reference spectra from laboratory characterized clinical isolates, allowing the user to expand the database with dedicated materials. This is helpful for the identification of single strains from human infections. Moreover, MALDI-TOF MS also has the potential to characterize strains at, and even below, the species level and hence could provide a useful confirmatory tool in taxonomy and may have sufficient discriminatory power for epidemiological purposes [17,18].

Thus far, only few studies have shown the usefulness of MALDI-TOF MS in the clinical laboratory for *Fusarium* identification, including medically important species [19–21]. *Fusarium* species of the *F. fujikuroi* complex are particularly hard to identify on the basis of

their morphological characteristics. According to Al-Hatmi *et al.* [22] this complex consists of more than 50 species, and so far 15 species have been reported to cause human infections [23]. Their complexity renders the *F. fujikuroi* complex an ideal candidate for testing the diagnostic power of MALDI-TOF MS.

To this aim, a reference spectra database was constructed for *F. fujikuroi* complex and subsequently we challenged the robustness of the database using a subset of well-identified strains. In the second part of the study, a dendrogram was created with the main spectrum profiles (MSPs) of the species studied and used for the construction of the spectral database. A comprehensive comparison of MALDI-TOF MS and DNA sequence based approaches was conducted for identification of all opportunistic species within the *F. fujikuroi* complex.

Materials and methods

Strains and cultivation

In this study, we used two sets of isolates. The first set, used to build the reference database, included 24 strains (10 type strains, and 14 clinical and environmental strains of *F. fujikuroi* complex) (Table 1). Species designations were confirmed via molecular identification by multilocus sequencing comprising the partial translation elongation factor-1a (*TEF1*) and the RNA polymerase subunit (*RPB*2) [24]. Each strain was subcultured on five Sabouraud Gentamicin Chloramphenicol agar plates (SGCA; Oxoid, France) and incubated for 2–3 days at 30°C before analysis. Four out of the five plates were selected to build up reference spectra (see below). The fifth plate was used as a challenge test to assess quality and specificity of the reference spectra before including them in the database. A second set, including 60 clinical and environmental isolates from Centraalbureau voor Schimmelcultures (CBS) reference collection, Utrecht, The Netherlands (Table 2), was used to assess the reference database.

Sample preparation for MAL DI-TOF MS analysis

Protein extraction was performed according to Cassagne *et al.* [13]. Briefly, the surface of the *Fusarium* colonies was scraped with a sterile scalpel, and mycelia were suspended in 1.5 ml microtubes containing 300 µl distilled water and 900 µl ethanol and centrifuged for 10 min at 13,000 ×*g* in a tabletop microcentrifuge. The pellet was incubated for 5 min in 10 µl of 70% formic acid (Sigma-Aldrich, Lyon, France). Then 10 µl of 100% acetonitrile (Prolabo BDH) was added for 10 min of incubation before centrifugation (13,000 ×*g*, 2 min) and the supernatant was removed and placed on a ground steel target (MTP384 polished steel target, Bruker Daltonics, Bremen, Germany) and air-dried at room temperature. Sample spots were overlaid with 1 µl matrix solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile HPLC (VWR, Fontenay-sous-Bois, France) and 2.5% trifluoroacetic acid HPLC (TFA) matrix (Sigma-Aldrich, Lyon, France) and air dried at room temperature.

Reference spectra and database creation

Measurement of the 24 reference strains was performed with the MALDI BioTyper 2.0 (library version 3.0) and FlexControl software on a Microflex LT20 mass spectrometer (20 Hz nitrogen laser, Bruker Daltonics); the spectra were acquired after 650 shots in linear mode with an Autoflex speedTM II over a mass range of 3–20 kDa. According to Normand *et al.* [25], for each isolate, ten raw mass spectral profiles (MSPs) were created from each of the four different subcultures. A bacterial test standard (Bruker Daltonics) was used for calibration. The data were automatically acquired by the FLEXCONTROL version 2.4 software, using the autoexecute default settings. The newly created database was implemented in the existing in-house database, which already contained the reference spectra of 880 other validated strains comprising 467 different fungal species from BCCM/IHEM Biomedical Fungi and Yeasts Collection and Centre Hospitalo-Universitaire (CHU) Timone, Marseille, Parasitology- Mycology Laboratory.

Identification test

To test the created database, spectra of 60 additional isolates of *F. fujikuroi* complex were generated and deposited in quadruplicates using the same protocol as for the references. Results of the pattern-matching process were expressed as proposed by the manufacturer with score range from 0 to 3. The identification process of the *Fusarium* strains was challenged against the reference database using the following criteria. First, spectra of 60 challenge isolates were analysed against the database *F. fujikuroi* complex alone (24 entries). Then, the mass spectra of the same isolates were compared with the mass spectra of *Fusarium* species in the existing database (39 entries) and finally compared with the largest library, which includes the reference spectra of various molds and dermatophyte species (880 entries). Log score of each deposit against the 13 species of *F. fujikuroi* complex in the reference database was recorded and used to build a box plot graph (R software). The mass data for each test were successively matched to the three reference libraries, and the resulting ‘best match’ log score values were calculated using MALDI Biotyper software. The MS based identificationwas considered correct only if at least three out of the four replicates displayed the same identification in accordance with the log score values. According to the manufacturer, log scores of ≥2.0 were recorded as identifications to the species level, and log scores of ≥1.7 as identifications to the genus level. Species represented by three strains such as *F. subglutinans* and *F. temperatum* in the dataset were not included in the calculations of boxplots. For these two species, it was checked whether they were identified by MALDI-TOF MS and multilocus sequence analysis as a phylogenetically closely related species.

Table 1. Reference strains used to create database for *F. fujikuroi* complex for MALDI-TOF MS analysis

Species	CBS number	Sequencing	Status	Source
F. ananatum	CBS 118516	*F. ananatum*	Type	Environmental
	CBS 184.29	*F. ananatum*		Environmental
F. verticillioides	CBS 139.40	*F. verticillioides*		Environmental
	CBS 576.78	*F. verticillioides*	Type	Environmental
F. sacchari	CBS 223.76	*F. sacchari*		Environmental
	CBS 135144	*F. sacchari*		Clinical
F. napiforme	CBS 748.97	*F. napiforme*	Type	Environmental
	CBS 135141	*F. napiforme*		Clinical
F. andiyazi	CBS 119857	*F. andiyazi*	Type	Environmental
	CBS 125177	*F. ficicrescens*		Environmental
F. anthophilum	CBS 245.59	*F. anthophilum*		Environmental
	CBS 222.76	*F. anthophilum*		Clinical
F. fujikuroi	CBS 221.76	*F. fujikuroi*	Type	Environmental
	CBS 262.54	*F. fujikuroi*		Environmental
F. proliferatum	CBS 480.77	*F. proliferatum*	Type	Environmental
	CBS 131391	*F. proliferatum*		Environmental
F. acutatum	CBS 402.97	*F. acutatum*	Type	Environmental
	CBS 136480	*F. acutatum*		Clinical
F. thapsinum	CBS 733.97	*F. thapsinum*	Type	Environmental
	CBS 130176	*F. thapsinum*		Clinical
F. nygamai	CBS 119850	*F. nygamai*		Environmental
	CBS 749.97	*F. nygamai*	Type	Environmental
F. temperatum	CBS 135541	*F. temperatum*		Clinical
F. subglutinans	CBS 747.97	*F. subglutinans*	Type	Environmental

Clustering of MAL DI-TOF spectra

The clustering approach is based on similarity score values of each individual peak which was treated as follows: baseline subtraction with a rolling disc width of 201 pt, smoothing with a beta of 10, a rolling disc width of 40, and peak detection with a S/N ratio of 10. The obtained distance values were calculated using Biotyper 3 and exported to the BioNumerics software (Applied Maths, Belgium). In order to visualize the relationship between species within *F. fujikuroi* complex, the dendrogram was generated based on these distance values. For each strain, a summary spectrum comprising all 40 deposits was built using the following criteria: import similarity filter 80%, minimum peak detection rate 50%, and minimum intensity filter of 800. A dendrogram was obtained using a Pearson similarity correlation coefficient based on curves. Tree nodes were considered significant when the cophenetic coefficient was greater than 70%. The topology of the dendrogram was compared with that of the Bayesian Inference consensus tree.

Reliability of the database

The reliability (percentage of correctly identified isolates according to the log score values) was assessed in a clinical routine by screening different clinical isolates with *Fusarium* identity based on morphological and molecular approaches. The isolates were recovered from patient samples in the CHU Timone, Marseille University Hospital Parasitology-Mycology Laboratory. We subjected them to MALDI-TOF MS identification, according to the criteria described above, and also *TEF1* and *RPB*2 target regions of these strains were sequenced.

Molecular identification

Molecular identification of isolates was performed by amplification and sequencing of the*TEF1* and *RPB*2 target regions of all 84 *Fusarium* isolates. The sequences obtained were used to identify the *Fusarium* isolates in the GenBank database using BLAST software and also to construct the phylogeny tree including 13 species of *F. fujikuroi* complex and one strain of *F. polyphialidicum* as an outgroup. PCR protocols and sequencing were done as previously described by Al-Hatmi *et al.* [26]. The sequences were aligned using MAFFT v. 7.127 [27], followed by manual adjustment with MEGA v. 6.2. Bayesian analysis was done using the online version of MrBayes v.3.1.2 on the CIPRES portal [28].

Table 2. Identification results for the 60 clinical and environmental isolates of *F. fujikuroi* complex obtained by MALDI-TOF MS in comparison with those obtained by multi-locus sequence analysis.

			Sequencing[a]	MALDI-TOF MS		
CBS No.	**Source**	**Origin**	**Species ID**	**Log (score)[b]**		**Species ID[c]**
				1st	2nd	
119831	Environmental	South Africa	*F. subglutinans*	2.41	2.23	*F. subglutinans*
136481	Clinical	Italy	*F. subglutinans*	2.265	2.138	*F. subglutinans*
118519	Environmental	South Africa	*F. ananatum*	2.22	2.074	*F. ananatum*
118517	Environmental	South Africa	*F. ananatum*	2.177	2.024	*F. ananatum*
118518	Environmental	South Africa	*F. ananatum*	2.088	2.03	*F. ananatum*
130392	Clinical	Italy	*F. ananatum*	1.935	1.923	*Funsarium*. spp.
115135	Clinical	Sweden	*F. verticillioides*	1.96	1.89	*Funsarium*. spp.
102699	Clinical	Germany	*F. verticillioides*	1.923	1.89	*Funsarium*. spp.
108922	Clinical	Germany	*F. verticillioides*	2.032	2	*F. verticillioides*
114579	Clinical	Austria	*F. verticillioides*	2.248	2.135	*F. verticillioides*
116665	Environmental	Unknown	*F. verticillioides*	2.1	2.09	*F. verticillioides*
123670	Environmental	USA	*F. verticillioides*	2.154	2.003	*F. verticillioides*
579.78	Clinical	USA	*F. verticillioides*	2.238	2.03	*F. verticillioides*
122159	Environmental	Spain	*F. verticillioides*	2.06	2.05	*F. verticillioides*
131390	Environmental	Australia	*F. verticillioides*	2.06	2.041	*F. verticillioides*
135142	Clinical	India	*F. sacchari*	2.158	2.046	*F. sacchari*
135145	Clinical	India	*F. sacchari*	2.111	2.067	*F. sacchari*
135143	Clinical	India	*F. sacchari*	2.09	2.038	*F. sacchari*
121683	Clinical	India	*F. sacchari*	2.178	2.06	*F. sacchari*
136482	Clinical	Italy	*F. sacchari*	2.127	2.02	*F. sacchari*
134.73	Environmental	Guyana	*F. sacchari*	2.269	2.028	*F. sacchari*
131369	Environmental	Australia	*F. sacchari*	2.25	2.1	*F. sacchari*
135139	Clinical	India	*F. napiforme*	2.15	2.09	*F. napiforme*
135140	Clinical	India	*F. napiforme*	2.16	2.01	*F. napiforme*
135141	Clinical	India	*F. napiforme*	2.594	2.518	*F. napiforme*
673.94	Environmental	Unknown	*F. napiforme*	2.202	2.146	*F. napiforme*
535.95	Environmental	Unknown	*F. napiforme*	2.733	2.596	*F. napiforme*
119856	Environmental	Ethiopia	*F. andiyazi*	2.2	2.012	*F. andiyazi*

Table 2. Continued						
134430	Clinical	Turkey	*F. andiyazi*	2.2	2.13	*F. andiyazi*
737.97	Environmental	Germany	*F. anthophilum*	2.17	2.121	*F. anthophilum*
119858	Environmental	USA	*F. anthophilum*	2.07	2.01	*F. anthophilum*
119859	Environmental	New Zealand	*F. anthophilum*	2.286	2.161	*F. anthophilum*
130402	Clinical	USA	*F. fujikuroi*	2.187	2.177	*F. fujikuroi*
119855	Environmental	Unknown	*F. fujikuroi*	2.05	2	*F. fujikuroi*
121864	Environmental	Unknown	*F. fujikuroi*	2.44	2.423	*F. fujikuroi*
449.95	Environmental	France	*F. fujikuroi*	2.001	2	*F. fujikuroi*
257.52	Environmental	Japan	*F. fujikuroi*	2.453	2.422	*F. fujikuroi*
184.33	Environmental	Guyana	*F. proliferatum*	2.229	2.162	*F. proliferatum*
183.29	Environmental	Unknown	*F. proliferatum*	2.151	2.031	*F. proliferatum*
116324	Clinical	Spain	*F. proliferatum*	2.193	2.128	*F. proliferatum*
182.32	Environmental	USA	*F. proliferatum*	2.435	2.405	*F. proliferatum*
133030	Clinical	Iran	*F. proliferatum*	2.1	2.02	*F. proliferatum*
125014	Clinical	USA	*F. proliferatum*	2.027	2.017	*F. proliferatum*
130179	Clinical	USA	*F. proliferatum*	2.086	2.021	*F. proliferatum*
131573	Environmental	Iran	*F. acutatum*	2.553	2.551	*F. acutatum*
401.97	Environmental	India	*F. acutatum*	2.232	2.232	*F. acutatum*
739.97	Environmental	India	*F. acutatum*	2.184	2.13	*F. acutatum*
113964	Environmental	Egypt	*F. acutatum*	2.36	2.32	*F. acutatum*
130548	Clinical	Iran	*F. acutatum*	2.117	2.106	*F. acutatum*
109077	Environmental	Ethiopia	*F. thapsinum*	2.314	2.245	*F. thapsinum*
113963	Environmental	Yemen	*F. thapsinum*	2.508	2.442	*F. thapsinum*
119833	Environmental	Unknown	*F. thapsinum*	2.255	2.23	*F. thapsinum*
120995	Environmental	Australia	*F. nygamai*	2.358	2.333	*F. nygamai*
140.95	Clinical	Egypt	*F. nygamai*	2.297	2.188	*F. nygamai*
131377	Environmental	Australia	*F. nygamai*	2.113	2.067	*F. nygamai*
454.97	Environmental	Sudan	*F. nygamai*	1.752	1.721	*Funsarium.* spp.
483.94	Environmental	Australia	*F. nygamai*	2.404	2.386	*F. nygamai*
125178	Environmental	Iran	*F. ficicrescens*	2.2	2.17	*F. ficicrescens*
125181	Environmental	Iran	*F. ficicrescens*	2.226	2.212	*F. ficicrescens*
135540	Clinical	Mexico	*F. temperatum*	2.32	2.21	*F. temperatum*

Results

Reference database

A set of 24 reference strains were used to create the reference database belonging to the clinically relevant species of *F. fujikuroi* complex represented by 13 species. Each species was represented by one or two isolates such as *F. acutatum* (n = 2), *F. ananatum* (n = 2), *F. andiyazi* (n = 1), *F. anthophilum* (n = 2), *F. fujikuroi* (n = 2), *F. napiforme (*n = 2), *F. nygamai* (n = 2), *F. proliferatum* (n = 2), *F. verticillioides* (n = 2), *F. sacchari* (n = 2), *F. subglutinans* (n = 1), *F. temperatum* (n = 1) and *F. thapsinum* (n = 2) (Table 1). Strains belonging to the *F. subglutinans* and *F. temperatum* were considered in the identification test but there was not enough data to build the boxplot charts because only 3 and 2 strains, respectively, were positively subcultured. The extended in-house reference database for clinical strains of *F. fujikuroi* complex was included in an existing database including 880 fungal strains of clinical relevance. To ascertain that variability between the reference spectra was suitable for discrimination between the different species, an MSP was generated from ten technical replicates of the same sample, and each MSP was checked for its quality and specificity before oasis:entry into the database. The mass spectra of 13 species showed clear differences among them (Figure 1). In Figure 1, the similarity of the inquired spectra with the reference of the same species was higher than other species within the *F. fujikuroi* complex, and the obtained log score was higher than for species from other *Fusarium* complexes such as *F. solani* and *F. oxysporum* and finally much higher than any other fungal species references.

Validation of the database

To prove the suitability of the extended MALDI Biotyper 3.0 database for routine identification and discrimination between members of *F. fujikuroi* complex, spectra of 60 strains were tested blindly and were matched against it. The set of challenge strains included clinical and environmental isolates (5 *F. acutatum*, 4 *F. ananatum*, 2 *F. andiyazi*, 3 *F. anthophilum*, 5 *F. fujikuroi*, 2 *F. musae*, 5 *F. napiforme*, 5 *F. nygamai*, 7 *F. proliferatum*, 7 *F. verticillioides*, 7 *F. sacchari*, 2 *F. subglutinans*, 1 *F. temperatum*, 3 *F. thapsinum*, and 2 *F. ficicrescens*). Using log score 2.0 as a cutoff, 56 of 60 isolates (93.3%) were correctly identified up to the species level whereas the four remaining isolates (with log scores ≥1.7 but <2.0) were within the correct species complex. The results of MALDI-TOF MS were compared with those of multilocus sequence analysis (Table 2) and also to the constructed phylogenetic tree which includes 13 species of *F. fujikuroi* complex and one strain of *F. polyphialidicum* as an outgroup (Figure 2).

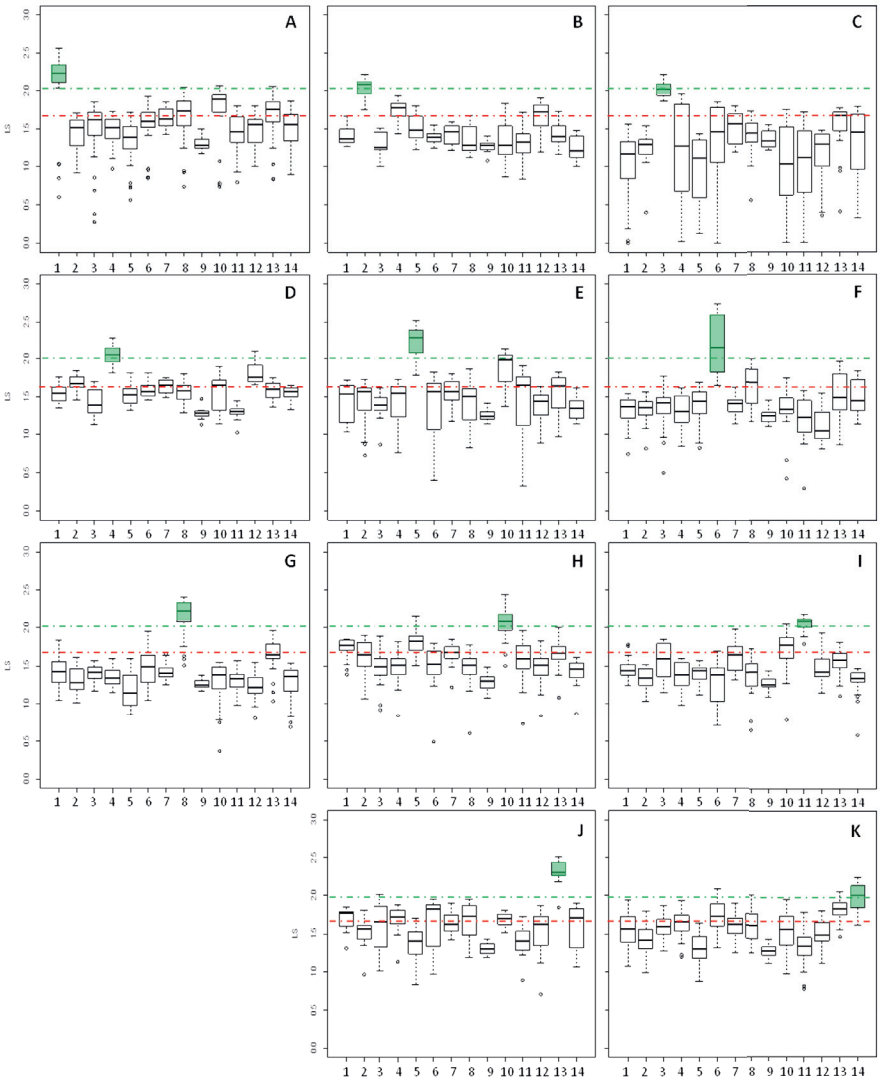

Fig 1. Log score repartition depending for each species belonging to the *F. fujikuroi* complex against the other species composing the complex (see facing page). Each graph represents the log score results for the tests of one species (**A**: *F. acutatum*; **B**: *F. ananatum;* **C**: *F. andiyazi*; **D**: *F. anthophilum;* **E**: *F. fujikuroi*; **F**: *F. napiforme*; **G**: *F. nygamai*; **H**: *F. proliferatum*; **I**: *F. sacchari*; **J**: *F. thapsinum*; **K**: *F. verticillioides*) against various references exposed in abscissa (**1**: *F. acutatum*; **2**: *F. ananatum*; **3**: *F. andiyazi*; **4**: *F. anthophilum*; **5**: *F. fujikuroi*; **6**: *F. napiforme*; **7**: non *F. fujikuroi* (other *Fusarium* spp.); **8**: *F. nygamai*; **9**: other filamentous fungal species; **10**: *F. proliferatum*; **11**: *F. sacchari*; **12**: *F. subglutinans*; **13**: *F. thapsinum*; **14**: *F. verticillioides*). The green dotted line represents the manufacturer identification at the species level (log score = 2) while the red dotted line represents the lower limit of identification at the genus level (log score = 1.7).

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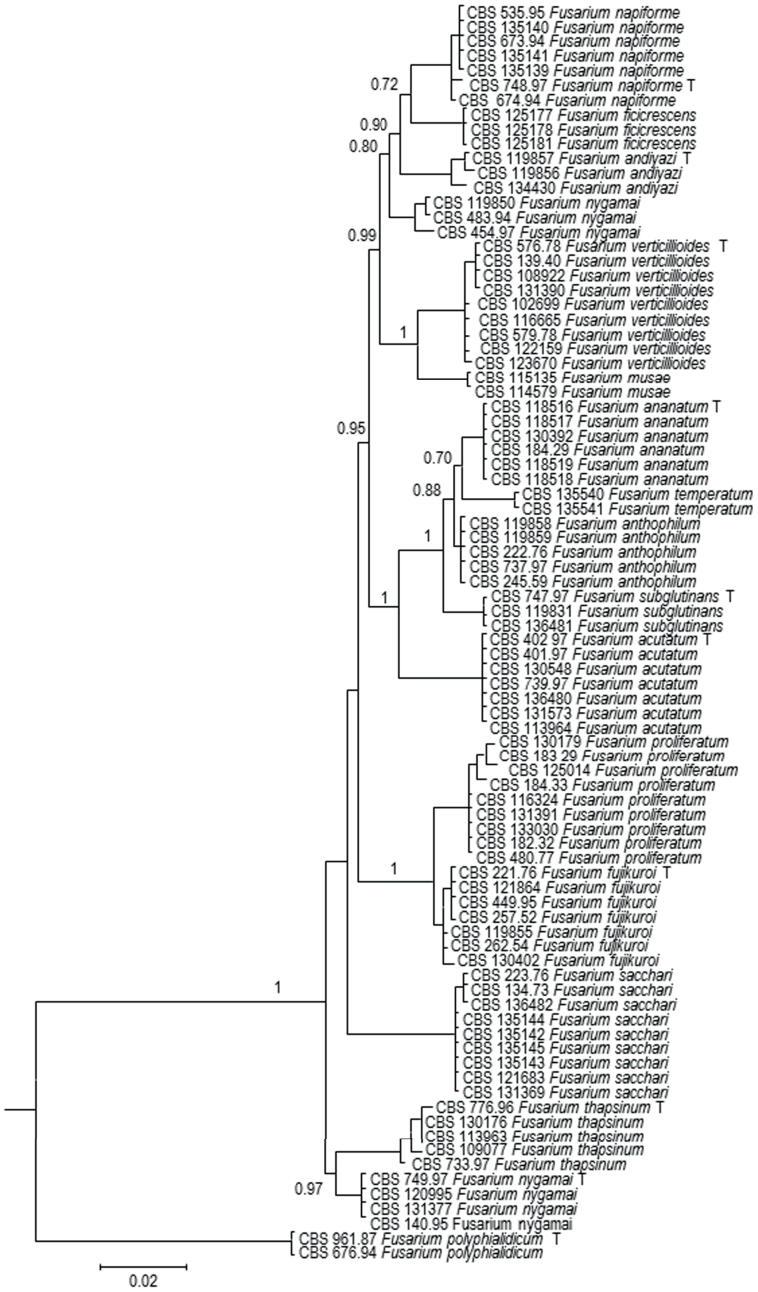

Fig 2. Phylogenetic tree based on the analysis of *TEF1* gene sequences. Total alignment length is 564 bp. Numbers above branches are posterior probabilities support values ≥ 0.70 shown.

Discussion

The increasing incidence of *Fusarium* spp. in clinical microbiology laboratories and their abundance in the hospital environment require the development of sensitive, rapid and automated methods of accurate identification of strains that are implicated in patients' health. The usefulness of MALDI-TOF MS for the identification of common yeasts and filamentous fungi has already been demonstrated [29–32]. Our main aim is to improve the identification of *Fusarium* species of the *F. fujikuroi* complex, which has traditionally been the most difficult species group to identify [33]. Infections due to species of the *F. fujikuroi* complex are increasingly reported [9,34], with 15 potentially opportunistic species known to date as shown by Al-Hatmi *et al.* [35]. This is of particular concern because *Fusarium* species frequently possess intrinsic resistance against different classes of antifungals, notably fluconazole, itraconazole and micafungin. The number of multidrugresistant isolates within the *F. fujikuroi* complex is growing, while strains show considerable variation between and within species [22]. Such a diversity of emerging *Fusarium* species poses a diagnostic challenge when traditional methods are used.

Two studies extensively evaluated the use of MALDI-TOF MS with other genotypic methods for the identification of *Fusarium* species. Marinach-Patrice *et al.* [19] analyzed 62 *Fusarium* strains most of which were isolated from human infections, including *F. solani, F. oxysporum, F. dimerum, F. verticillioides*, and *F. proliferatum,* the last two being the most frequently encountered members of the *F. fujikuroi* complex in human infections. Triest *et al.* [21] studied a large collection of 289 strains representing 40 species mainly from *F. solani, F. oxysporum, F. dimerum* and *F. fujikuroi* complexes and showed that identification was 100% correct at the complex level and 82.8% at the species level compared with sequencing, using score 2.0 as a cut-off value in MALDI-TOF MS. The same authors included five clinical species of *F. fujikuroi* complex (*F. proliferatum, F. verticillioides*, *F. sacchari*, *F. subglutinans* and *F. musae*) for inhouse database construction, but did not include *F. acutatum*, *F. ananatum*, *F. andiyazi*, *F. anthophilum*, *F. fujikuroi*, *F. napiforme*, *F. nygamai*, *F. temperatum* and *F. thapsinum* for which the distinction from other *Fusarium* species would have been the most challenging.

Our study included 60 strains belonging to 13 rare clinically relevant species and we documented that MALDI-TOF MS is an accurate, fast and competent tool for rapid identification of species within *F. fujikuroi* complex. Using standard criteria of the MALDI Biotyper 3, our database allowed a 93.3% correct identification rate at the species level. Apart from the high accuracy of MALDI-TOF MS, the rapidity of the analysis is advantageous for the application in clinical microbiology laboratories. Obtaining the mass spectra of *Fusarium* isolates, including sample preparation, takes less than 30 min, compared with several hours or even several days for molecular or phenotypic analysis.

Accurate identification of *Fusarium* isolates by MALDI-TOF MS is limited by species heterogeneity in the reference spectrum database differing from that generated from sequence

data. Most of the deviating results between the two methods can be explained. Strains CBS 115135 and CBS 102699 were identified as *F. verticillioides* by MALDI-TOF MS with log scores 1.96 and 1.92, respectively (Table 2), indicating uncertain species identification. In the course of our study, the strains were sequenced and gained a new taxonomic status as they were found to be conspecific with *F. musae,* a close relative of *F. verticillioides*. *Fusarium musae* was not included in our study because the first clinical case was published in October 2014 by Triest *et al.* [36], when our work had already been concluded. CBS 130392 was selected as *F. ananatum* on phenotypic grounds and identified by MALDITOF with log score 1.9. Molecular identification by *RPB*2 showed that this strain belonged to *F. guttiforme*, a species closely related to *F. ananatum*. Blast queries in GenBank with *TEF1*, however, yielded 100% similarity with *F. ananatum*, which was in apparent conflict with *RPB*2 results. Similarly, strain CBS 454.97 was selected from the collection as *F. nygamai* on the basis of morphological identification and was identified by MALDI-TOF MS (with log score 1.75) as belonging to the *F. fujikuroi* complex. Molecular identification showed that this strain was nested in a separate clade remote from *F. nygamai*; an unidentified cryptic species might be concerned.

Strains CBS 125177, CBS 125178 and CBS 125181 had initially been identified morphologically as *F. andiyazi*. Two separate clades using MALDI-TOF MS, one comprising three strains including the type strain of *F. andiyazi* (CBS 119857, CBS 119856 and 134430) and another (CBS 125178 and CBS 125181), with log scores 2.20 and 2.226, respectively, matched the mass spectra of CBS 125177, which was used as a reference with the type strain CBS 119856 of *F. andiyazi*. Further polyphasic assessment of these isolates led to definitive species designation of strains CBS 125177, CBS 125178 and CBS 125181 as *F. ficicrescens* [37]. When comparing the log score of MALDI-TOF MS with multilocus sequence analysis, high concordance was found (Table 3). These results indicate that MALDI-TOF MS has a potential to distinguish closely related *Fusarium* species and might help to find the source of infection. This outcome of mass spectrometry might be improved by extending the BioTyper reference database, as indicated by Ranque *et al.* [38].

Table 3. Concordance results between MALDI-TOF MS and multi-locus sequence analysis for 60 clinical and environmental *Fusarium* isolates belonging to *F. fujikuroi* complex

Species (No)	Sequencing	MALDI-TOF MS	Concordance [%]
F. subglutinans (2)	2	2	100
F. ananatum (4)	4	3	75
F. verticillioides (7)	7	5	71
F. musae (2)	2	0	-
F. sacchari (7)	8	8	100
F. napiforme (5)	5	5	100
F. andiyazi (2)	2	2	100
F. anthophilum (3)	3	3	100
F. fujikuroi (5)	7	7	100
F. proliferatum (7)	8	8	100
F. acutatum (5)	6	6	100
F. thapsinum (3)	3	3	100
F. nygamai (5)	5	4	80
F. temperatum (1)	1	1	100
F. ficicrescens (2)	2	2	100

As well as the full discrimination between the majorities of tested strains with the log score approach, which is sufficient for clinical purposes, we were interested in analyzing the capability of MALDI-TOF MS to display phylogenetic relationships between strains. Therefore, a cluster analysis based on a pairwise correlation values for spectra of all reference and challenge strains resulted in partial separate clusters at the species level. The resulting dendrogram shows discrete clusters for *F. ananatum*, *F. ficicrescens*, *F. subglutinans* and *F. thapsinum*. Strains of *F. anthophilum*, *F. fujikuroi*, *F. napiforme*, *F. nygamai*, *F. proliferatum*, *F. verticillioides* and *F. sacchari* did not form individual clusters, but only partially grouped with their respective reference isolate. Overall, 72% of the tested isolates grouped with the corresponding reference strains, in agreement with reference method, and their clusters were distinct from those of other species, but 28% of the strains were deviant from their respective reference. Thus, in contrast to the full discrimination between the *Fusarium* species by matching spectra with reference spectra in the MALDI Biotyper 3.0 database, cluster analysis does not resolve the *F. fujikuroi* complex and strains of the same species were often not clustered together (Figure 3). Intraspecific heterogeneity in almost a third of the analyzed samples was larger than distances between species, barcoding gaps being absent. On the other hand, this suggests a certain epidemiological potential. Extended analyses with many genera of bacteria and fungi have demonstrated that MALDITOF MS is a feasible approach for (sub) species classification [39].

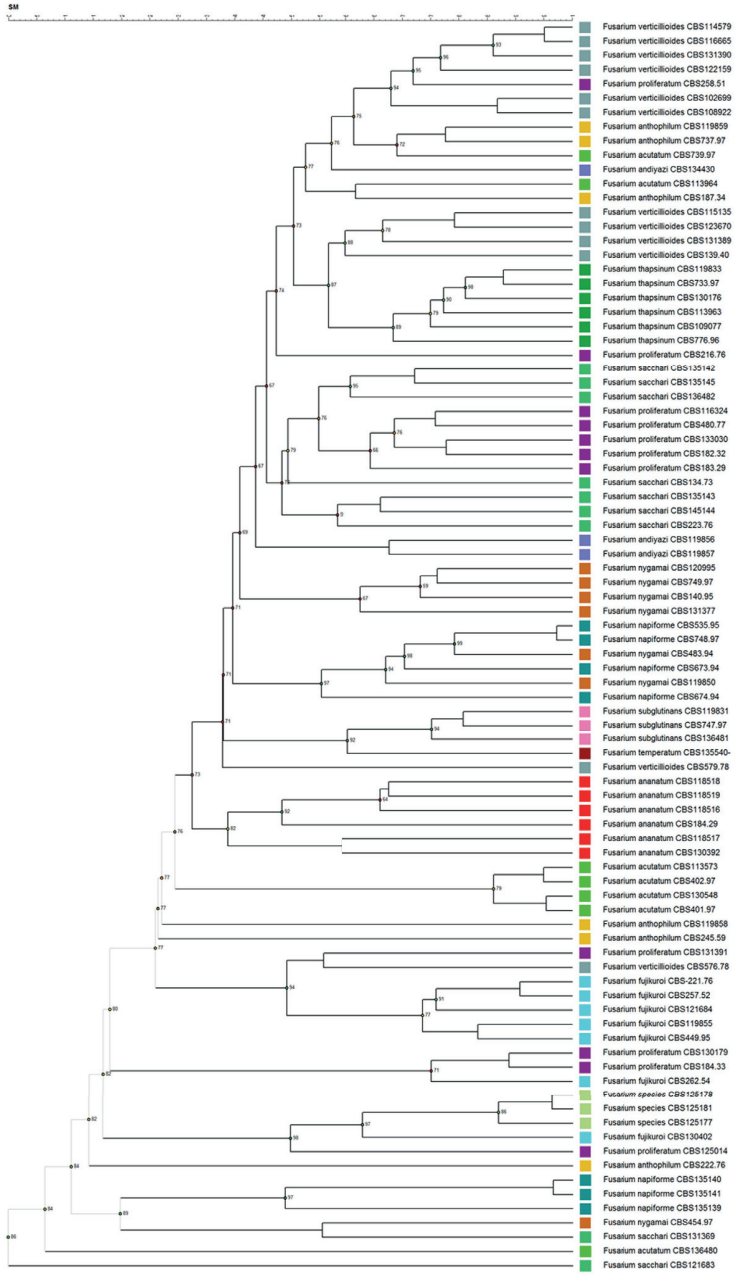

Fig 3. UPGMA tree based on MAL DI TOF MS summarized spectra created using the BioNumerics software, showing the relationship among the various strains of *Fusarium fujikuroi* species (see facing page). Cophenetic correlation coefficients are presented at the nodes.

Despite the widespread use of MALDI-TOF MS in clinical microbiology laboratories, the availability of low numbers of the MALDI Biotyper library (MBL) reference spectra for particular species may not be sufficient to capture the extractable protein variability that may exist within isolates of a given species. Two studies reported that MALDI-TOF MS spectra are considered to be composed of conserved proteins which have functions that are minimally affected by environmental conditions [40,41]. These limitations are partly being mitigated by continued improvement of databases and with the users' supplementation of the MBL with reference spectra of well-characterized, correctly identified isolates. On the other hand, the quality of spectra influenced by parameters such as culture medium, incubation time, the quality of materials and nature of samples can affect MALDI-TOF MS results [42]. We think that such factors make it important to carefully control all variables in experiments aimed at typing with mass spectrometry.

Conclusion

Conventional identification of *Fusarium* species by culture methods takes up to 1 week and sequencing takes up to 48 h, depending on the species and the availability of the sequencing system used. By contrast, MALDI-TOF MS can be performed with minimal amounts of young colonies and takes only a few minutes, with comparable levels of resolution. MALDI-TOF MS has the potential to become an important tool for the routine identification of pathogenic *Fusarium* species, provided that the availability of extended reference databases is guaranteed, although technical reproducibility in *Fusarium* may be a limiting factor. Maldi also revealed some possible flaws in the taxonomy of *Fusarium* species, which needs to be solved with high urgency.

Acknowledgements

We acknowledge Katleen Vranckx from Applied Maths in Gand, Belgium, for kindly providing us with the Bionumerics software to analyze our results. Abdullah Mohammed Said Al-Hatmi was financially supported by the Ministry of Health, Muscat, Oman.

Financial & competing interests disclosure

The work of Abdullah Mohammed Said Al-Hatmi was financially supported by Ministry of Health, Oman. Formal Agreement number 28/2014. The authors have no other relevant affiliations or financialinvolvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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CHAPTER 6

Chapter 6

The concept of ecthyma gangrenosum illustrated by a *Fusarium oxysporum* infection in an immunocompetent individual

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Mycopathologia 2016; 181: 759-763.

Abstract

Ecthyma gangrenosum (EG) involves necrotic cutaneous lesions caused by bacteria, mainly *Pseudomonas aeruginosa*, and is usually seen in immunocompromised patients with septicemia. However, clinically similar infections have been published with fungi as etiologic agents. We present a case of an EG-like lesion due to *Fusarium oxysporum* confirmed by clinical diagnosis, culture and molecular identification and discuss the definition of EG.

Key words: *Fusarium oxysporum*, Ecthyma gangrenosum, Definition, Immunocompetent

Introduction

Ecthyma gangrenosum (EG) is generally defined as an infection with multiple necrotic cutaneous ulcers caused by species of *Pseudomonas*, mainly *P. aeruginosa*, or *Aeromonas*. The disorder is usually seen in immunocompromised patients, particularly thosewith underlying malignant disease. Occurrence in immunocompetent individuals is highly exceptional [1]. Since 1980s, data have accumulated on a wider bacterial spectrumof gram negative agents like *Escherichia coli*, *Citrobacter freundii*, *Klebsiella pneumonia*, *Morganella morganii* and various *Pseudomonas* species [2, 3]. Also some fungi have been reported to cause clinically similar lesions, e.g., species of *Candida*, *Aspergillus*, and *Curvularia* [4]. Thus, the disease definition for some authors is determined by the etiologic agent and for others by clinical features. Nevertheless, the name is infrequently applied to fungal infections. This confusion may mask a wider prevalence of EG-like infections, which otherwise may have been reported under a wide spectrum of fungal diseases.We present a fungal case of an EG-like infection in an immunocompetent patient caused by *Fusarium oxysporum* and discuss the definition of EG.

Case presentation

A 17-year-old male from southwestern China with epilepsy presented with a painless ulcer on his right leg which was clinically suspected as being dry gangrene. The lesion started 2 months earlier when he was injured during an epileptic insult by a wooden bench. Initial lesions were in the form of small, painless papules which quickly progressed to a painless ulcer with a black eschar over a period of 2 weeks. All laboratory investigations were within normal ranges, and in particular, no immune deficiency was apparent. However, the patient had a history of epilepsy for which he was on carbamazepine for 7 years. Examination of the skin lesion showed a 5 x 13 cm skin ulcer with black eschar, i.e., the ulcer edge appeared to ‘‘roll over’’ and took the shape of a tough, erythematous and fibrous ring. The black eschar adhered tightly without discharge of pus or granules (a1, a2). Upon first visit, the case was diagnosed as EG (Fig. 1).

Identification of the fungal etiological agent

A skin biopsy was taken from the lesion and direct examination (KOH 10 %) showed multiple thin hyaline hyphae (b1, b2). Histopathological examination showed irregular, hyaline hyphae in the H&E stained tissue (c1, c2). Culture on Sabouraud’s glucose agar (SGA) showed white colonies with a light brown pigment excreted into the medium. Phenotypic identification of this isolate resulted in a *Fusarium* species. Since fungal hyphae were visible in histopathological slides, the medical judgment of the case was changed from EG to an EG-like fungal infection. Subsequently, voriconazole (400 mg/day orally) was added to the therapeutic plan at day 14 after admission. Two weeks after the start of antifungal treatment, the patient received surgical debridement and continued on oral voriconazole for another 2 weeks with noticeable

improvement. The patient did not return for follow-up visits. The isolate was preserved and deposited in the CBS-KNAW reference collection with accession number CBS 140424.

Subculturing of the isolate for a more thorough morphological study was performed on Malt Extract Agar (MEA) and Oatmeal Agar (OA) with incubation times of 1 week at 25, 33 and 37 °C. Rapidly growing, hairy, pink–violet colonies were observed after incubation on MEA after 5 days at 28 °C. Microscopic morphology showed short, single, lateral monophialides (flask-shaped projections) in the aerial mycelium. Macroconidia were fusiform, slightly curved, 1–3 septate, 21.4–38.5 µm in length and 3.2–4.5 µm in width. Microconidia were abundant, not arranged in chains, 0–1 septate, 6.4–14.3 µm in length and 2.8–4.3 µm in width (d1–d4). Optimal growth was observed at 27 °C; maximum growth temperature was 37 °C. On the basis of culture and microscopy, the fungus was provisionally identified as *F. oxysporum*.

Genomic DNA was extracted from a culture grown on MEA following a cetyltrimethyl ammonium bromide protocol as described previously [5]. The rDNA internal transcribed spacer region (ITS) was analysed as routine marker using primers ITS1 and ITS4. Since ITS is not a recommended locus for reliable identification of *Fusarium* species, the partial translation elongation factor 1-alpha (*TEF1*) and the partial RNA polymerase second largest subunit (*rPB2*) were also analyzed. PCR amplification and sequencing of the partial fragments were, respectively, done for *TEF1* with primers EF1 and EF2R and for *RPB2* with primers *RPB2*-7cR and *RPB2*-5F2 [5]. To perform identification, a similarity search with the sequences of each gene was done using the BLAST tool of the NCBI database, the FUSARIUM-ID database and the *Fusarium* MLST database. These BLAST searches yielded 100 % similarity scores with several submissions under the name *F. oxysporum*. The ITS, *TEF1* and *RPB2* nucleotide sequences were deposited in GenBank with accession numbers KT794176, KT794174 and KT794173, respectively.

Antifungal susceptibility testing

Antifungal susceptibility testing was performed according to CLSI M38A (Clinical and Laboratory Standards Institute 2008) method, slightly modified by Al-Hatmi et al. [6] and demonstrated that the fungus had a low minimum inhibitory concentrations (MICs) of 1 µg/ml against amphotericin B and high MICs above published epidemiological cut off values (ECVs) [7], for itraconazole (>16 µg/ml), voriconazole (8 µg/ml), posaconazole (>16 µg/ml), isavuconazole (8 µg/ml), anidulafungin (>16 µg/ml), micafungin (>16 µg/ml), and fluconazole (>64 µg/ ml).

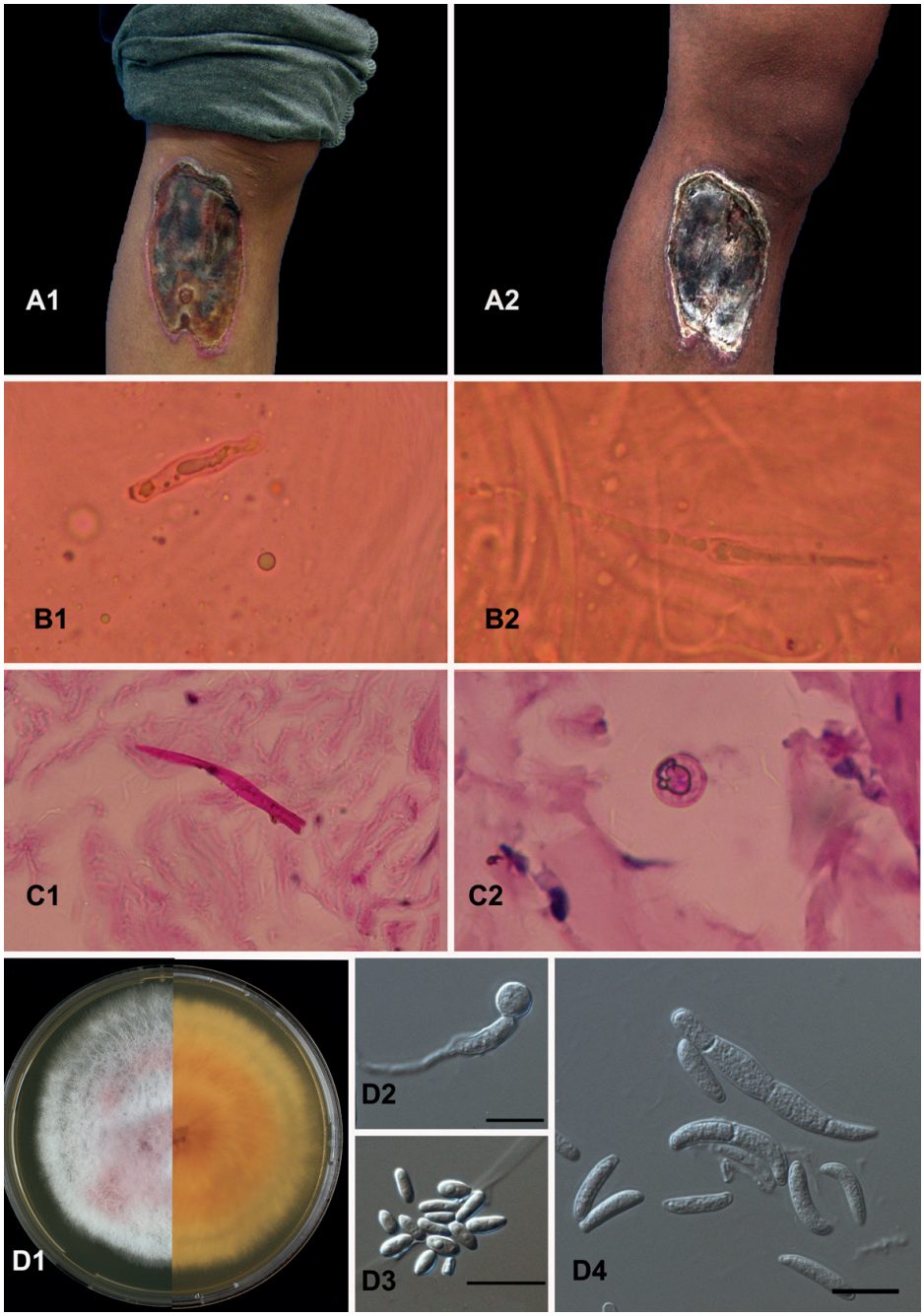

Fig 1. a1 Initial infection; a2 After 2 weeks of treatment; b1, b2 KOH mount of the swab collected from the lesion showing fungal hyaline hyphae (magnification, 940); c1, c2 fungal hyphae seen with H&E stain from the biopsy; d1 growth of white colonies and purple pigmentation on MEA at 25 °C; d2– d4 septate macroconidia and microconidia. All scale bars 10 μm.

Discussion

At present EG is generally defined as a condition pathognomonic for *Pseudomonas aeruginosa* or related bacteria in immunocompromised patients. In the current literature, a much wider definition is often maintained, including variable etiology and variable host factors, also including immunocompetent. In our view, the gangrenous clinical appearance is the unique feature of the infection and should determine the definition, as follows: (1) Pathogens, which may be bacteria but also fungi, yeasts or mycobacteria are isolated from blood cultures and skin biopsies [8], (2) The host is immunocompromised. (3) Clinically, the lesions demonstrate hemorrhagic pustules that lead to necrotic ulcers which evolve into gangrenes with black scab and in a later stage become surrounded by a red halo. (4) Histopathology shows a necrotic epidermis and papillary dermal edema; venules are congested and red blood cell extravasation is noted in the papillary dermis; secondary thrombosis of the arterioles, tissue edema and separation of the epidermis leads to the specific picture of EG.

Consequently, our case was interpreted as EG-like, with presence of fungal hyphae, but a difference was noted in the fact that our patient was an immunocompetent individual. Although fungal agents are mostly reported from immunocompromised patients with EG, an immunocompromised status was judged not necessary [8]. This makes the clinical appearance of local gangrene even less specific. While originally *Pseudomonas* bacteria were part of the definition, now also other agents including fungi such as the general opportunist genus *Fusarium* can be involved, and the immune status of the host also appears variable. For all these reasons, it seems to us that a definition of EG by medical aspects of clinical features, and histopathology is most appropriate.

Reported fungal opportunists of EG-like and EG infections are *Aspergillus fumigatus*, *Candida albicans*, *C. tropicalis*, *Curvularia* sp., *Exserohilum* sp., *Fusarium solani*, *Metarhizium anisopliae*, *Mucor pusillus*, *Scedosporium boydii* and *Neoscytalidium dimidiatum* [4]. To this list, *F. oxysporum* can be added. Histopathology showed that local invasion with fungal elements was present in epidermis and dermis. An extensive literature search revealed six cases since 1975 in neutropenic patients due to *F. solani* or other *Fusarium* species that were listed as EG [9–14]. Our patient, without known immune deficiency, had a history of trauma during which the fungus could have been implanted in the skin. The eschar was tightly attached to the ulcer, suggesting that fungal EG might be different from bacterial EG, which would favor reference to fungal infections as ‘‘EG-like.’’ Though, more cases have to be studied to confirm this clinical difference. In most EG and EGlike cases, the clinical picture is specific; differential diagnosis should consider warfarin-induced skin necrosis, cocaine-induced skin necrosis, calciphylaxis, septic emboli, loxoscelism, diabetic microangiopathy, disseminated intravascular coagulation, paraneoplastic extensive necrotizing vasculitis, pyoderma gangrenosum, livedoid vasculopathy, antineutrophil cytoplasmic antibody -associated vasculitis, cutaneous necrotizing vasculitis due

to familial Mediterranean fever, and necrosis secondary to the use of vasoactive drugs [15, 16].

Treatment of our patient was only for 2 weeks with voriconazole, but the beginning of a curative effect was seen during this short period. The loftiness around the lesion became flatter and the surface of the eschar became dry. EG is an ominous sign in immunocompromised patients, especially with *P. aeruginosa*. However, dermatologists should be aware that other pathogens such as fungi can cause clinically indistinguishable lesions. In most cases, the cutaneous appearance constitutes an important physical clue to the clinical diagnosis as an EG or EG-like lesion, that is black scab and surrounded by a red halo. Given the fact that the etiologic agent can be of bacterial and of fungal nature, it is essential that the pathogen is identified.

Acknowledgments

This study was partially funded by the Ministry of Health, Oman (Formal Agreement No. 28/2014). Abdullah M. S. Al-Hatmi received a Ph.D. scholarship from the Ministry of Health, Oman. We acknowledge Hao Chen M.D., for helping us with the histopathological diagnosis.

Conflict of interest

The authors declare that they have no competing interests.

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CHAPTER 7

Chapter 7

Fusarium ramigenum, a novel human opportunist in a patient with common variable immunodeficiency and cellular immune defects: case report

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BMC Infectious Diseases 2016; 16:79.

Abstract

Background: *Fusarium* species are ubiquitous environmental fungi that occasionally provoke serious invasive infections in immunocompromised hosts. Among *Fusarium* species, *Fusarium ramigenum*, belonging to the *Fusarium fujikuroi* species complex, has thus far never been found to cause human infections. Here we describe the first case of invasive fusariosis caused by *Fusarium ramigenum* in a human and also identify immunological deficiencies that most likely contributed to invasiveness. **Case presentation**: A 32-year-old Caucasian male with a seemingly insignificant medical history of mild respiratory illness during the preceding two years, developed invasive pulmonary fusariosis. Detailed immunological assessment revealed the presence of common variable immunodeficiency, complicated by a severe impairment of the capacity of T-cells to produce both gamma-interferon and interleukin-17. In-depth microbiological assessment identified the novel human opportunistic pathogen *Fusarium ramigenum* as cause of the infection. **Conclusion**: This report demonstrated that an opportunistic invasive fungal infection may indicate an underlying cellular immune impairment of the host. The unexpected invasive infection with *Fusarium ramigenum* in this case unmasked a complex combined humoral and cellular immunological deficiency.

Key words: *Fusarium ramigenum* infection, Immune deficiency, Gamma-interferon, IL-17 deficiency.

Background

Fusarium species are common saprophytic fungi that globally represent the third cause of invasive mould infection in humans, after *Aspergillus* and after Mucorales. This opportunistic infection is common in Brazil but rare in other parts of the world. The important *Fusarium* species implicated in human pathology belong to the *F. solani, F. oxysporum*, and *F. fujikuroi* species complexes [1]. In immunocompetent hosts, clinical manifestations are relatively mild and mostly result from accidental trauma (e.g. keratitis and contact lens-related infections, onychomycosis, osteo-arthritis, but also peritonitis after peritoneal dialysis). Invasive infections are almost exclusively found in immunodeficient hosts, particularly those with severe dysfunction of cellular immunity [2, 3]. In those patients, infections of the respiratory tract are commonly encountered. Mortality due to invasive fusariosis can be above 50 %, even when appropriate and intensive therapeutic management is applied [1, 4]. Here we describe a case of invasive fusariosis caused by a hitherto unknown opportunist, *Fusarium ramigenum*, and report on the immunological causes most likely contributing to this infection.

Case report

A 32-year-old Caucasian male, an outdoor worker (borderguard), with mild, recurrent respiratory infections during two preceding years was admitted to the infectious diseases clinic with a 5-day history of high fever, chills, chest pain, dry cough and myalgia. Physical examination showed a good general condition, 38.5 °C fever, no crackles on auscultation, and a palpable spleen (15 cm length). Pulmonary imaging (chest X-ray and lung CT-scan) demonstrated bilateral pulmonary micro-nodular infiltrations and satellite mediastinal lymphadenopathies with a maximum diameter of 16 mm (Fig. 1). Laboratory investigations revealed leukocytosis (15000/mm^3) with neutrophilia (11700/mm^3), mild thrombocytopenia (120000/mm^3), and elevated inflammatory markers (CRP 51 mg/L, ESR 17 mm/h, fibrinogen 416 mg/dl). Serological tests for atypical pathogens (*Chlamydia, Mycoplasma, Coxiella, Legionella*) and Quantiferon for tuberculosis were negative. Blood cultures were also negative.

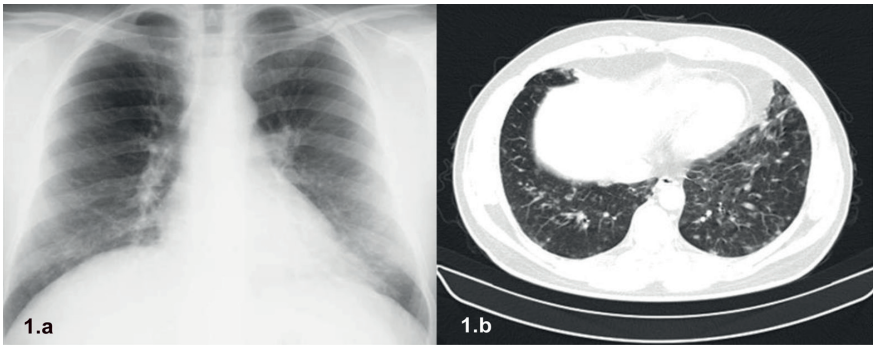

Fig. 1 a Chest X-ray: bilateral micro-nodular alveolar infiltrates, predominantly in inferior areas; b Chest CT-scan: same aspects.

The initial empirical therapy consisted of moxifloxacin for 2 weeks and non-steroidal anti-inflammatory drugs. The clinical course was unsatisfactory except for a partial decline of fever in the first days but a persistent lowgrade fever remained. A broncho-alveolar lavage (BAL) was performed 10 days after admission. Cytology of the BAL fluid was consistent with hemorrhagic lymphocytic alveolitis. No microorganisms were observed during direct microscopical examination. However on the Sabouraud's glucose agar (SGA) there was a growth of colonies with cottony aerial hyphae which were white, with a light shade of purple and which grew from a pinkish submerged mycelium. The colonies were phenotypically identified as *Fusarium* spp. on the basis of curved, septate conidia (Fig. 2).

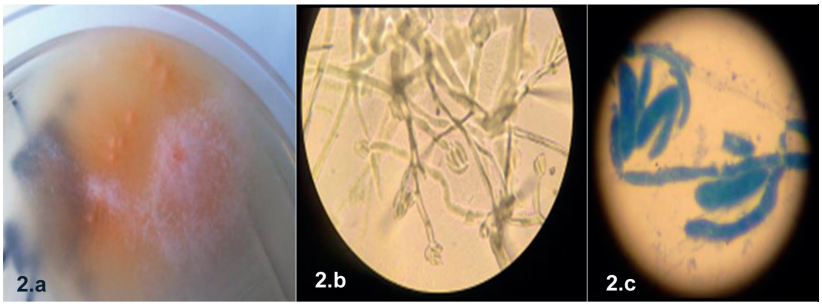

Fig. 2. a Culture on SGA plate: *Fusarium* colonies; b Direct microscopic examination of *Fusarium* with segmented hyphae and conidia x200; c Methylene blue stained slide from *Fusarium* culture with banana-shaped conidia, x1200.

At this point, invasive fungal infection was not demonstrated and the positive *Fusarium* culture was interpreted as fungal colonization in an apparently immunocompetent patient. Subsequent examination of the patient's immune system showed a severe hypogammaglobulinemia (0.13 g/l) involving all three analyzed lines: IgM < 0.17, IgG < 0.89, and IgA < 0.24 (g/l). CD4 T-cells were moderately decreased to 468 per cubic mm (33 %), while CD8 T-cells were 745 per cubic mm (53 %), with a low CD4/CD8 ratio (0.63).

Investigations regarding a possible acquired hypogammaglobulinemia (autoimmune diseases, viral infections including HIV, hematologic malignancies) failed to give a clue, suggesting the final clinical diagnosis as being common variable immunodeficiency (CVID). Bone marrow biopsy was normal. The patient was substituted intravenously with immunoglobulins (25 g/day, 5 days). The diagnosis of the patient's immune deficiency changed the medical judgment of the case, and now an invasive fungal disease being taken into account. Subsequently, voriconazole was added to the therapeutic plan at day 14 after admission (6 mg/kg IV q12h for first 24 h, then 4 mg/kg IV q12h for 2 weeks, then 200 mg orally q12h, with a total duration of six weeks). A voriconazole E test showed an MIC of 2 mg/L. The patient responded with an initial good clinical improvement.

Three weeks after cessation of voriconazole, the patient was re-admitted with productive cough, without fever. Physical examination revealed bilateral, rough vesicular murmurs and a CT-scan showed progressive pulmonary lesions. A significant increase of alveolar infiltrates with extension to the superior regions of the lungs and multiple new spherical dense masses (<5 mm diameter) were observed. A new BAL was performed and the cytology showed the same aspect as few weeks previously (hemorrhagic alveolitis), while the culture was again positive for a *Fusarium* species. IgA, IgG and IgM had again very low values and needed substitution. A second antifungal treatment course with voriconazole was started (same protocol as first course).

A lung biopsy was performed at day 8 after voriconazole reinitiation (3 months after first admission). Immunohistochemical examination excluded lung lymphoma and confirmed a reactive cell pattern (interstitial lymphoid infiltrate). Hyaline hyphae were detected in smears from lung tissue imprints (Fig. 3), suggesting an invasive pulmonary fungal disease.

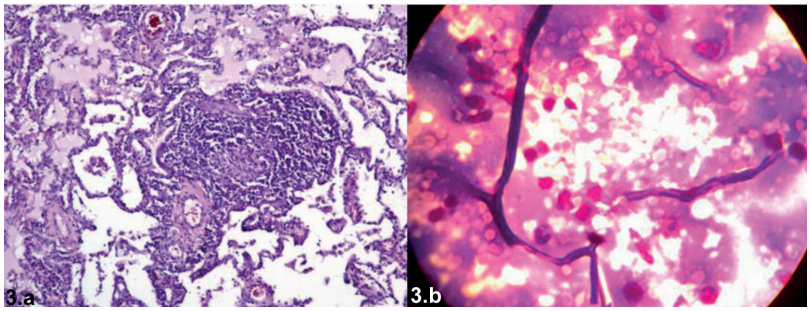

Fig. 3. a Inflammatory lymphocytic nodular and focal infiltrate with fibrosis (HE stain × 100); b Inflammatory reaction and hyphae on pulmonary biopsy smear (Gram stain × 1200).

The patient's immunological status, i.e. the CVID, is a humoral deficit and even in severe forms invasive fungal diseases are rare. Therefore the cellular immune profile was further analyzed and an important qualitative cellular deficiency was additionally found: a defective production of both gamma-interferon- γ (IFN-γ) and IL-17. Deficient cytokine production was demonstrated using a method of whole blood stimulation with specific antigens [5]. The patient's whole blood IFN-γ production, 72 h after stimulation with heat-killed *Candida albicans* yeast cells (CA), phytohemagglutinin (PHA) and staphylococcal antigen (SA), was 16, 1000 and 12 pg/ml, respectively, and was much lower than the production of 7160, >10000 and 1620 pg/ml of healthy volunteers. IL-17 production after stimulation with PHA was 465 and 300 pg/ml in the volunteers, while values were below detection limit in our patient in both in-duplo stimulations. *Candida albicans* and *S. aureus* did not stimulate IL-17 production in the whole blood stimulation system (Table 1).

Table 1. Cytokine production after whole blood stimulation at 24 h (for TNF and for IL-6) and at 72 h (for IFNγ and IL-17)

24 hours		**contr.1**	**contr.2**	**pat.1**	**pat.2**
RPMI		<78	<78	<78	<78
CA	**IFNγ**	3270	3130	1120	890
PHA		960	1360	170	220
SA		4810	7480	2430	2560
24 hours		**contr.1**	**contr.2**	**pat.1**	**pat.2**
RPMI		32	63	48	26
CA	**IL 6**	4700	4100	1730	1560
PHA		1535	1900	265	325
SA		12500	14000	9500	8700
72 hours		**contr.1**	**contr.2**	**pat.1**	**pat.2**
RPMI		10	9	9	<8
CA	**IFNγ**	8940	7160	16	<8
PHA		8300	>10000	1000	834
SA		1280	1620	12	8
72 hours		**contr.1**	**contr.2**	**pat.1**	**pat.2**
RPMI		<40	<40	<40	<40
CA	**IL 17**	<40	<40	<40	<40
PHA		300	465	<40	<40
SA		<40	<40	<40	<40

Whole blood was stimulated either with RPMI culture medium (unstimulated control), with heat-killed *C. albicans* (CA), phytohemagluttinine (PHA) or heatkilled S. aureus (SA). Concentrations of the cytokine produced are expressed as pg/mL.

The evolution was favorable under prolonged antifungal therapy with voriconazole for 6 months and continuous immunoglobulin substitution with 25 g/day, 5 days per month. A CT-scan after 6 months showed regression of the pulmonary lesions. The subsequent BAL was culture-negative for *Fusarium* and no signs of hemorrhagic lymphocytic alveolitis were seen. Antifungal treatment was stopped and during two years of follow-up (CT-scan, respiratory functional tests) no further progression was noted.

Further identification of the fungus was undertaken at the CBS-KNAW Fungal Biodiversity Centre in Utrecht, The Netherlands, under accession number CBS 140388. Sequencing of partial elongation factor 1 alpha (*TEF1*) and β-tubulin (*BT2*) genes was performed. Blast results with sequences in GenBank revealed that this fungus belonged to the *Fusarium fujikuroi* complex. In order to establish the phylogenetic position of this clinical isolate, a general tree was made with MrBayes v. 3.1.2 on the Cipres Portal based on the sequenced *BT2* (500 bp) and *TEF1* (600 bp) regions. Thirty-six species within the *Fusarium fujikuroi* species complex were selected for phylogenetic analyses of combined *BT2* and *TEF1* fragments. Our strain was nested with a *F. ramigenum* subclade (Fig. 4). Sequences of this novel human opportunistic fungus (CBS 140388) were deposited in GenBank with accession numbers KT794172 for BT2 and KT794175 for *TEF1*, respectively.

Antifungal susceptibility testing performed with broth microdilution according to CLSI M38A resulted in the following MICs/MECs: amphotericin B, 1 mg/L; posaconazole, 1 mg/L; itraconazole, >16 mg/L; voriconazole, 2 mg/L; isavuconazole, 4 mg/L and anidulafungin and micafungin both > 8 mg/L.

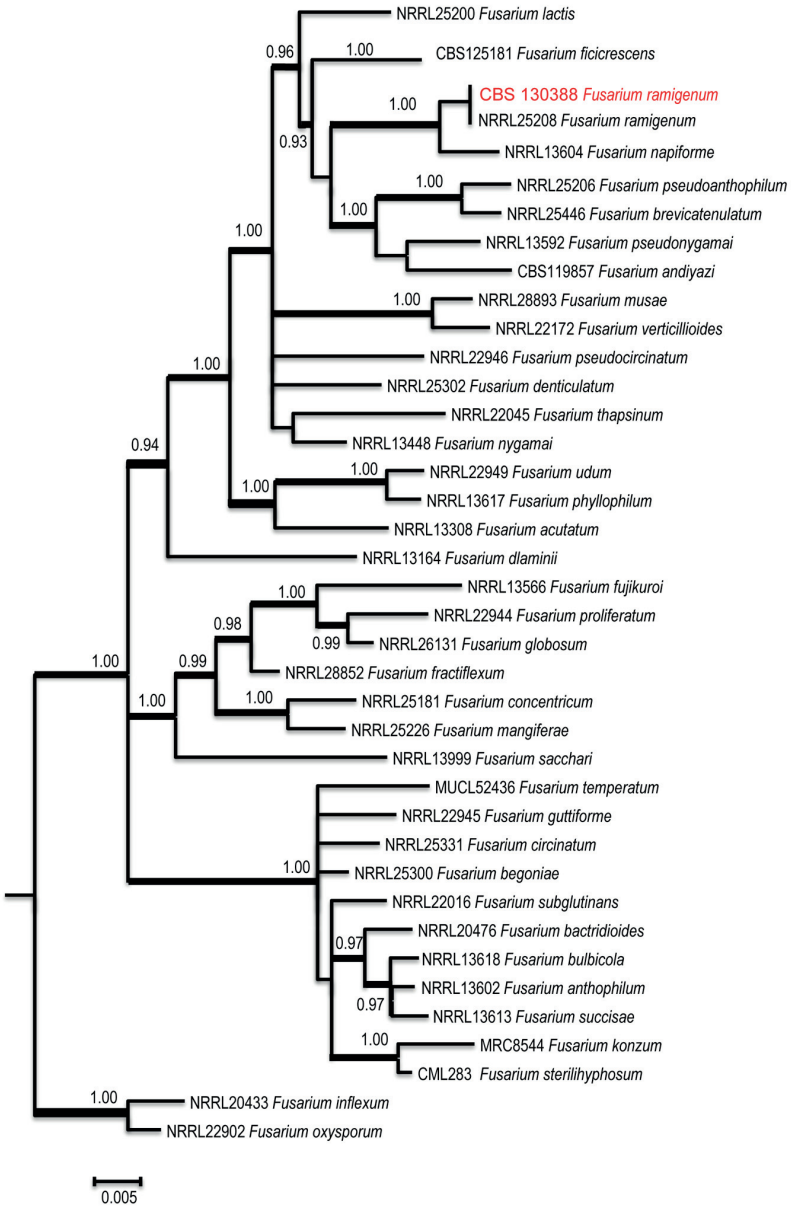

Fig. 4 Phylogenetic analysis of *Fusarium ramigenum*. Phylogenetic reconstruction obtained from Bayesian inference of two combined loci (*TEF1* and *BT2*) using MrBayes v. 3.1.2. Values at branch node indicate branch support with posterior probabilities (PP; values ≥ 0.80 shown) and branches in boldface = bootstrapping percentages based on Maximum Likelihood (ML). The tree was rooted with two strains of *F. inflexum* NRRL20433 and *F. oxysporum* NRRL22902.

Discussion

We describe a patient with CVID and cellular Thelper- defects who developed an invasive infection with *Fusarium ramigenum*. After long-term treatment with voriconazole (6 months) and immunoglobulin substitution, the patient recovered from this opportunistic infection. To the best of our knowledge, this is the first case in which *F. ramigenum* was described as a cause of invasive infection in a human patient, reinforcing the significance of the *F. fujikuroi* species complex as opportunistic pathogens in immunocompromised hosts. The fungus cultured from BAL specimens of this patient with atypical pneumonia and no significant medical history first suggested fungal colonization rather than infection. Moreover, the observed BAL cytology, indicating hemorrhagic lymphocytic alveolitis, could neither prove nor exclude a fungus induced inflammatory reaction. However, the relapse of *Fusarium* infection after a short 6-week course of antimycotic therapy (6 weeks) raised the suspicion of an invasive infection. The microscopic findings of hyaline hyphae in the lung biopsy confirmed the invasive fusariosis. This was further supported by the identification of combined humoral (severe pan-hypogammaglobulinemia) and cellular (defective IFN- γ and IL-17 production capacity) immune defects that are known to be crucial for antifungal host defense [6, 7].

The initial humoral immunological deficit identified, i.e. severe pan-hypogammaglobulinemia, was not consistent with an invasive fungal infection. The slightly lowered CD4 T-cell counts, combined with a reduced T-cell CD4:CD8 ratio, could not explain this opportunistic infection either, and therefore we embarked on functional assays to test the T-helper functions. Subsequent analysis revealed an important deficiency, with very low levels of IFN-γ and a deficit in IL-17 production capacity. Cellular defects in CVID patients have been reported previously, and this is the most likely explanation of the observed infection [8, 9]. The results are in consensus with the latest data in the literature, describing the possibility of complex T-cell abnormalities in association with CVID. T-cell abnormalities associated with CVID generate a slight quantitative deficit of CD4 lymphocytes, an abnormal CD4/CD8 ratio, and a qualitative deficit in cytokine production [10–14]. The exact mechanisms and genetic causes of these defects in CVID remain to be elucidated. Alternatively, a different explanation may be represented by defects in genes known to be crucial for antifungal host defense, such as the CARD9 adaptor [6, 7].

An important aspect of this clinical case is the first identification of a novel human opportunistic fungus, *F. ramigenum* as cause of the infection. This fungus belongs to the relatively frequently encountered *F. fujikuroi* complex, but molecular analysis identified *F. ramigenum*, a species not figuring on the list of species known to occur in human or animal infections [15]. *Fusarium ramigenum* was first described in 1998 from inedible wild Capri figs in California, U.S.A. [16]. The species produced fusaric acid, beauvericin and fumonisin [17]. Its pattern of susceptibility to antimycotic therapy showed potential activity of amphotericin

B, voriconazole and posaconazole and no activity of itraconazole and the echinocandins which is similar to a previous study [18]. The relevance of these *in vitro* data is not clear because a correlation between MICs/ MECs and clinical outcome has not been documented for fusariosis [2]. The MIC of voriconazole of 2 mg/L is below the mode of 4 mg/L as described for *F. fujikuroi* [19] and a retrospective analysis of 73 patients with invasive fusariosis showed 47% success rate [20]. Indeed recently published guidelines recommend voriconazole (AII) or amphotericin B (BII) as treatment option for invasive fusariosis [21].

Conclusion

In summary, this report demonstrated that an opportunistic invasive fungal infection may indicate an underlying cellular immune impairment of the host. The unexpected invasive infection with *F. ramigenum* in this case unmasked complex combined humoral and cellular immunological deficiencies. Moreover, this paper provides evidence indicating *F. ramigenum* as a potential human opportunist especially in immunocompromised patients.

Consent

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor of this journal.

Abbreviations

CT-scan: Computed tomography; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; BAL: Broncho-alveolar lavage; SGA: Sabouraud's glucose agar; IgM: Immunoglobulin M; IgG: Immunoglobulin G; IgA: Immunoglobulin A; CD4: Cluster of differentiation 4; CD8: Cluster of differentiation 8; HIV: Human immunodeficiency virus; CVID: Common variable immunodeficiency; CA: Candida albicans yeasts; PHA: Phytohemagglutinin; SA: Staphylococcal antigen; IL-17: Interleukin 17; *TEFI1*: Translation elongation factor1; *BT2*: Beta-tubulin; CLSI: Clinical and Laboratory Standards Institute; CLSI M38-A: CLSI microtiter mould testing standard methods for antifungal susceptibility; MIC: Minimum inhibitory concentration; IFN γ: Gammainterferon; CARD9: Caspase recruitment domain containing protein 9.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

We thank Dr. Florin Alexandru Caruntu, the Chief of Adults I Department Matei Bals Institute, where the patient is in care. We thank also Dr. Mona Popoiu and Dr. Daniela Talapan, from Bacteriology Department – Matei Bals Institute, who provided technical support. MGN was supported by an ERC Consolidator Grant (#310372). The work of Abdullah M. S. Al-Hatmi was financially supported for his PhD study by Ministry of Health, Oman (Formal Agreement no. 28/2014).

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CHAPTER 8

Chapter 8

Keratitis by *Fusarium temperatum*, a novel opportunist

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BMC Infectious Diseases 2014; 14:588.

Abstract

Background: *Fusarium* species are among the most common fungi present in the environment and some species have emerged as major opportunistic fungal infection in human. However, in immunocompromised hosts they can be virulent pathogens and can cause death. The pathogenesis of this infection relies on three factors: colonization, tissue damage, and immunosuppression. A novel *Fusarium* species is reported for the first time from keratitis in an agriculture worker who acquired the infection from plant material of maize. Maize plants are the natural host of this fungus where it causes stalk rot and seeding malformation under temperate and humid climatic conditions. The clinical manifestation, microbiological morphology, physiological features and molecular data are described. **Methods:** Diagnosis was established by using polymerase chain reaction of fungal DNA followed by sequencing portions of translation elongation factor 1 alpha (*TEF1*) and β-tubulin (*BT2*) genes. Susceptibility profiles of this fungus were evaluated using CLSI broth microdilution method. **Results:** The analyses of these two genes sequences support a novel opportunist with the designation *Fusarium temperatum*. Phylogenetic analyses showed that the reported clinical isolate was nested within the *Fusarium fujikuroi* species complex. Antifungal susceptibility testing demonstrated that the fungus had low MICs of micafungin (0.031 μg/ml), posaconazole (0.25 μg/ml) and amphotericin B (0.5 μg/ml). **Conclusion:** The present case extends the significance of the genus *Fusarium* as agents of keratitis and underscores the utility of molecular verification of these emerging fungi in the human host.

Key words: Keratitis, *Fusarium temperatum*, Maize, Molecular phylogenetics, Infection

Background

Fungal keratitis was first reported by Theodor Leber in 1879 in a farmer who had an eye trauma due to blades used for cutting wheat. Today the infection is known to occur worldwide, particularly in warmer climates, and is caused by a large diversity of fungal species. In temperate regions, fungal keratitis is most commonly caused by *Fusarium* species [1]. Under (sub) tropical conditions, filamentous fungi are prevalent as causes of infection. Particularly *Fusarium* and *Aspergillus* predominate, with up to one-third of cases of traumatic keratitis [2,3]. On a global scale, fusariosis is one of the most common causes of fungal corneal ulcers [4-6].

Keratitis caused by *Fusarium* is a serious infection and occurs especially among farmers and workers with agricultural occupations. Corneal abrasions occur commonly during harvest, when labor handling decayed and dried plant products is a major risk factor for ocular trauma [7-9]. Fungi are one of the possible causes of keratitis and are differentially susceptible to commonly used antifungals. Therefore misdiagnosis potentially leads to visual loss and devastating ocular damage if the infection remains untreated [10].

Fusarium is a genus of more than 200 species of molds that are widely distributed in soil, on terrestrial plants, in plant debris and on other organic substrates. Numerous agents of diseases of plants and coldblooded animals are known [11], but only a few have been recognized as causing infections in humans [12]. *Fusarium* infections in immunocompetent hosts are mostly associated with superficial mycosis such as onychomycosis and keratitis; the first reported case of a *Fusarium* eye infection dates back to 1958 [13]. Recently, deep and systemic infections are observed in immunocompromised patients, with increasing frequency [14].

Here we present an extraordinary case of keratitis in a worker who acquired the infection from plant material of maize. The case is worth reporting not only by its rareness but also its unusual infection in a human. In the present report, this case was initially ascribed to *Fusarium oxysporum* based on the morphological characters. Since morphological studies are insufficient to determine the correct taxonomic position at the species level in *Fusarium*, a multilocus DNA sequence study followed by phylogenetic analysis was applied to identify the agent of this case. As a result, *Fusarium temperatum* is reported as a new causative agent of human keratitis.

Case report

An agriculture worker presented with a corneal ulceration. The disorder had started 12 days earlier when patient suffered from a trauma in the eye with maize plant materials during harvest. Before presenting to the hospital, the patient was seen by a local practitioner who prescribed neomycin, polymyxin B, phenylephrine and dexamethasone eye drops for eight days, supposing that a bacterial infection was concerned. On presentation, visual acuity was 20/50 by using Snellen chart. Slitlamp examination of the right eye showed a 12 mm white-yellowish central corneal epithelial defect with irregular and raised edges, along with intense hyperemia of the

conjunctiva, photophobia and pain (Figure 1A).

Corneal scrapings for direct microscopic examination with 10% potassium hydroxide demonstrated multiple irregular, septate, hyaline hyphae (Figure 1A). Samples were inoculated onto Sabouraud's glucose agar (SGA) plates and incubated for 5 days at 28 °C. Fluffy, pink-violet colonies rapidly developed, which microscopically revealed abundant sickle-shaped, septate macroconidia (4–5 μm in length) in addition to microconidia (2–3 μm in length). On the basis of culture and microscopy, the fungus was provisionally identified as *F. oxysporum*. Antifungal treatment was initiated with topical natamycin 5% (Laboratorios Grin, Distrito Federal, Mexico) ophthalmic solution, as follows: 2 drops each hour during eight days, then one drop each 4 hours, in addition to itraconazole 200 mg daily. Noteworthy improvement was achieved, and the patient did not return for follow up visits.

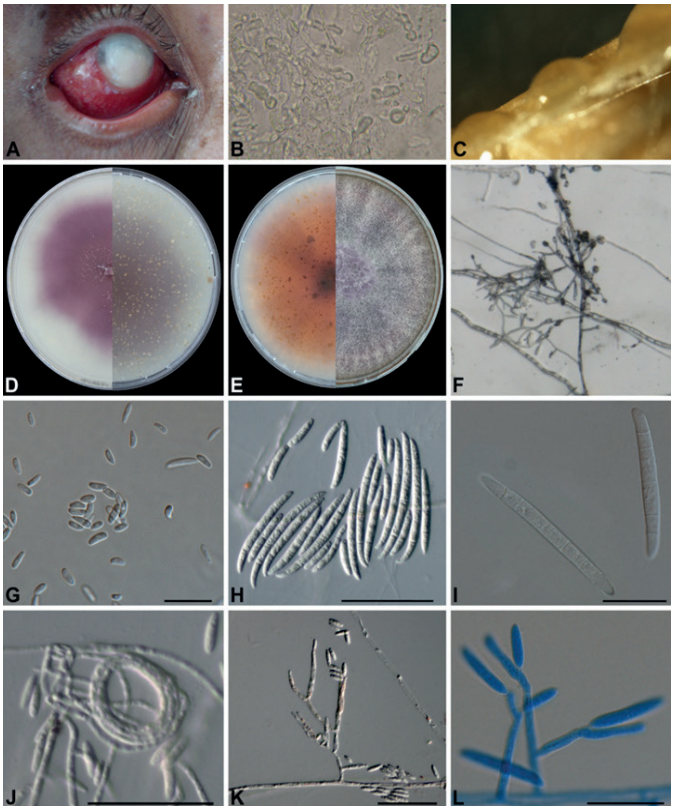

Fig 1. Morphological description of *Fusarium temperatum*. (A) Slit lamp photograph showing infected cornea involving regions of sclera; (B) KOH mount of the scraping material showing fungal hyaline and nonseptate hyphae (magnification, ×40); (C) Sporodochia present in yellowish orange on CLA; (D) Growth of the isolate *F. temperatum* on OA, agar pigmentation ranges from colorless to dark purple on; reverse pigmentations in light pink; (E) Growth of isolates on PDA at 25 °C; (F) In situ conidiophores with false heads; (G) Microconidia on CLA; (H-I) Macroconida; (J) Coild hyphae; (K-L) Monophialidic and polyphialidic conidiogenous cells. All scale bars, 10 μm.

Methods

Ethics statement

The study protocol was approved by the Scientific and Ethics Committees of the Hospital General de México (approval number DIC/12/102/3/23) and was performed in accordance with the ethical principles described in the 1964 Declaration of Helsinki. Informed written consent was obtained from the patient prior to their inclusion in the study.

Clinical specimen

Corneal scrapings were collected for microbiological studies from a patient seen in Hospital General de México, O.D, Mexico City, Mexico.

Fungal isolation

Corneal scrapings were inoculated onto Sabouraud's glucose agar (SGA) plates and incubated for 5 days at 28 °C. Subcultures of the causative agent were deposited in the reference collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands under accession number CBS 135540, where further identification was undertaken.

Morphology

The fungus grew on culture plates of malt extract ager (MEA; Oxoid, U.K.), oatmeal agar (OA; home-made at CBS), potato dextrose agar (PDA; Oxoid), synthetic nutrient agar (SNA) [15], and carnation leaf agar (CLA) [16]. Culture plates were incubated in the dark for one week at 25 °C. Microscopic mounts in lactic acid with cotton blue were made from cultures grown on a PDA plate. Slide cultures were observed after 5 days of incubation at 25 °C. Slides were examined and measured with a light microscope (Nikon Eclipse 80i), and pictures were taken using a Nikon digital-sight DS-5 M camera attached to the microscope.

Physiology

Cardinal growth temperatures were determined on PDA with isolates incubated in the dark for one week at 25, 27, 30, 33, 35, 36, 37 and 40 °C.

DNA extraction

DNA was extracted following the Quick CTAB protocol. 1–10 mm^3 fungal material was transferred to two mL screw-capped tubes filled with 490 µL CTAB-buffer 2×and 6–10 acid-washed glass beads. 10 µL Proteinase K were added and mixed thoroughly on a MoBio vortex for 10 min. 500 µL Chloroform: isoamylalcohol (24:1) was added and shaken for 2 min after incubation for 60 min at 60 °C. Tubes were centrifuged for 10 min at 14,000 r.p.m. The supernatant was collected in a new Eppendorf tube. To ~400 µL DNA sample 2/3 vol

(~270 µL) of ice-cold iso-propanol was added and centrifuged again at 14,000 r.p. m. for 10 min and the upper layer was dissolved in 1 mL ice-cold 70% ethanol. Tubes were centrifuged again at 14,000 r.p.m. for 2 min, air-dried and re-suspended in 50 µL TE-buffer. The quality of genomic DNA was verified by running 2–3 µL on a 0.8% agarose gel. DNA was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher, Wilmington, U.S.A.). Samples were stored at −20 °C until use.

DNA amplification and sequencing

Two gene regions were amplified directly from the genomic DNA for multilocus sequence typing. The primer pairs for the genes were EF1 and EF2 [17], BT-2a [18] and BT-2b [19] (primers listed in Table 1). PCR reaction mixture (12.5 µL final vol) contained 10 × PCR buffer 1.25 µL, water 7.5 µL, dNTP mix (2.5 mM) 0.5 µL, 0.25 µL of each primer (10 pmol), Taq polymerase (5 U/µL) 0.05 µL, DMSO 0.7 µL, and template DNA (100 ng/µL) 1 µL. Amplification was performed in an ABI PRISM 2720 (Applied Biosystems, Foster City, U.S.A.) thermocycler as follows: 95 °C for 4 min, followed by 35 cycles consisting of 95 °C for 45 sec, 52 °C for 30 sec and 72 °C for 2 min, and a delay at 72 °C for 7 min. Annealing temperature was changed to 58 °C for the *BT2* gene. PCR products were visualized by electrophoresis on a 1% (w/v) agarose gel. Amplicons were purified using exoSAP. Both strands of the PCR fragments were sequenced with the above-mentioned primers. The ABI PrismH Big DyeTM Terminator v. 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) was used for sequencing PCR. Sequences were determined with an ABI PRISM™ 3,100 Genetic Analyzer (Applied Biosystems). Sequencing PCR was performed as follows: 1 min at 95 °C, followed by 30 cycles consisting of 10 sec at 95 °C, 5 sec at 50 °C and 2 min 60 °C. Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems) with ABI PRISM BigDyeTM terminator cycle sequencing kit.

Table 1. PCR primers used for amplification

Locus	Primers	Primer sequence (5'-3')	Size (bp)	Ref
TEF1	EF1	ATGGGTAAGGARGACAAGAC	600	[17]
	EF2	GG ARGTACCAGTSATCATGTT	600	[17]
BT2	BT-2a	GGTAACCAAATCGGTGCTGCTT	500	[18]
	BT-2b	TTACGTCCCTGCCCTTTGTA	500	[19]

Phylogenetic analyses

A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNAstar, Madison, WI). Thirty eight sequences of species of the *Fusarium fujikuroi* species complex (FFSC) were included and retrieved from GenBank, including two sequences of the *F. oxysporum* species complex (FOSC) for *TEF1* and *BT2* markers as an outgroup. The sequences were aligned using MAFFT v.7.127 (http://mafft.cbrc.jp), followed by manual adjustments with MEGA v. 5.2. A combined alignment was constructed for both *TEF1* and *BT2* markers. The best-fit model of evolution was determined by ModelTest v. 0.1.1. Bayesian analysis was performed with MrBayes v. 3.1.2. Four MCMC chains were run simultaneously for 1×10^7 generations. Bayesian phylogenetic tree was constructed. Sequences of CBS 135540 were deposited in GenBank under the accession numbers [GenBank:KF956084] for *TEF1* and [GenBank:KF956080] for *BT2*.

Antifungal susceptibility

Antifungal susceptibility testing (AFST) of CBS 135540 was performed by the CLSI broth microdilution method, M38-A2 [20]. The antifungals tested were amphotericin B (Sigma, St. Louis, MO), fluconazole (Pfizer, Groton, CT), itraconazole (Janssen Pharmaceutica, Tilburg Netherlands), voriconazole (Pfizer), posaconazole (Merck, Whitehouse Station, NJ), isavuconazole (Basilea Pharmaceutica, Basel, Switzerland), micafungin (Astellas, Ibaraki, Japan), anidulafungin (Pfizer) and natamycin (DSM, Delft, the Netherlands). For the broth microdilution test, RPMI 1640 medium with glutamine without bicarbonate (Sigma) buffered to pH 7 with 0.165 mol/liter 3-N-morpholinepropanesulfonic acid (Sigma) was used. Isolates were grown on PDA for 5 days and incubated at 25 °C and for sporulation. Final inoculum was adjusted to a density of 1.0 to 5.0×10^4 hyphal fragments/spores per ml by adjusting an optical density of 0.13 to 0.18 at 530 nm using a spectrophotometer. Drug-free and mold-free controls were included, and microtiter plates were incubated at 35 °C for 72 to 96 h. Three reference strains. *Paecilomyces variotii* ATCC 22319, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included as quality controls. The MIC endpoints were read visually, which, for azoles and amphotericin B, were defined as the lowest concentration at which there was 100% inhibition of growth compared with the drug-free control wells. For echinocandins, minimal effective concentrations (MEC) were defined as the lowest concentration of drug that led to the growth of small, rounded, and compact hyphal forms.

Results

Morphology

The clinical isolates grew and sporulated well on PDA, OA, CLA and SNA at 25, 27, 30 and 33 °C and growth was apparent within 2 days on all agar plates. Yellowish orange sporodochia

were produced on CLA (Figure 1C). Agar pigmentation ranged from colorless to dark purple on OA; pigmentation of colony reverse was in shades of light pink (Figure 1D). Aerial mycelium was cottony, initially white, becoming pinkish white, turning violet in the colony center in a later stage. Subsequently, colonies spread rapidly, filling the culture plate within 1 week (Figure 1E). Conidiophores in the aerial mycelium were erect, branched, terminating in 1–3 phialides (Figure 1F). On SNA with filter paper, colonies were colorless, later changing the color of the filter papers to pale pink. Microconidia were oval, abundant, grouped in masses; hyaline and non-septate (Figure 1G). Macroconidia hyaline, with 3–6 (mostly 4–5) septa, slender, slightly falcate, with a beaked, curved apical cell and a foot-like basal cell with a thin cell wall (Figure 1H, I). Polyphialides and monophialides were observed (Figure 1K, L). Chlamydospores were not found over 10 days of incubation.

Physiology

Cardinal growth temperatures tests showed optimal development at 25 − 27 °C (Figure 2), with a maximum growth temperature at 36 °C. No growth was observed at 37 and 40 °C. 37 °C proved to be fungistatic, but regrowth was observed after incubation at 25 °C. Colonies on PDA attained a diameter of approximately 65 mm, and those on OA covered the entire agar surface after 5 days at 25 ° C. Colonies attained a diameter of about 68 mm at 27 °C in the dark on PDA. Colonies on PDA plates incubated at 33 °C showed slow growth and attained a diameter of about 32 mm after 5 days.

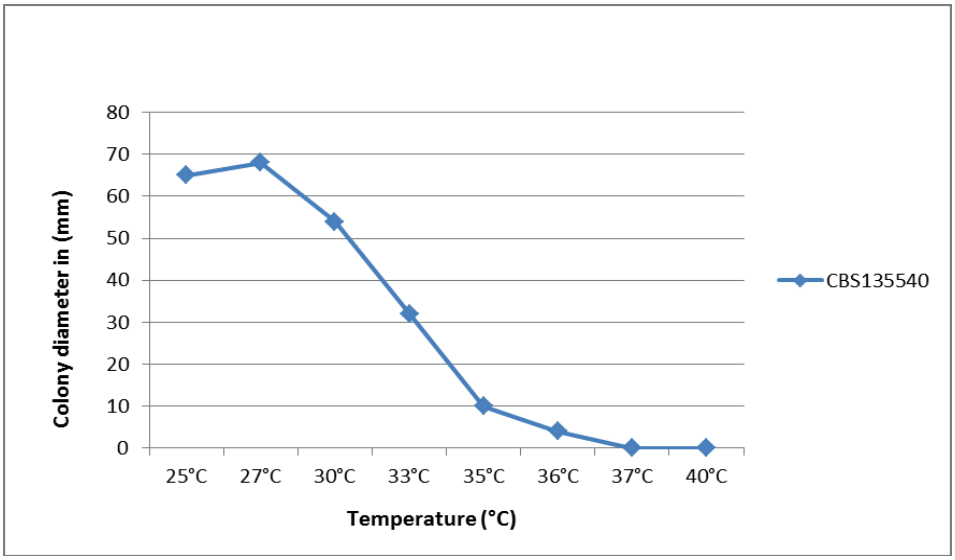

Fig 2. Average growth of *F. temperatum*. Colony diameters (mm) at different temperatures ranging from 25 °C to 40 °C, measured after 5 days of incubation on 2% MEA, were calculated for *F. temperatum*, CBS135540.

Phylogeny

BT2 and *TEF1* partial genes (NCBI JX987074.1 for *TEF1* and KC964140.1 for *BT2*) were used for identification of clinical isolate CBS 135540. Both genes possessed enough polymorphism, and therefore, were excellent markers with 99–100% accuracy for the identification of *Fusarium* species to be *Fusarium temperatum* within the *Fusarium fujikuroi* species complex. No data were available in the *Fusarium* MLST database (http:// www.cbs.knaw.nl/fusarium) for this isolate.

In order to establish the phylogenetic position of the *F. temperatum* clade, a general tree was made with MrBayes v. 3.1.2 on the Cipres Portal based on the *BT2* (500 bp) and *TEF1* (600 bp) regions. Fifteen species within the *Fusarium fujikuroi* species complex clade were selected for phylogenetic analyses and sequences of the *BT2* and *TEF1* genes were aligned among the sequences available from GenBank. Bayesian analysis was done by using Metropolis-coupled Markov chain Monte Carlo sampling approach to calculate posterior probabilities. Four simultaneous Markov chains, three heated and one cold, were run under a mixed model of sequence evolution and gamma approximation for rate variation among sites. Chains were analysed with random starting trees for 10^7 generations, sampling from trees every 1000^{th} generation. The burn-in period was set at 25%. Topologies of the trees generated with either gene (*TEF1* and *BT2*) were concordant. Partition Homogeneity Test (PHT = 0.97) did not detect conflict between loci, and therefore, these two genes were combined to investigate species delimitation using PSR (Figure 3). The *BT2* and *TEF1* phylogenetic analyses showed that the reported clinical isolate was nested within the *Fusarium fujikuroi* species complex and was found to be identical to four environmental strains of *F. temperatum*.

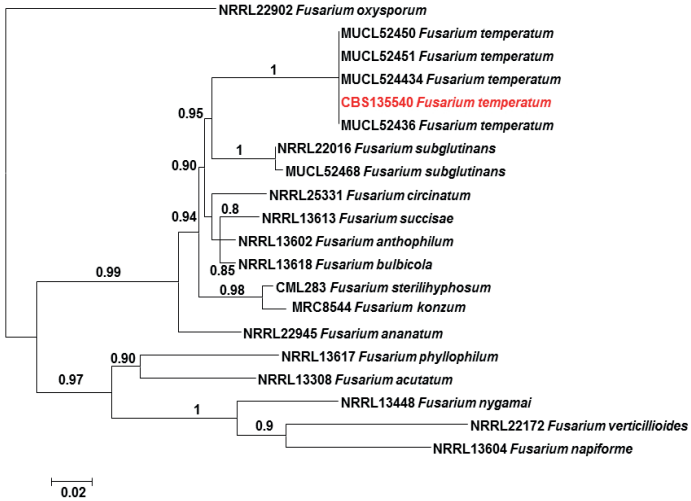

Fig 3. Phylogenetic analysis of *F. temperatum*. Phylogenetic tree resultingfrom Bayesian analysis for the *TEF1* and *BT2* genes (values of 0.8 for Bayesian probability are shown). *F. oxysporum* (NRRL 22902) was used as the outgroup.

Antifungal susceptibility

Susceptibility testing was performed according to the guidelines of the Clinical and Laboratory Standards Institute document M38-A2. Our strain was highly susceptible to micafungin with an MIC of 0.031 µg/ml, followed by posaconazole with an MIC of 0.25 µg/ml and amphotericin B with an MIC of 0.5 µg/ml. Low MICs were also found for voriconazole (1 µg/ml) followed by isavuconazole, natamycin and anidulafungin, with MICs of 4 µg/ml. The azoles for which high MICs were found were fluconazole (>64 µg/ml) and itraconazole (>16 µg/ml) (Table 2).

Table 1. MIC values of clinical isolate *F. temperatum*, CBS 135540

Drug	**AMB**	**FLC**	**ITC**	**VOR**	**POS**	**NATA**	**ISA**	**ANI**	**MICA**
MIC values(µg/ml)	0.5	>64	>16	1	0.25	4	4	4	0.031

AMB: Amphotericin B, FLC: Fluconazole, ITC: Itraconazole, VOR: Voriconazole, POS: Posaconazole, ISA: Isavuconazole, NATA: Natamycin, ANI: Anidulafungin, MICA: micafungin

Discussion

Mycotic keratitis is an important ophthalmologic problem with slow progression that must be distinguished from its bacterial counterpart [21] in view of appropriate treatment. Despite developments in diagnostics and therapy, the infection remains a significant public health problem, occasionally leading to significant visual disability [22]. *Fusarium* species are among the most common etiologic agents of the disorder [23-25] and are problematic because of their therapy-refractive nature. In direct microscopy *Fusarium* species are indistinguishable from *Aspergillus* because both produce hyaline, septate hyphae (Figure 1B), and therefore supplementary diagnostics are necessary. For accurate identification of the causative agent, multi-locus analysis involving of parts of *TEF1* and *BT2* genes, known to be informative at the species level in *Fusarium* [19,26-28] was performed. Identification of the etiological agent as *F. temperatum* was unambiguous. The present case extends the significance of genus of *Fusarium* as agents of keratitis and underscores the utility of molecular methods in verification of these emerging fungi in the human host [29].

Fusarium species are distributed worldwide in a wide diversity of habitats such as soil, plant debris, and as pathogens on a wide diversity of plant hosts [30]. Some species synthesize mycotoxins, which may accumulate in infected plant tissue before harvest or in stored agricultural products where they can be harmful for humans [31]. *Fusarium* in agricultural products should not only be considered as a food spoilers, but also are a risk factor for farmers and harvesters dealing with infected farming material [32], causing traumatic infections.

A remarkable feature in *Fusarium* is the apparent combination of plant pathogenicity and the ability to cause infections in humans. A study of members of the *Fusarium* solani species complex demonstrated that strains from clinical specimens, sewage, and plants were all capable of infecting zucchini and growing at 37 °C [33]. Zhang et al. [34] noted that a group of clinical isolates were identical to *F. solani* f. sp. *cucurbitae* race 2, which infects squashes [35]. Conversely, *F. oxysporum* f. sp. *lycopersici* race 2 killing tomato plants was also able to cause disseminated, fatal infection in a murine model [36]. Several studies reported *Fusarium subglutinans* as an important pathogen of maize, causing stalk and seeding malformation [36,37], but the species has been reported as a human opportunist [38,39].

Scauflaire et al. [40] examined the taxonomic status of 30 *Fusarium* strains isolated from maize fields in Belgium including three isolates named *F. subglutinans* and described them as a new species, *F. temperatum* [40]. Subsequently the authors [41] used different pathogenicity tests such as toothpick inoculation to obtain better understanding of the infection process in maize plants and concluded that *F. temperatum* is a host-specific plant pathogen. The species causes seedling blight chlorosis and forms necrotic lesions in maize stalks; in addition, the species produces mycotoxins. Pintos et al. [42] reported *F. temperatum* seedling malformations, chlorosis, shoot reduction and stalk rot in maize growing in inoculated soil.

The present case concerns a further example of a plant pathogen in addition to 70 other *Fusarium* species causing human infection. Such examples are expected to be rare, because in general degradation of plant versus animal components requires entirely different enzymatic machinery. Van Baarlen et al. [43] noted molecular similarity between hypothetical virulence factors in plant and human pathogens, but in practice such species are extremely uncommon [44]. Our patient developed a mycotic keratitis after traumatic introduction with maize leaves during harvest. Its clinical diagnosis was made by the ophthalmologist. At the clinical laboratory, it was first misdiagnosed as *F. oxysporum* using morphology. These results indicate that morphologic identification has some limitations.

Cardinal growth temperatures showed optimal development at 27 °C (Figure 2), with a maximum growth temperature at 36 °C. No growth was observed at 37 °C and 40 °C; these temperatures proved to be fungistatic as regrowth was observed after incubation at 25 °C. The relatively low maximum growth temperature allows superficial infections such as keratitis. Our patient's infection was first clinically diagnosed as a reactional or bacterial ophthalmitis, leading to the use of a preparation that contained two antibiotics, a decongestant (vasopressor), and a synthetic glucocorticoid. It is noteworthy to record that most cases of keratitis are treated on the basis of clinical features with antibiotics and steroids, which are compounds that stimulate fungal growth [45], while co-inoculation of a fungal agent is common. At observation of fungal hyphae in tissue, therapy was changed to natamycin and itraconazole.

The high susceptibility of CBS 135540 was unexpected because of the intrinsic resistance against antifungal drugs in *Fusarium* in general [46-48]. The *F. temperatum* isolates had low MICs for micafungin (0.031 μg/ml), posaconazole (0.25 μg/ml) and amphotericin B (0.5 μg/ml). Antifungal susceptibility testing proved that itraconazole and fluconazole were ineffective, and therefore clinical improvement of our patient was probably entirely due to natamycin because the present study shows that the natamycin had good activity against *F. temperatum* (4 μg/ml). Patient was not available for follow-up. In similar cases of keratitis, natamycin, anidulafungin, micafungin, voriconazole and amphotericin B were found to be effective [49].

Conclusion

Fusarium species in general show high degrees of resistance to most antifungals. Using molecular identification to identify a number of rare medically important fungal genera and species, including *Fusarium*, is very important to predict therapeutic outcome and the emergence of these Fungi. Moreover, this paper provides evidence indicating *F. temperatum* as a potential human opportunist that may have decreased susceptibility to azoles and other antifungal agents.

Abbreviations

TEF1 : Translation elongation factor 1 alpha; *BT2*: Beta-tubulin; MLST: Multilocus sequence typing; MEA: Malt extract agar; OA: Oatmeal agar; PDA: Potato dextrose agar; SNA: Synthetic nutrient agar; CLA: Carnation leaf agar; PCR: Polymerase chain reaction; MIC: Minimum inhibitory concentration; AMB: Amphotericin B; FLC: Fluconazole; ITC: itraconazole; VOR: Voriconazole; POS: Posaconazole; NATA: Natamycin; ISA: Isavuconazole; ANI: Anidulafungin; MICA: Micafungin; MEC: Minimal effective concentrations.

Competing interests

The authors declare that they have no competing interest.

Acknowledgment

We thank Kasper Luijsterburg for providing the photographic plates. The work of Abdullah Mohammed Said Al-Hatmi was financially supported by Ministry of Health, Muscat, Oman.

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CHAPTER 9

Chapter 9

Fusarium species causing eumycetoma: Report of two cases and comprehensive review of the literature

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Mycoses 2016; (in press).

Abstract

Recently mycetoma was added to the World Health Organization's list of neglected tropical disease priorities. One of the causative agents of eumycetoma is *Fusarium*, a genus known to be resistant to most antifungal agents. The aim of the present study was to review *Fusarium* agents of cases where molecular data are available. The partial translation elongation factor 1-alpha (*TEF1*) gene was applied as diagnostic parameter. Two additional cases of eumycetoma, due to *F. keratoplasticum* and *F. pseudensiforme*, respectively, are presented. A systematic literature review was performed to assess general features, identification, treatment and outcome of eumycetoma infections due to *Fusarium* species. Of twenty reviewed patients, the majority (75%) were male. Most agents belonged to the *F. solani* species complex, i.e. *F. keratoplasticum*, *F. pseudensiforme*, and undescribed lineage of *F. solani*. In addition, *F. thapsinum,* a member of another species complex was encountered. The main antifungal drugs used were itraconazole, ketoconazole, and amphotericin B but cure rates were low (15%). Partial response or relapse was observed in some cases and a case ended in amputation. Medical management of eumycetoma due to *Fusarium* is complex and a decision of combination therapy might be required to increase cure rates.

Key words: *Fusarium*, mycetoma, *TEF1*, molecular diagnostics, review

Introduction

Mycetoma is an infectious disease which can be caused by bacteria (actinomycetoma) or by fungi (eumycetoma). The disease develops after traumatic implantation of the etiologic agent in cutaneous or subcutaneous tissues. Pedal mycetoma is the most common, but also other body parts can be affected.[1,2] Main characteristics of mycetoma are the triad of tumefaction, draining sinuses and presence of grains in the exudates. In eumycetoma, the grains are compacted hyphae of the infecting agent and are covered with a cement-like matrix. Grain color is determined by pigments present in or excreted by the causative agent.[3] Grains of eumycetoma are colorless to yellow, or black, which is due to hyaline or melanized agents, respectively.[4]

Reports of human mycetoma are increasing from African countries, India and the Middle East.[5] The infection is uncommon in temperate climate zones, but does occur in the southern USA and in Mexico.[6] The most affected population are those who lives in rural areas and their work activities pose them at risk of traumatic injury and thus, greater exposure to the causal agents, besides having limited access to services of health.[7] In endemic areas in Africa, eumycetoma is commonly caused by *Madurella mycetomatis,* which produces black grains. The high prevalence of disease in these areas has been explained by environmental exposure to the pathogen, to insufficiently protective footwear, as well as to certain host immune-factors influencing susceptibility to the infection.[8] In Mexico and in Latin American countries the most common cause of mycetoma is of bacterial origin (actinomycetoma).[9-11]

To date, many fungal agents of eumycetoma have been reported in the literature, belonging to an array of the unrelated genera *Aspergillus*, *Biatriospora, Cladophialophora, Curvularia*, *Exophiala*, *Falciformispora*, *Fusarium*, *Madurella*, *Medicopsis*, *Neotestudina*, *Phaeoacremonium*, *Pleurostomophora*, *Pseudochaetosphaeronema, Scedosporium,* and *Trematosphaeria*.[5,8] Correct diagnosis and prognosis of eumycetoma cases depend on culture and identification of the causative agent, and determination of its susceptibility to antimicrobial agents.[4]

Fusarium is a large genus of filamentous fungi with more than 300 species. Numerous species are important plant pathogens on economically important crops.[12] They reside in soil and enter host plants as endophytes leading to fulminant plant disease. Melanin is absent from hyphae and therefore human infections are classified in the hyalohyphomycosis group with white grains.[13] Reports of human fusariosis are increasing globally due to improved recognition in the laboratory, and also because of increasing immunocompromised host populations.[13] However, mycetoma due to *Fusarium* is typically found among immunocompetent individuals.

Peloux and Segretain[14] documented the first case of *Fusarium* causing white grain eumycetoma in Senegal. Katkar *et al*.[15] stated that some earlier reports of white grain mycetoma due to '*Cephalosporium*' or '*Acremonium*' species actually might have been *Fusarium* cases, because some species might fail to produce macroconidia leading to misidentification.[16] This

hypothesis was confirmed by Ajello *et al*.[17] who restudied such a case and found it to be caused by *F. moniliforme* (syn. *F. verticillioides*).

The genus *Fusarium* is divided into twenty two species complexes, ten of which are known to contain members causing human fusariosis.[13] To date, only members of the *F. solani* and *F. fujikuroi* species complexes have been confirmed to cause mycetoma.[17-20] Here we describe two cases of eumycetoma, due to *F. keratoplasticum* and *F. pseudensiforme,* in immunocompetent individuals. In addition, we sequenced agents of *Fusarium* mycetoma cases of which voucher strains are maintained in the reference collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands, and review the respective literature.

Case presentation

Case 1: A 64-year-old male farmworker from Villaflores, Chiapas (southern Mexico), attended the Department of Dermatology with an occasionally painful lesion of two years duration located on the right ankle which was characterized by a single sinus draining bloody material. The lesion developed after a trauma. Laboratory findings were within normal ranges. X-rays of the ankle showed radiolucent areas in the periosteum of tibia and fibula. Presumptive clinical diagnosis was osteosarcoma *versus* mycetoma. The diagnostic protocol included a biopsy for histopathology, which showed a granuloma, with lymphohistiocytic infiltration and eosinophilic eumycetoma grains (H & E and PAS staining). A preliminary diagnosis of white grain mycetoma was made (Fig. 1). KOH examination of samples from fistula exudates showed grains consisting of multiple, interwoven, hyaline hyphae. Cultures on Sabouraud's glucose agar showed whitish colonies which became dirty brown; microscopically, short and long macroconidia, spindle-shaped microconidia and multiple chlamydospores were observed. The preliminary morphological identification of this fungus was *F. chlamydosporum*. Molecular identification revealed that *F. keratoplasticum* was concerned (CBS 135530).

Based on this diagnosis and on phenotypic studies, treatment with itraconazole 200 mg/day for 14 months was started, with significant improvement of the inflammatory process, however a relapse occurred and thus it was decided to switch to terbinafine 250 mg/day for 10 months. Clinical and mycological cure was achieved and no relapse was observed during follow up.

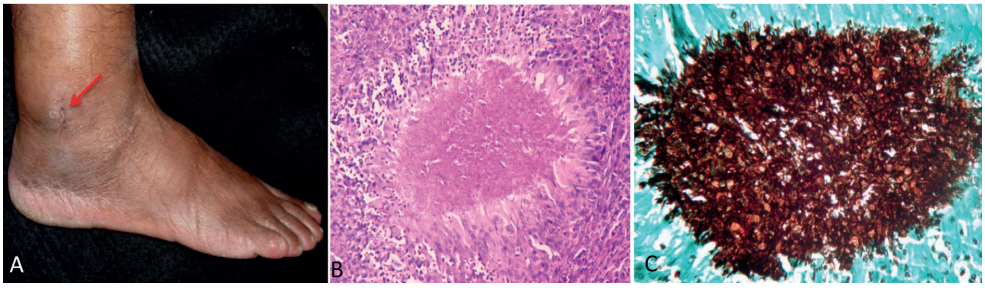

Fig 1. Mycetoma due to *F. keratoplasticum*. (a) Eumycetoma of the right foot of the patient (case 1) after treatment and surgery. (b) Inflammatory lesion characterised by lymphohistiocytic infiltrate and eosinophillic eumycetoma grains (H&E). (c) Fungal grain (Grocott).

Case 2: A 37-year-old male patient, corn and sugarcane farmworker from Morelos Alpuyeca (Central Mexico) attended the Department of Dermatology with a 3-year duration of left foot inflammation. Previous treatment included significant surgical debridement, extending to the distal third of the leg. On physical examination, a 15 × 12 cm exophytic lesion with multiple draining sinuses was observed; patient complained of pain. Laboratory results were in normal ranges. Presumptive clinical diagnosis of actinomycetoma *versus* eumycetoma was made.

Study protocol included foot X-ray with no periostal reaction and no heterogenic aspect; no extra imaging studies (CT, MR) were needed. Direct examination with KOH showed multiple white grains composed of thin, septate hyphae. On Sabouraud's glucose agar, a hairy white colony that became brown with violet tones was seen. Microscopically, short conidiophores with macro- and microconidia were observed, which was compatible with species of the *F. solani* complex. A biopsy of the lesion showed a lymphohistiocytic infiltrate with an eosinophilic eumycetomic grain and good PAS staining, allowing the diagnosis of white grain eumycetoma (Fig. 2). The strain was sent to the Centraalbureau voor Schimmelcultures, (CBS) Utrecht, The Netherlands for further molecular identification and was identified as *F. pseudensiforme* (CBS 135554) by *TEF1* sequencing. Based on this diagnoses treatment with itraconazole (oral solution, 200 mg / twice a day) for six months was started, leading to significant reduction of the inflammatory process; however, the patient withdrew from treatment for unknown reasons.

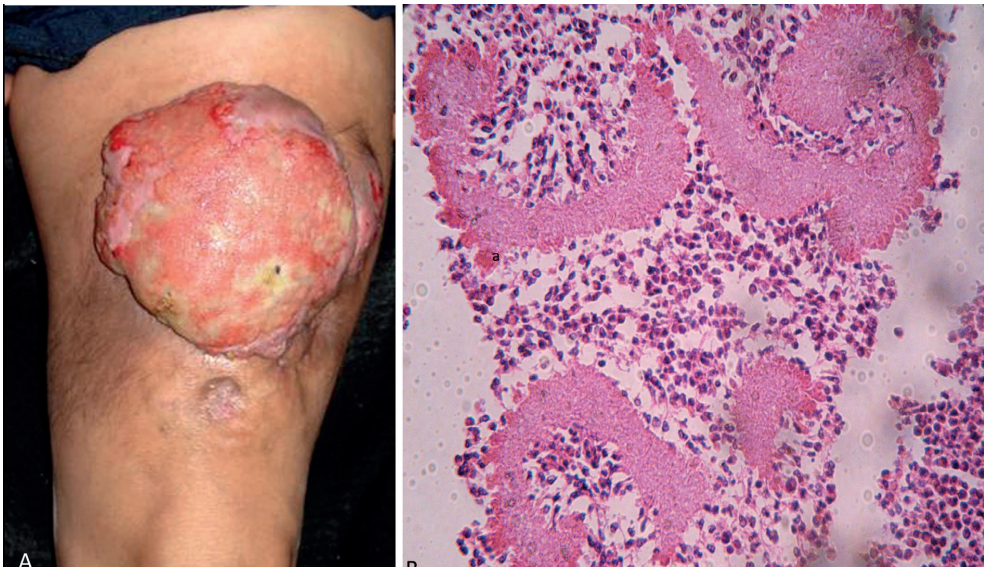

Fig 2. Mycetoma due to *F. pseudensiforme*. (a) Tumour like lesions of the left tigh that have softened and ruptured forming sinus tracts (case 2). (b) Biopsy showing a lymphohistiocytic infiltrate with an eosinophilic eumycotic grain and good affinity with PAS staining.

Materials and methods

Case reports

The existing medical literature was reviewed for human cases of mycetoma due to *Fusarium* species. The following search terms were used in electronic database of PubMed and the Atlas of Clinical Fungi.[16] Mycetoma AND *Fusarium*, Eumycetoma AND *Fusarium*, Madura foot AND *Fusarium*. Studies in any language were considered and reviewed. The search was supplemented by reviewing references from previous studies and review articles. Cases were analysed for: (i) their underlying disease, (ii) the origin of the isolate, (iii) the clinical relevance of *Fusarium* species, (iv) antimycotic treatment, (v) and the clinical outcome (Table 1).

Table1: Reported cases of mycetoma infections caused by *Fusarium* species.

No	Species	Identification/ Strain number	Grain colour	Gender/Age	Origin	Site of infection	Duration	Underlying condition	Treatment	Outcome	Reference
1	*F. solani*	Morphology	No grain observed	30 / Female	India	Right buttock	4 year	No	–	–	[15]
2	*F. moniliforme*	Morphology / CBS 539.79	White	78 / Male	Italy	Right foot & ankle	5 year	Pneumoconiosis	Antibiotics	Improvement	[17]
3	*F. subglutinans*	Molecular / D1/ D2 in text LSU	No grain	29 / Male	México	Left foot (leg & ankle)	8 year	No	TMP/SMX +DDS+ AMK (as bacteria) and ITR (4 months)	Improvement	[18]
4	*F. solani*	Morphology	–	71 / Male	Brazil	Left hand	6 year	No	KTA	Improvement	[19]
5	*F. solani*	Molecular / ITS	–	51 / Male	From Mauritius diagnosed in France	Left foot	6 month	N/A	ITR	Complete cure	[20]
6	*Fusarium sp.*	Morphology	–	Preterm infant	–	Renal pelvis	–	–	AMB + 5FC + surgery	Cure	[27]
7	*Fusarium sp.*	Morphology	–	50 / Male	Surinam	–	30 year	–	surgery + ITR	–	[28]
8	*F. solani*	Morphology	–	Male	–	Right ankle	7 year	–	Antifungals	Improvement	[29]
9	*A. falciforme*	Morphology	White	46 / Male	China	Left foot	15 year	No	TER	Improvement, relapse after 1 year	[30]
10	*F. cf. solani*	Morphology / CBS 241.93	No grains observed	–	Surinam	Foot	36 year	Diabetes mellitus	ITR	Improvement	[31]
11	*F. solani*	Morphology	White grain	28 / Male	Thailand	Left ankle	1 year	No	Antibiotics	Unsatisfactory	[32]
12	*A. falciforme*	Morphology	White to pale-yellow	60 / Male	USA	Hands and forearms	Approx. 1 year	Renal failure / diabetes mellitus	AMB / KTA	Died	[33]
13	*Fusarium sp. / F. solani var. cerulens*	Morphology	Non-pigmented	30	Nigeria	Left foot	1 year	No	KTA	Improvement	[34]
14	*Fusarium solani*	Morphology	No grain observed	45 / Male	India	Right and left foot	15 year	No	ITR	Improvement	[35]
15	*Fusarium sp.*	Morphology	-	66 / Male	Romania	Left foot	22 year	–	KTA+PCN+ surgery	Amputation	[36]
16	*Fusarium sp.*	Morphology	White	72 / Male	Cuba	Left foot	18 year	No	ITR	Improvement	[37]
17	*Fusarium solani*	Morphology	Present	24 / Male	Argentina	Right foot	–	–	–	–	[38]
18	*Fusarium sp.*	Morphology	–	–	Somalia	–	–	–	–	–	[39]
19	*Fusarium sp.*	Morphology	–	40 / Male	Somalia	Right foot		Kidney transplant	Surgery	Complete cure	[40]
20	*Fusarium solani*	PCR	White-yellowish	45 / Male	Guinea-Bissau	Right forearm	9 year	No	Treated as TB +surgery + ITR +AMB	–	[41]

Strains analyzed

Two isolates (CBS 135530 and CBS 135554) were recovered from the patients reported in the present paper. Additional strains (CBS 101427, CBS 637.82, CBS 241.93, CBS 130176 and CBS 539.79) deposited at the CBS with phenotypic identification as '*Fusarium* sp.' were also analyzed. Stock cultures were maintained on slants of 2% Malt Extract Agar (MEA; Difco) and Oatmeal Agar (OA; Difco) and incubated at 24 °C for two weeks. Identification was done using the translation elongation factor 1-alpha (*TEF1*) gene.

DNA extraction and sequencing

DNA extraction was performed using glass beads (Sigma G9143, Sigma-Aldrich, Missouri, USA) according to a protocol described by Al-Hatmi *et al*.[21] The (*TEF1*) was amplified and sequenced following the methods published by Al-Hatmi *et al.*[22] with primers EF1 and EF2.

Identification

DNA sequences were edited and consensus sequences were assembled by the SeqMan package of Lasergene software (DNAStar, Madison, WI, U.S.A.). Retrieved alignments were manually corrected to avoid mis-paired bases. Sequences were exported as FASTA files. For preliminary identification, a homology search for the sequences of *TEF1* was done using the BLAST tool in NCBI database, the CBS database, FUSARIUM-ID[23] and the *Fusarium* MLST[24] database down to species and haplotype level. For conclusive identification, sequences of *TEF1* were aligned with MAFFT program (www.ebi.ac.uk/Tools/msa/mafft/) and adjusted in MEGA6.[25] The best-fit model of evolution was determined by MEGA6. Maximum likelihood (ML) analysis was done with RAxML-VI-HPC v. 7.0.3 with non-parametric bootstrapping using 1000 replicates. GenBank accession numbers are shown in Fig. 3.

Antifungal susceptibility testing

Antifungal susceptibility testing of (CBS 135530 and CBS 135554) was performed using the M38-A2 broth microdilution method of CLSI (Clinical and Laboratory Standards Institute), Wayne (2008).[26]

Results

Types of articles

Using a literature search, twenty evaluable cases of eumycetoma caused by *Fusarium* species were retrieved and included in our analysis from 1964 till 2016[15, 17-20, 27-41], (Table 1), while three articles could not be acquired.[14, 42, 43] Cases were reported from the following continents: Africa (*n*=5; 25%), Asia (*n*=4; 20%), North America (*n*=3; 15%), South America (*n*=4; 20%), and Europe (*n*=2; 10%). We found that the fungal etiology was *F. solani* (*n*=11; 55%), *Fusarium*

spp. (*n*=5; 25%), *Acremonium falciforme* (syn. *F. falciforme*) (*n*=2; 10%), *F. subglutinans* (*n*=1; 5%) and *F. moniliforme* (syn. *F. verticillioides*) (n=1; 5%).

Patient characteristics

The mean age of the patients was 47 years, ranging from 24 to 72 years. Most patients were male (*n*=15; 75%); one female (5%), while four (20%) were of unknown origin. In 13 (65%) cases the infection involved the foot; other body sites included hand (*n*=3; 15%), buttock (*n*=1; 5%) and renal pelvis (*n*=1; 5%). Three (15%) patients had an underlying disease when the diagnosis mycetoma infection was made: diabetes mellitus (n=1), kidney transplant (n=1), renal failure and diabetes mellitus (n=1). Mean duration of the infection was 11 years, with shortest duration of 6 months and maximum up to 36 years.

Identification

In all published cases identification was done using morphological parameters of the culture. The description of microscopic characteristics was minimal and mostly insufficient for determination. In three cases additional molecular methods were applied, but with markers (rDNA ITS or LSU) which were insufficient for correct *Fusarium* species identification. The reported species in 10 cases (50%) was *F. solani,* while *F. falciforme* was isolated from 2 cases, *F. subglutinans* from 1 case, *F. moniliforme* (= *F. verticillioides*) from 1 case, and in 6 cases identification down to species level was not achieved. The re-identified species were *F. keratoplasticum* (CBS 101427) and *F. thapsinum* (CBS 130176 and CBS 539.79) based on (*TEF1*) and BT respectively. Two undescribed species were recovered, based on (*TEF1*), labeled as *Fusarium* cf. *solani* (CBS 637.82 and CBS 241.93). In our patients, *F. keratoplasticum* (CBS 135530) and *F. pseudensiforme* (CBS 135554) were identified by polyphasic identification. The GenBank accession numbers for the studied strains are shown in Fig 3.

Treatment

Overall, seven patients (35%) received itraconazole either alone or in combination with other drugs. The second most common therapy was ketoconazole (*n*=4; 20%), followed by amphotericin B (*n*=2; 10%). Other antifungal therapies were terbinafine and 5FC (each *n*=1; 5%, respectively). Combinations of antifungal drugs were used in two cases (10%), i.e. amphotericin B with itraconazole, and amphotericin B with ketoconazole. Besides the antifungal therapy, surgery alone (*n*=1; 5%) or with antibiotics in eight patients (40%).

In our patients, antifungal susceptibility testing showed that *F. keratoplasticum* had a low MIC of 1 µg/ml against amphotericin B and high MICs above published ECV values.[44] for itraconazole (4 µg/ml), voriconazole (4 µg/ml), posaconazole (>16 µg/ml), isavuconazole (8 µg/ml), anidulafungin (>16 µg/ml), micafungin (16 µg/ml), and fluconazole(>64 µg/ml).

F. pseudensiforme had the following MICs; 0.5, >16, 8, 4, 8, >16, >16 and >64 µg/ml for amphotericin B, itraconazole, voriconazole, posaconazole, isavuconazole, anidulafungin, micafungin, and fluconazole, respectively.

Chapter 9

ml). *F. pseudensiforme* had the following MICs; 0.5, >16, 8, 4, 8, >16, >16 and >64 µg/ml for amphotericin B, itraconazole, voriconazole, posaconazole, isavuconazole, anidulafungin, micafungin, and fluconazole, respectively.

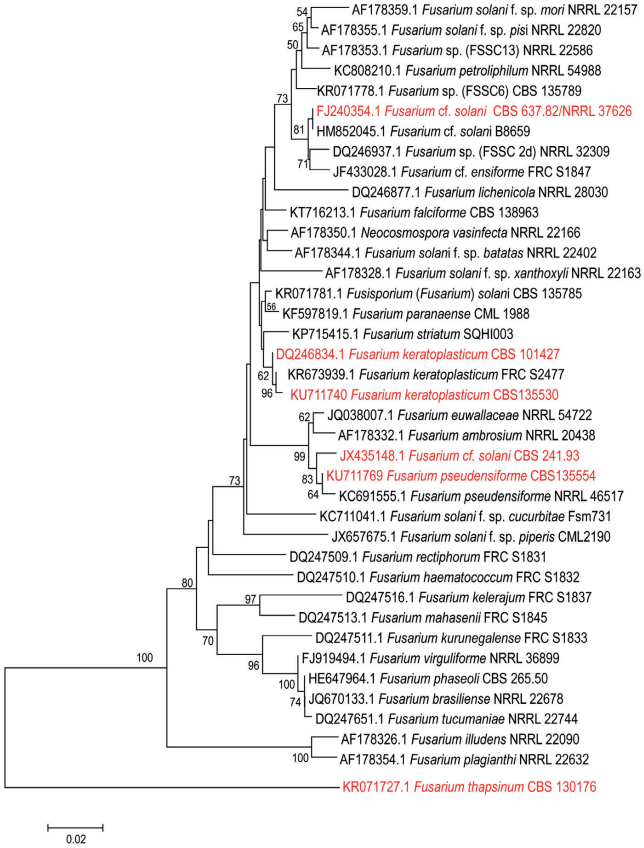

Fig 3. Maximum likelihood (ML) phylogenetic tree created from *TEF1* sequences of 39 *Fusarium* sequences. Total alignment length is 689 bp. 1,000 bootstrap-replications. The tree was rooted with *F. thapsinum* CBS 130176. The red in color are the CBS strains involved in mycetoma cases. The numbers before the species' name are the GenBank numbers

170

Fig 3. Maximum likelihood (ML) phylogenetic tree created from *TEF1* sequences of 39 *Fusarium* sequences. Total alignment length is 689 bp. 1,000 bootstrap-replications. The tree was rooted with *F. thapsinum* CBS 130176. The red in color are the CBS strains involved in mycetoma cases. The numbers before the species' name are the GenBank numbers.

Outcome

The duration of follow-up was not specified in most published cases (*n*=19; 95%). In one case (5%) the follow-up time was 12 months. Three patients (15%) were completely cured after treatment. Partial responses and relapses were noted in ten patients (50%). Amputation was done in one case (5%) and no data were available in four cases (20%). One patient (5%) died, either related directly to the fungal infection or related to preexisting medical conditions. Our patient (case 1) responded well to sequential treatment with itraconazole and terbinafine, allowing a clinical and mycological cure. The second patient (case 2) showed a good initial response to treatment with itraconazole with the lesion reducing in size by 30%, but the patient showed poor treatment compliance.

Discussion

Recently, mycetoma was recognized by the World Health Organization (WHO) as a neglected disease and officially added to the list of neglected tropical disease.[2] Mycetoma is a chronic and slowly progressive infection characterized by a clinical syndrome of localized, swollen lesions and sinuses discharging grains at the site of inoculation.[5] There is usually a considerable time gap between traumatic implantation and disease expression and often the patient could not recall the inoculation event.[45] Since mycetoma has a prolonged incubation period, it goes undiagnosed for a long time and, especially outside endemic regions, is hardly included in the differential diagnosis. When patients remain without early treatment, the infection may lead to severe tissue destruction.[46]

Eumycetoma is an infection caused by several types of hyaline and dematiaceous fungi. The genus *Fusarium* belongs to the hyaline fungi[8] but is a rare cause of mycetoma. Of the about 300 described species in the genus *Fusarium*[47], about a quarter[13] have been involved in human infections, with *F. solani* as the most frequently identified species. Phylogenetic analyses have shown that *F. solani* represents a series of species known as the *F. solani* species complex (FSSC), which consists of three major clades encompassing more than 60 phylogenetic lineages, some carrying names but the majority remaining unnamed.[48] Members of FSSC with a significant role in clinical infections are nested in clade 3[48], which involves *F. falciforme*, *F. keratoplasticum*, *F. lichenicola, F. petroliphilum*, *F. pseudensiforme and F. solani* spp.[13] Sequencing and phylogenetic analysis of partial *RPB2* and *TEF1* genes sequences showed high variability and sufficient difference to distinguish *Fusarium* species within FSSC.[49] Therefore, in the present study we utilized the *TEF1* sequencing to identify *Fusarium* strains that has been recovered from cases of human eumycetoma. In addition extensive review of reported *Fusarium* eumycetoma cases in the medical literature was performed.

In this study, we reviewed 20 cases of mycetoma patients caused by *Fusarium* and were published between 1964–2016. The most frequent species identified phenotypically in

these series was *F. solani* (n=11;55%). Other species identified were *Fusarium* spp. (25%), *Acremonium falciforme* (syn. *F. falciforme*) (10%), *F. subglutinans* (5%) and *F. moniliforme* (syn. *F. verticillioides*) (5%). To gain insight into the *Fusarium* species involved in mycetoma and which were properly identified, seven strains which were deposited at the CBS-KNAW collection were sequenced. Six strains of *Fusarium* including our two cases reported in this paper (CBS 101427, CBS 637.82, CBS 241.93, CBS 130176, CBS 135530 and CBS 135554) were re-identified using the (*TEF1*) and one strain (CBS 539.79/ GB: AM933124.1) using β-tubulin sequences. With this molecular revision, it is important to note that only few phylogenetic species in Clade 3 of the FSSC[48], are connected to names.

Our phylogenetic analysis based on *TEF1* gene shows that five of our isolates belong to FSSC: *F. keratoplasticum* (CBS 135530 and CBS 101427)*, F. pseudensiforme* (CBS 135554)*.* The last two were clustering within FSSC and blasting results gave *Fusarium cf.* solani (CBS 637.82 and CBS 241.93)*,* but both did not match with any earlier described molecular sibling in this group. Two strains identified in the present study (CBS 130176 and CBS 539.79) belonged to the *F. fujikuroi* species complex (FFSC) and both were identified as *F. thapsinum*.

The detailed phylogenetic analysis in the present study clearly showed that previous identification of many *Fusarium* strains from eumycetoma cases was insufficient or not correct. An example, the strain CBS 539.79 has been reported by Ajello *et al*.[17] as *F. moniliforme* (syn. *F. verticillioides*) whereas according to the *TEF1* gene sequence, *F. thapsinum* was found. Furthermore, inaccurate diagnosis of the disease has been reported as an actinomycetoma caused by bacterial agent where the patient was initially treated with antibacterial agents.[18] This is most probably due to the phenotypic misinterpretation of different colors of discharged granules to make an etiological diagnosis.[50] Most of the *Fusarium* species reported in this study are otherwise known as occurring in the environment, often as opportunists[13], and are poorly adapted to the human host. In addition, *F. oxysporum* has been reported in the literature as one of the causative agents for mycetoma[50], but this could not be confirmed in our study because of lack of sequence data and unavailability of the strains.

In agreement with reports on mycetoma overall, we found that the foot was the most frequently involved site of infection (65%). Eumycetoma is mostly seen in otherwise healthy individuals, because the innate immune response triggers the compacted hyphal growth of the fungus. However, three cases (15%) of published *Fusarium* eumycetomata had underlying diseases when mycetoma was diagnosed, with diabetes mellitus slightly more common than other immune and metabolic disorders. Diabetes could be one of the risk factors for the infection in those patients especially with opportunistic fungus like *Fusarium*. Ahmed *et al*.[51] also noted a high frequency of diabetic patient with white grain eumycetoma caused by the opportunistic fungus *Aspergillus*. However, in a large collection of 6,792 mycetoma cases only 33 patients had diabetes.[52] Studies are required to investigate whether the diabetes is one of the major risk

factors among white grain eumycetoma patients or other immune disorders are predisposing to the infection.

Treatment for fusariosis is challenging, recent European guidelines generally proposed amphotericin B followed by voriconazole for the treatment of hyalohyphomycosis.[53] The reported antifungal agents used to treat *Fusarium* mycetoma included amphotericin B in deoxycholate or lipid form, azoles (ketoconazole, itraconazole), terbinafine and flucytosine (5FC), but the ideal antifungal treatment for mycetoma is uncertain. Although itraconazole shows high MICs *in vitro*[54], our results of the literature revealed that the most frequently used drug was itraconazole in 35% of cases. The second common therapy was ketoconazole (20%), followed by amphotericin B (10%). Although, amphotericin B has been recommended as the first line of treating patients with invasive fusariosis[13], its toxicity is undesirable for treating eumycetoma as it requires prolonged therapy.[55] Alternatively, recent data suggest that voriconazole or posaconazole are more effective *in vitro* than other azoles[13,56], therefore both could be an option for treating eumycetoma.[55] These compounds show moderate activity with MICs of 2–8 mg/L and 0.5–8 mg/L, respectively, depending on the *Fusarium* species.[13] Voriconazole or posaconazole might decrease the need for surgical interventions due to their efficacy, good penetration, and bioavailability. However, long-term usage may be hampered by cost constrains. Our review of the published cases showed there was improvement in about 35% of the patients although in 90% of these patients survived whether or not antifungal therapy was successful.

Our patient with *F. keratoplasticum* responded well to the sequential treatment with itraconazole and terbinafine, resulting in clinical and mycological cure. The second patient presented with an atypical form of eumycetoma due to *F. pseudensiforme* with an exophytic tumor lesion. This is not a natural evolution of mycetoma, but probably due to the previous surgery which stimulated the growth of it. The geographical area from which this patient originates is subtropical and most mycetoma cases are actinomycetoma due to *Nocardia*. This case reinforces the importance of proper identification of the cause of mycetoma before initiating medical treatment. The second mycetoma case which is caused by *F. pseudensiforme* is of interest because this species is a novel cause of mycetoma as the first case of human infection.

In conclusion, although sequencing of *TEF1* gene is not available to most clinical laboratories, we reinforce that it is important to get a correct identification of *Fusarium* spp. and antifungal susceptibility tests should be performed to optimize medical treatment. FSSC includes the three closely related species *F. keratoplasticum*, *F. pseudensiforme*, and *Fusarium cf. solani* are involved in development of mycetoma. In addition, *F. thapsinum* a member of FFSC also appeared to be involved in mycetoma. The literature search emphasized that FSSC is the most involved complex in mycetoma infections.

Acknowledgements

The work of Abdullah Mohammed Said Al-Hatmi was financially supported by Ministry of Health, Oman. Formal Agreement number 28/2014.

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CHAPTER 10

Chapter 10

Proximal subungual onychomycosis caused by *Fusarium falciforme* successfully cured with posaconazole

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Adapted from British Journal of Dermatology 2015; 173: 253-255.

Abstract

We report a case of proximal subungual onychomycosis (PSO) in a male HIV-AIDS patient. The mould recovered in multiple cultures was identified as *Fusarium falciforme* by morphology and multi-locus sequence typing based on partial transcription elongation factor 1 alpha (*TEF1*) and the second largest subunit of RNA polymerase (*RPB2*). After initial failure of terbinafine and itraconazole therapy, clinical and mycological cure was achieved with three-month course with posaconazole oral sultion. The patient was followed during one year and four months without relapse.

Key words: onychomycosis, toenails, infection, *Fusarium falciforme*, posaconazole, HIV AIDS, intermittent dosages

Introduction

Proximal subungual onychomycosis (PSO) is a fungal infection on the inner layer of the nail plate. The infection is very rare because it starts from the eponychium and nail matrix.[1] Onychomycosis in general can be caused by a variety of dermatophytes, yeasts and non-dermatophyte molds (NDMs). The NDMs causing onychomycosis include species of *Aspergillus*, *Acremonium*, *Scopulariopsis*, *Chaetomium*, and *Fusarium*.[2] *Fusarium* and *Acremonium* species require special attention, because of their ability to disseminate, using the nail as a potential source of infection, and their intrinsically low susceptibility to most common antifungal agents.[3]

Since the application of molecular methods to the taxonomy of *Fusarium*, a large hidden diversity has been found. At least 71 molecular species have been reported to be involved in mycoses of humans and animals, most of which belong to eight species complexes.[4] Many members of the FSSC are phenotypically indistinguishable and rely for identification on multi-locus sequence analysis.[5] In a phylogenetic study using the large-subunit (LSU) region of ribosomal DNA (rDNA) genes, Summerbell *et al*[6] showed that several FFSC species do not display their typical *Fusarium solani* morphology. Therefore, also species which consistently lacking typical banana-shaped macroconidia but with a phylogenetic relationship to *Fusarium* were nevertheless accepted as members of the genus. Currently *Fusarium falciforme* is an increasingly recognized FSSC species recovered from clinical samples from superficial locations. Here, we report a rare proximal subungual onychomycosis in an HIV-AIDS patient caused by *F. falciforme* which was successfully treated with posaconazole.

Case report

A 64-year-old male, pedagogue and horticulturist, presented with a 9-month history of proximal leukonychia of the first toenails of both feet: one of them with slight paronychia affecting the proximal fold, and the 3rd and 4th toenails of the right foot with distal leukonychia (Figure 1, A1-A3). He had not received previous treatment for fungal infection. The patient history was relevant for HIV infection diagnosed 8 years ago, classified as AIDS A2 (488 CD4+/ml and undetectable viral load), treated with atazanavir, with good compliance. Complete blood count was normal. The first clinical diagnosis was proximal subungual onychomycosis (POS) due to dermatophytes. Direct examination (KOH 10%) showed multiple thin motley hyphae (Figure 1, B). Culture on Sabouraud's glucose agar (SGA) led to the growth of white colonies with light brown pigment diffusing into the agar. Microscopical examination showed septate hyaline hyphae with erect hyphal bundles and long conidiophores with fusiform microconidia. The phenotypic identification resulted in an *Acremonium* or a *Fusarium* species.

The strain was deposited at the CBS-KNAW Fungal Biodiversity Centre in Utrecht, The Netherlands, under accession number CBS 135512, for further identification. The fungus grew on malt extract ager (MEA; Oxoid, U.K.) Growth of aerial white, fluffy to floccose colonies was

seen with pale yellow, diffuse reverse pigmentation (Figure 1, C). Microscopically, microconidia were abundant, often in false heads (Figure 1, D-E), (0-1) septate, oval to fusiform microconidia (arrows) were seen on carnation leaf agar (CLA) (Figure 1, F). Septate macroconidia with (1-4) septa (Figure 1, G), were scarce and were produced on relatively short monophialides (Figure 1, H). Long and septate conidiophores (Figure 1, H) had two septa (arrows) bearing monophialides with fusiform microconidia. Chlamydospores were frequently observed (Figure 1, I). Cardinal growth temperatures showed optimal development at 25−27 °C (Figure 2), with a good growth at 37 °C.

For species identification, partial sequences of transcription elongation factor 1 alpha (*TEF1*) and of the second largest subunit of RNA polymerase (*RPB2*) were compared with the *Fusarium* MLST database and GenBank and aligned with *Fusarium* strains maintained at CBS including type strains of *Fusarium* species. The isolate showed 99% similarity for *TEF1* with CBS 132312 and 100% similarity for *RPB2* with CBS132313 (both *F. falciforme*). This species is a member of the *Fusarium solani* species complex. Sequences of isolate CBS 135512 were deposited in GenBank with accession numbers KM401895 KM401893, KM401894 and KM401892 for ITS, LSU, *TEF1* and *RPB2*, respectively.

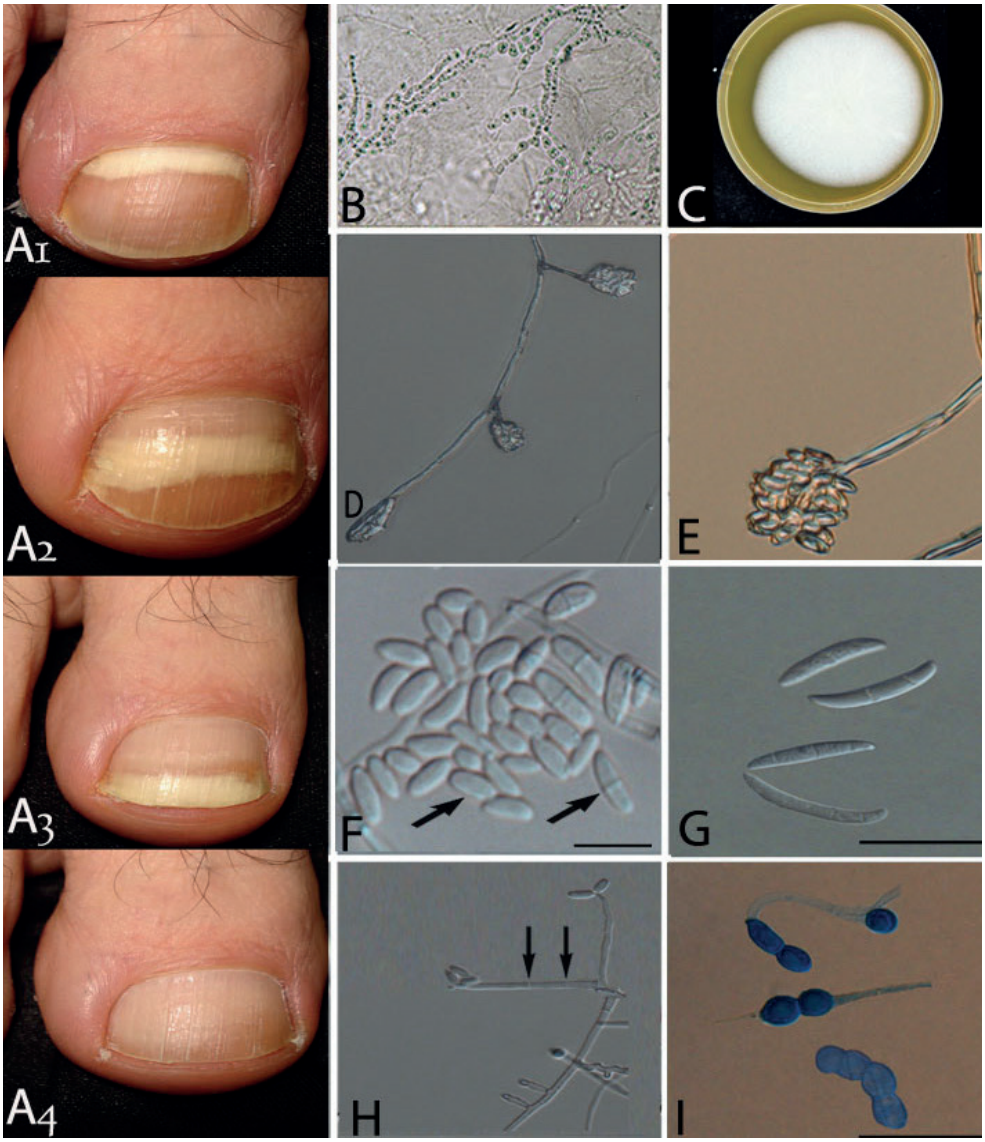

Fig 1. (a1) Initial infection; (a2) After 3rd pulse of posaconazole treatment; (a3) 1 year later; (a4) 1 year and 4 months; (b) KOH mount of the nail scraping material showing fungal hyaline hyphae (magnification, 940); (c) Growth of aerial white colonies on MEA at 25 °C; (d) conidiophores with false heads; (e) Microconidia abundant, in false heads; (f) Microconidia and fusiform cells (arrows) on CLA; (g) Septate macroconidia; (h) Conidiogenous cells as septate monophialides of *Fusarium falciform*e (arrows); (i) Chlamydospores over 10 days of incubation. All scale bars 10 μm.

Antifungal susceptibility testing (AFST), demonstrated that the fungus had low MICs of posaconazole (0.5 µg/ml) and amphotericin B (0.5 µg/ml) (Table 1). Initially, the patient received itraconazole 200 mg/day. He complained of severe dyspepsia and medication was changed to terbinafine 250 mg/day during the first two and a half months, without clinical improvement. After molecular identification and AFST, intermittent therapy was started with posaconazole oral solution 800 mg/day for one week each month, and this schedule was maintained during four months. Clinical and mycological cure was achieved with this regimen and the patient was followed during 1 year and 4 months without relapse (Figure 1, A4).

Table1. Minimum inhibitory concentration (MIC) values of clinical isolate *Fusarium falciforme* CBS 135512

Drugs	AMB	FLC	ITC	VOR	POS	MICA
MIC values (µg/ml)	0.5	>64	>16	8	0.5	>8

AMB, amphotericin B; FLC, fluconazole; ITC, itraconazole; VOR, voriconazole; POS, posaconazole; MICA, micafungin.

Discussion

Non-dermatophyte mold infections (NDM) account for almost 10% of onychomycosis worldwide and the main types of NDM onychomycosis are distal and lateral subungual onychomycosis (DLSO), and superficial white onychomycosis (SWO); proximal subungual onychomycosis (PSO) represents a less common type.[7] The main etiological agents encountered are *Scopulariopsis brevicaulis*, *Aspergillus niger*, *A. fumigatus*, *A. flavus, Acremonium* species, and *Fusarium solani*.[2] PSO is considered to be an HIV infection marker[8] and is usually caused by dermatophytes such as *Trichophyton rubrum* or sometimes *T. mentagrophytes*. When caused by NDM, the disorder may be indistinguishable of dermatophyte PSO.[9] Onychomycosis due to *Fusarium* species usually affects toenails[10] while paronychia or periungual inflammation and purulent discharge are associated features.[2] The route of entry in our patient was possibly related to horticulture. Importantly, onychomycosis in patients with underlying immune disorders constitutes a portal of entry for invasive mold infections[11]. Nucci *et al*[12] noted that patients with neutropenia had a higher rate of cutaneous lesions, while infections were more frequently disseminated, when compared to a non-neutropenic immunocompromised population.

Therefore, patients with immunosuppression, such as leukemia or HIV-AIDS, who present with onychomycosis due to *Fusarium* or other molds, carry an increased risk of systemic fungal infection therefore, tailored therapy is essential.[13] Success rates of treatment of NDM infection vary with the clinical type of onychomycosis and the etiological. Some patients with infections due to *Fusarium* species respond to terbinafin or itraconazole, but others fail to achieve clinical or mycological cure[14]. Krishna *et al*[15] demonstrated that posaconazole diffuses into the nail plate with levels remaining high after treatment discontinuation and the intermittent therapy

with posaconazole is indicated similar to itraconazole based on pharmacokinetic properties of these drugs and also new antifungals like posaconazole have wider spectra and administration is easier (i.e., with oral suspension).

Here we report the first case of *F. falciforme* successfully treated with posaconazole which could be a successful alternative treatment for patients presenting with onychomycosis due to *Fusarium* species. Posaconazole treatment was chosen considering the poor clinical response with the initial medication and the low MICs of posaconazole. Additionally the strain CBS 135512 morphologically deviates from the textbook descriptions. This is important for morphological diagnostics in which *F. falciforme* usually produces long, slender, cylindrical conidiophores and integrated, terminal phialides and while the other *Fusarium* group tend to form shorter ones. However, in our case we found that both long conidiophores and shorter, discrete monophialides were present which suggest that *F. falciforme* conidiogenesis may vary from strain to strain.

Acknowledgements

The work of Abdullah Mohammed Said Al-Hatmi was financially supported by Ministry of Health, Oman.

Conflicts of interest

J.F.M. received grants from Astellas, Basilea and Merck. He has been a consultant to Astellas, Basilea and Merck and received speaker's fees from Merck and Gilead. All other authors: none to declare.

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CHAPTER 11

Chapter 11

Emergence of fusariosis in a university hospital in Turkey during a 20-year period

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European Journal of Clinical Microbiology & Infectious Diseases 2015; 34:1683-1691.

Abstract

Fusarium species have started appearing increasingly as the main cause of infections, particularly in immunocompromised patients. In this study, we aimed to present the first epidemiological data from Turkey, analyze fusariosis cases that have been monitored in a university hospital during the past 20 years, identify the responsible *Fusarium* species, and determine antifungal susceptibilities. A total of 47 cases of fusariosis was included in the study. *Fusarium* isolates were identified by multilocus sequence typing (MLST). Antifungal susceptibility was tested by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) methodology. Of the *Fusarium* infections, 23.4 % were superficial, 44.7 % were locally invasive, and 31.9 % were disseminated. A significant increase was observed over the years. The *Fusarium fujikuroi* species complex (FFSC) proved to be the most frequent agent group (17 cases; 51.5 %), followed by the *Fusarium solani* species complex (FSSC) (14 cases; 42.4 %), the *Fusarium dimerum* species complex (FDSC), and the *Fusarium oxysporum* species complexes (FOSC) (one case each). Amphotericin B had the highest *in vitro* activity against all species. Voriconazole and posaconazole showed interspecies variability across and within *Fusarium* species complexes. In conclusion, our data support the fact that regional differences exist in the distribution of the *Fusarium* species and that species-specific differences are observed in antifungal susceptibility patterns. The monitoring of local epidemiological data by determining fungal identity and susceptibility are of importance in guiding the clinical followup of patients.

Key words: Acute leukaemia; disseminated fusariosis; emerging fungal infections; *Fusarium solani* species complex; *Fusarium fujikuroi* species complex; antifungal susceptibility.

Introduction

The large genus *Fusarium* contains mainly species with a saprobic or plant-pathogenic lifestyle, but also contains species infamous for their adverse health effects: some have mycotoxigenic properties and/or have the ability to cause opportunistic infections in humans and animals [1]. Most of the former *Fusarium* species are now known to be species complexes composed of clusters of cryptic species with little to no morphological differences. Multilocus sequence typing (MLST) is necessary to distinguish between such species [2–5]. Now that the 'Bone fungus = one name' rule has been adopted, a proposal was made to conserve the name *Fusarium* above linked sexual state names like *Haemonectria* and *Gibberella* [6], and a plea to keep the taxonomy of clinically important species as stable as possible [7]. In line with these proposals, we maintain a concept of the genus *Fusarium* in the broadest sense according to Geiser et al. [6].

Most of the opportunistic *Fusarium* pathogens identified in the literature are members of the *F. solani* species complex (FSSC), the *F. oxysporum* species complex (FOSC), and the *F. fujikuroi* species complex (FFSC), and less often of the *F. incarnatum-equiseti* species complex (FIESC), the *F. dimerum* speci es complex (FDSC), and the *F. chlamydosporum* species complex (FCSC) [5, 8, 9]. Some species and haplotypes causing infections have a worldwide distribution, while local variation also exists [10].

Prevalent fusariosis in otherwise healthy individuals mostly concern onychomycosis, skin infections, and keratitis, and an occasional mycetoma, especially in areas with warmer, drier climates [11]. Deep and disseminated infections are confined to severely immunocompromised patients. Disseminated infections seem predominantly related to hematological disorders [12], sometimes with a link to a pre-existing nail or cutaneous infection [13, 14]. The observed number of fusarioses has been reported to increase over the past several decades: this may be partly due to the growing number of immunocompromised patients and their prolonged average survival time, partly due to prophylactic therapeutic approaches against opportunistic fungal infections which have little impact on *Fusarium*, and partly to our improved ability to identify the infective agents [14, 15].

Most opportunistic *Fusarium* species are relatively to very resistant to antifungal drugs *in vitro*, although anti-fungal sensitivity profiles may differ between species [2, 16, 17]. While refractory onychomycoses are not life-threatening, timely and effective treatment for deep and disseminated fusariosis is very important. Joint guidelines on the treatment of hyalohyphomycoses recommend amphotericin B and voriconazole for the treatment of systemic fusariosis [18], despite variable minimum inhibitory concentration (MIC) test results for both drugs [2, 16, 17, 19]. Hence, accurate identification of the species involved is of importance for epidemiological studies and for guiding clinical follow-up of the patients [2, 13].

In this study, cases of fusariosis acquired in a tertiary-care university hospital in Turkey over the past 20 years were analyzed. All preserved *Fusarium* isolates were identified using an MLST approach suitable for the state-of-the-art identification of species complexes, species, and haplotypes, resulting in an overview of the *Fusarium* species occurring as etiological agents of disease. Antifungal susceptibility profiles were made for all these isolates to help clinicians develop appropriate therapies that have a high probability of successfully treating fusarioses.

Materials and methods

Setting and epidemiological data

Uludağ University Healthcare and Research Hospital is an 800-bed tertiary-care teaching institution in Bursa, Turkey. There are seven adult (Reanimation, Thoracic–Heart–Vascular Surgery, Cardiology, Plastic Surgery and Burns Unit, Neurology, Brain Surgery, and Lung Diseases) and two pediatric (Neonatal and Pediatric) intensive care units, as well as hematology and oncology wards and kidney, liver, and bone marrow transplantation units. The mycology unit of the medical microbiology laboratory receives specimens from patients admitted to the hospital and all fusariosis data regarding diagnosis and sites of infection were obtained retrospectively from laboratory records from 1 June 1995 till 31 December 2014. All cases were classified as superficial, locally invasive, or disseminated infections. The microscopic appearance of hypha and recovery of *Fusarium* spp. from specimens like skin, nails, and corneal scrapings were considered superficial infections. Disseminated disease was documented either by at least two positive blood cultures, or one positive blood culture together with skin or another organ involvement proven by culture and microscopy. Locally invasive pulmonary, sinonasal, and skin fusarioses were considered when *Fusarium* species were isolated from the respiratory tract, sinuses, or skin biopsies in the presence of risk factors, clinical signs, and symptoms, and when hyphae were seen at microscopy.

Isolates

Fusarium isolates have been collected and deposited in 10 % glycerol at −80 °C in the culture collection of Uludağ University Healthcare and Research Hospital from 1 June 1995 to 31 December 2014. The initial identification as *Fusarium* species was based on macroscopic and microscopic morphological features. Isolates which were able to grow after subculturing twice on Sabouraud dextrose agar (SDA) were also deposited in the reference collection of the CBS-KNAW Fungal Biodiversity Centre in Utrecht, the Netherlands, and these were characterized based on MLST.

DNA extraction and sequencing

The DNA of strains were extracted with the CTAB-based method of Möller et al. [20]. Tentative identification of species (complexes) was by nuclear ribosomal internal transcribed spacer (ITS)

sequencing with primers ITS1 and ITS4 [21] and a part of the translation elongation factor 1-alpha (*TEF1*) gene with primers EF1 and EF2 [22]. To determine the exact haplotypes within species, partial sequences of the *RPB2* gene [5], the intergenic spacer region IGS [3], the large ribosomal subunit LSU, and the β-tubulin gene [23] were obtained as needed. For sequence typing of members of the FSSC, LSU and *RPB2* [2] were used in addition to ITS and *TEF1*, for members of the FDSC and the FFSC, *RPB2* and β- tubulin [23], and for the FOSC, IGS and *RPB2* [3]. All strains could, thus, unambiguously be assigned to the species or haplotype level. All polymerase chain reaction (PCR) assays were done with Bioline Taq Polymerase in 12.5-µl volumes. Amplicons were purified with Sephadex G-50 Fine (GE Healthcare, Uppsala, Sweden), then subjected to direct sequencing with the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and analyzed on an ABI PRISM 3730xl sequencer. Sequences were edited using SeqMan in the Lasergene software (DNASTAR, Madison,WI, USA).

Identification

Strains were identified by BLAST identification of the obtained sequences inGenBank, the CBS database, the *Fusarium* ID [22] and *Fusarium* MLST [5] databases. Besides phylogenetic placements with species/haplotypes within species, complexes were checked with the above mentioned available databases specific for the found species complexes.

Antifungal susceptibility testing

Isolates were tested for *in vitro* susceptibility to fluconazole (FLC; Pfizer Central Research Sandwich, Tadworth, Surrey, UK), itraconazole (ITC: Janssen Research Foundation, Beerse, Belgium), posaconazole (POS: Merck, Whitehouse Station, NJ, USA), voriconazole (VOR: Pfizer Central Research, Sandwich, Tadworth, Surrey, UK), amphotericin B (AMB; Bristol-Myers Squibb, Woerden, the Netherlands), caspofungin (CAS; Merck Sharp & Dohme BV, Haarlem, the Netherlands), and anidulafungin (AND; Pfizer Central Research, Sandwich, Tadworth, Surrey, UK) by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) methodology [24]. The MICs of amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole, and posaconazole were determined visually: an inverted mirror was used for comparing the growth in wells containing the drugs with that in the drug-free control well. The results were also read using a microtitration plate spectrophotometric reader (Anthos htIII; Anthos Labtec Instruments, Salzburg, Austria). The minimum effective concentrations (MECs) of anidulafungin and caspofungin were read using a plate microscope (Olympus SZX9; Olympus Nederland, Zoeterwoude, the Netherlands), at 25x to 50x magnification. *Paecilomyces variotii* (ATCC 22319), *Candida parapsilosis* (ATCC 22019), and *C. krusei* (ATCC 6258) were used for quality controls in all experiments. The

ranges and geometric means (GMs) of the MICs and MECs were determined for each species and drug after 48 h of incubation. If no growth was observed or the growth was not adequate, the incubation was extended to 72 h. All experiments on each strain were performed using three independent replicates on different days.

Statistical analysis

Differences between years andMIC/MEC distributions between the groups were compared using the Chi-square test; differences were considered statistically significant at a p-value of ≤0.05.

Results

We identified 47 cases of *Fusarium* infection from 1995 to 2015: 11 (23.4 %) were superficial, 21 (44.7 %) were locally invasive, and 15 (31.9 %) were disseminated. A significant increase in the number of fusarioses in 5-year intervals was observed over the past several years ($p < 0.001$; Fig 1). Patient data on underlying disorders are provided in Table 1. Disseminated infections were mainly associated with hematological malignancies (80 %; 12 out of 15 cases). In the remaining patients with disseminated fusariosis, two were severely immunosuppressed due to malign melanoma and Wegener's granulomatosis, respectively, and a third was a low-birthweight newborn (Table 1). Local invasive infections (21/47; 44.7 %) were seen in patients with diverse underlying disease conditions (Table 1).

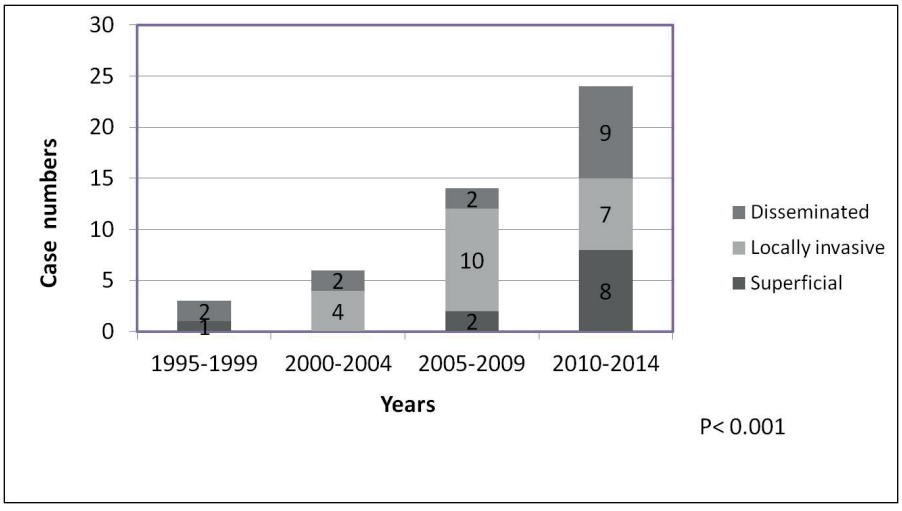

Fig 1. Increasing incidence of *Fusarium* infections over the past 20 years in the studied university hospital in Turkey.

Table 1. Underlying diseases of patients with *Fusarium* infections			
	***Fusarium* infections, no. of patients**		
Underlying disease (no. of patients)	**Superficial**	**Locally invasive**	**Disseminated**
Hematological malignancy[a] (19)	-	7	12
Solid organ carcinoma[b] (5)	-	4	1
Chronic disorder[c] (12)	2	9	1
Keratitis (4)	4	-	-
Onychomycosis (4)	4	-	-
Others (3)	1[d]	1[e]	1[f]
Total (47)	**11**	**21**	**15**

[a]Acute myeloid leukemia (11), acute lymphoblastic leukemia (5), multiple myeloma (1), myelodysplastic syndrome (1), T-cell lymphoma (1) [b]Larynx cancer (1), lung cancer (1), malign melanoma (1), adrenal cortex cancer (1), pancreas cancer (1) [c]Peripheral artery disease (2), aplastic anemia (2), Wegener's granulomatosis (1), cerebrovascular disease (1), renal transplant (1), chronic renal failure (1), hepatic cirrhosis (1), paraplegia (1), chronic obstructive pulmonary disease (1), diabetes (1) [d] Burn, [e]Hydatid cyst, [f]Low-birth-weight newborn

Fusarium spp. grew from respiratory tract specimens in ten patients who suffered from hematological malignancy (n = 1), solid organ carcinoma (n = 3), and various chronic diseases (n = 6). Local sinonasal infections, proven by nasal biopsy samples with positive microscopy and culture, were seen in five patients, four of whom had hematological malignancies and one had aplastic anemia. In six patients, *Fusarium* was grown from skin biopsy samples with no growth in blood cultures. Two of these patients had a hematological malignancy, while one had solid carcinoma. *Fusarium* isolates were repeatedly cultured from skin biopsies of lower extremity lesions in a paraplegic patient [25]. Of the last two patients, one had chronic renal failure, while the other had hydatid cysts. The main superficial infections were keratitis and onychomycosis (72.7 %; 8 out of 11). In two chronically ill patients and one patient suffering from burn wounds, *Fusarium* growth was obtained from skin scrapings.

MLST was performed on 46 revivable isolates from 33 of the 47 patients (70.2 %) described above (Table 2). The FFSC proved to be themost frequent agent group (17 cases; 51.5 %), followed by the FSSC (14 cases; 42.4 %), and the FDSC and the FOSC (one case each). The distribution of the isolated species differed among the patients with various clinical presentations. Members of the FFSC were the most frequent etiological agents of disseminated infections (8/12; 66.7 %), followed by *Fusarium petroliphilum* of the FSSC (4/12; 33.3 %) (Table 3).

Table 2. List of molecularly identified *Fusarium* isolates during the period 1995-2014.

Patient	Specimen	Isolation date	Infection	Underlying disease	CBS number	Alternative number	*Fusarium* species
Patient 1	Blood	09-03-2010	D	Acute myeloid leukemia	139006	28623	*Fusarium petroliphilum* (FSSC)
Patient 2	Nasal biopsy	26-04-2013	LI	Myelodysplastic syndrome	138932	40009	*Fusarium petroliphilum* (FSSC)
Patient 3	Blood	16-07-2013	D	Acute lymphoblastic leukemia	139011	40686	*Fusarium petroliphilum* (FSSC)
	Nasal biopsy	08-07-2013			139009	40600	*Fusarium petroliphilum* (FSSC)
	Skin biopsy	12-07-2013			139010	40625	*Fusarium petroliphilum* (FSSC)
	Skin biopsy	12-07-2013			139019	40654	*Fusarium petroliphilum* (FSSC)
Patient 4	Blood	12-05-2014	D	Acute myeloid leukemia	139324	43248	*Fusarium petroliphilum* (FSSC)
Patient 5	Blood	20-05-2014	D	Acute myeloid leukemia	139013	43325	*Fusarium petroliphilum* (FSSC)
Patient 6	Nail scraping	02-01-2008	S	Onychomycosis	139016	22840	*Fusarium solani s.s.* (FSSC-5)
Patient 7	Sputum	04-03-2008	LI	Larynx cancer	138927	23239	*Fusarium solani s.s.* (FSSC-5)
	Sputum	05-03-2008			139205	23250	*Fusarium solani s.s.* (FSSC-5)
	Sputum	11-03-2008			138999	23292	*Fusarium solani s.s.* (FSSC-5)
Patient 8	Skin scraping	16-05-2012	S	Diabetes	139007	36403	*Fusarium solani s.s.* (FSSC-5)
Patient 9	Nasal biopsy	31-05-2013	LI	Acute myeloid leukemia	139008	40273	*Fusarium solani s.s.* (FSSC-5)
Patient 10	Cornea scraping	15-11-2013	S	Keratitis	139012	41672	*Fusarium solani s.s.* (FSSC-5)
Patient 11	Cornea scraping	04-08-2014	S	Keratitis	139199	43937	*Fusarium solani s.s. (FSSC-5)*
	Cornea scraping	08-08-2014			139200	43976	*Fusarium solani s.s.* (FSSC-5)
Patient 12	Nail scraping	05-02-2010	S	Onychomycosis	139005	28341	*Fusarium keratoplasticum* (FSSC)
Patient 13	Nail scraping	13-07-2012	S	Onychomycosis	139017	37057	*Fusarium keratoplasticum* (FSSC)
Patient 14	Skin biopsy	14-12-2009	LI	Acute myeloid leukemia	139197	27854	*Fusarium solani s.s.* (FSSC-6)
Patient 15	Blood	26-07-2000	D	Acute lymphoblastic leukemia	138998	7810	*Fusarium proliferatum* (FFSC)
Patient 16	Nasal biopsy	24-08-2004	LI	Acute myeloid leukemia	138924	14663	*Fusarium proliferatum* (FFSC)
Patient 17	Skin biopsy	01-12-2005	LI	Chronic renal failure	138925	17857	*Fusarium proliferatum* (FFSC)
Patient 18	BAL	02-05-2008	LI	Aplastic anemia	139000	23660	*Fusarium proliferatum* (FFSC)
Patient 19	Skin biopsy	07-05-2008	LI	Acute myeloid leukemia	139001	23690	*Fusarium proliferatum* (FFSC)
Patient 20	Blood	11-08-2008	D	Acute lymphoblastic leukemia	139003	24321	*Fusarium proliferatum* (FFSC)
Patient 21	Sputum	06-11-2008	LI	Lung cancer	139004	24861	*Fusarium proliferatum* (FFSC)
Patient 22	Cornea scraping	16-01-2012	S	Keratitis	138929	35142	*Fusarium proliferatum* (FFSC)

Table 2. (Continued)

Patient 23 Nasal biopsy	16-05-2012	LI	Aplastic anemia	138930	36392	*Fusarium proliferatum* (FFSC)
Nasal biopsy	22-05-2012			139203	36465	*Fusarium proliferatum* (FFSC)
Patient 24 Blood	14-06-2012	D	Malign melanoma	138928	29541	*Fusarium proliferatum* (FFSC)
Patient 25 Nasal biopsy	05-07-2013	LI	Acute myeloid leukemia	139198	40572	*Fusarium proliferatum* (FFSC)
Patient 26 Blood	03-06-2014	D	Acute lymphoblastic leukemia	139014	43498	*Fusarium proliferatum* (FFSC)
Nasal biopsy	09-05-2014			138933	43242	*Fusarium proliferatum* (FFSC)
Blood	05-06-2014			138934	43445	*Fusarium proliferatum* (FFSC)
Blood	09-06-2014			139201	43474	*Fusarium proliferatum* (FFSC)
Patient 27 Pleural fluid	28-11-2014	LI	Pancreas cancer	139325	45004	*Fusarium proliferatum* (FFSC)
Patient 28 Blood	30-10-2007	D	Acute myeloid leukemia	139015	22525	*Fusarium verticillioides* (FFSC)
Patient 29 Blood	17-09-2012	D	T-cell lymphoma	139018	37791	*Fusarium verticillioides* (FFSC)
Patient 30 Blood	21-10-2014	D	Acute lymphoblastic leukemia	139202	44597	*Fusarium verticillioides* (FFSC)
Skin biopsy	13-10-2014			139204	44536	*Fusarium verticillioides* (FFSC)
Patient 31 Blood	07-12-2012	D	Acute myeloid leukemia	138931	38603	*Fusarium andiyazi* (FFSC)
Blood	11-12-2012			139195	38621	*Fusarium andiyazi* (FFSC)
Patient 32 Sputum	20-06-2007	LI	Hepatic cirrhosis	138926	21689	*Fusarium oxysporum* (FOSC)
Patient 33 Skin biopsy	01-08-2008	LI	Paraplegia	139196	24262	*Fusarium dimerum* (FDSC)
Skin biopsy	01-08-2008			139002	24264	*Fusarium dimerum* (FDSC)

Although there was no growth from blood cultures, the isolation of *Fusarium proliferatum* from skin and nasal biopsy specimens in some severely immunocompromised patients with hematological malignancy (n = 3), aplastic anemia (n = 1), and chronic renal failure (n = 1) underlined the predominance of the FFSC in deep invasive diseases. In one aplastic anemia and in two cancer patients who had symptoms of respiratory tract infection, *F. proliferatum* was grown from respiratory specimens like bronchoalveolar lavage, pleural fluid, and sputum. The FSSC caused localized infections in four patients. Three of them suffered from hematological malignancy, while in a patient with larynx cancer, *F. solani* s.s. (FSSC-5) was obtained from three different sputum specimens. One isolate of *F. dimerum* (FDSC) and one of *F. oxysporum* (FOSC) grew from skin biopsies and sputum samples in patients with paraplegia and hepatic cirrhosis, respectively.

The FSSC was responsible for most (6/7; 85.7 %) of the superficial infections (Table 3): *Fusarium keratoplasticum* was isolated from two onychomycosis cases, and *F. solani* s.s. (FSSC-5) was isolated from nail, corneal, and skin scrapings in patients with onychomycosis (one patient), keratitis (two patients), and diabetes mellitus (one patient), respectively.

Table 3. *Fusarium* species in different types of infections.

	***Fusarium* infections**			
Species	**Superficial**	**Locally invasive**	**Disseminated**	**Total**
F. petroliphilum (FSSC)	-	1	4	5
F. solani s.s. (FSSC-5)	4	2	-	6
F. keratoplasticum (FSSC)	2	-	-	2
F. solani s.s. (FSSC-6)	-	1	-	1
F. proliferatum (FFSC)	1	8	4	13
F. verticillioides (FFSC)	-	-	3	3
F. andiyazi (FFSC)	-	-	1	1
F. oxysporum (FOSC)	-	1	-	1
F. dimerum (FDSC)	-	1	-	1
Total	**7**	**14**	**12**	**33**

Antifungal susceptibility tests were performed for 32 strains (isolated from 33 patients) which were identified by MLST. The overall results obtained from visual and/or spectrophotometric readings were similar for the MIC endpoints after 48 and 72 h of incubation. The GM values of MICs (mg/L) across all isolates used in this study were as follows (in increasing order): amphotericin B, 0.84 mg/L; voriconazole, 3.83 mg/L; posaconazole, 5.3 mg/L; caspofungin, 15.66 mg/L; anidulafungin, >16 mg/L; itraconazole, 59.97 mg/L; fluconazole, 61.29 mg/L; and flucytosine, >64 mg/L (Table 4). Of note, all of the species showed high MIC/MEC values for flucytosine, fluconazole, itraconazole, anidulafungin, and caspofungin.

Amphotericin B had the highest *in vitro* activity (ranging from 0.125 to 4 mg/L) against all species, with the MIC ranging from 0.125 to 4 mg/L. However, both voriconazole and posaconazole showed interspecies variability across and within *Fusarium* species complexes. The members of the FSSC (MIC ranging from 2 to 8 mg/L) and especially *F. petroliphilum* strains (MIC ranging from 8 to 16 mg/L) showed higher MIC values than other species for voriconazole. A similar pattern of susceptibility was also observed with posaconazole against the FSSC (MIC ranging from 0.125 to >16 mg/L).

Table 4. GM of MICs/MECs, MIC/MEC ranges values obtained by testing the susceptibility of *Fusarium* strains to antifungal agents

	MIC/MEC (mg/L)															
	Amphotericin B		**Voriconazole**		**Posaconazole**		**Fluconazole**		**Itraconazole**		**Anidulafungin**		**Caspofungin**		**5-Fluorocytosine**	
Species (n)	MIC Range	GM MIC	MIC Range	GM MIC	MIC Range	GM MIC	MIC Range	GM MIC	MIC Range	GM MIC	MEC Range	GM MEC	MEC Range	GM MEC	MIC Range	GM MIC
FSSC (14)	**0.25-2**	**0.86**	**2-16**	**5.94**	**0.12->16**	**11.2**	**>64**	**>64**	**>64**	**>64**	**>16**	**>16**	**>16**	**>16**	**>64**	**>64**
F. petroliphilum (5)	0.25-1	0.57	8-16	9.19	>16	>16	>64	>64	>64	>64	>16	>16	>16	>16	>64	>64
F. solani s.s (FSSC-5) (6)	0.25-1	0.79	2-8	4.49	>16	>16	>64	>64	>64	>64	>16	>16	>16	>16	>64	>64
F. keratoplasticum (2)	2	2	8	8	0.12-16	1.41	>64	>64	>64	>64	>16	>16	>16	>16	>64	>64
F. solani s.s. (FSSC-6) (1)	2	2	2	2	>16	>16	>64	>64	>64	>64	>16	>16	>16	>16	>64	>64
FFSC (16)	**0.12-8**	**0.88**	**1-8**	**2.71**	**0.12-16**	**2.36**	**16-64**	**58.69**	**8-64**	**56.20**	**>16**	**>16**	**8-16**	**15.32**	**>64**	**>64**
F. proliferatum (12)	0.12-1	0.56	1-8	3.56	0.12-16	5.02	>64	>64	>64	>64	>16	>16	>16	>16	>64	>64
F. verticillioides 3)	2-4	2.52	1	1	0.12-0.25	0.16	>64	>64	>64	>64	>16	>16	>16	>16	>64	>64
F. andiyazi (1)	8	8	2	2	1	1	16	16	8	8	-[a]	-[a]	8	8	-[a]	-[a]
FOSC (1) *F. oxysporum*	0.5	0.5	2	2	16	16	>64	>64	>64	>64	>16	>16	>16	>16	>64	>64
FDSC (1) *F. dimerum*	0.5	0.5	4	4	>16	>16	>64	>64	>64	>64	>16	>16	>16	>16	>64	>64
TOTAL (32)	**0.12-8**	**0.84**	**1-16**	**3.83**	**0.12-16**	**5.30**	**16->64**	**61,29**	**8->64**	**59.97**	**>16**	**>16**	**8->16**	**15,66**	**>64**	**>64**

[a] Not examined MIC: Minimum inhibitory concentration,MEC: Minimum effective concentration

Colour code:

≤1 mg/l	2 mg/l	4 mg/l	8 mg/l	≥16 mg/l

Discussion

In the present study, cases of fusariosis occurring in a tertiary care university hospital in Turkey over the last 20 years were evaluated. Both species distribution and antifungal susceptibility of the isolates were determined. These are the first epidemiological data on fusarioses from Turkey. Similar to recent surveys elsewhere, a statistically significant increase in the incidence of fusarioses over the years was also determined in this study [26, 27].

Fusarium species cause a broad spectrum of infections in humans, including superficial, locally invasive, and disseminated infections [13, 14]. Clinical manifestations largely depend on the immune status of the host and the portal of entry, which include paranasal sinuses, lungs, and skin [13]. Among immunocompetent hosts, keratitis and onychomycosis are the most common infections and, occasionally, mycetoma by *Fusarium* spp. is observed. Unlike infections in the normal host, fusariosis in the immunocompromised population is typically invasive and disseminated. Disseminated infections occur mainly in patients with hematological malignancies and have also occasionally been reported in the immunocompromised patients due to transplantation, solid tumors, and autoimmune disorders [13, 27–30]. In line with this, 80 % of the patients with disseminated fusariosis in the present study had hematological malignancy. The remaining patients with disseminated infections were also severely immunocompromised, e.g., due to solid tumor, autoimmune disease, or low birth weight.

The principal portal of entry for *Fusarium* species seems to be the airways or traumatic inoculation. Sinus and lung involvement is common and these foci may serve as sites for dissemination in immunocompromised hosts. Clinical manifestations of fusarial sinusitis and pneumonia are indistinguishable from those caused by *Aspergillus* spp., but especially lung involvement is associated with higher mortality [13]. Involvement of the skin is another important manifestation of fusariosis and can represent a primary site of infection or a manifestation of secondary metastasis in patients with disseminated fusariosis. Among immunocompromised patients, skin lesions may be the single source of diagnosis [13, 31].

In this study, nearly half (44.7 %) of the cases were localized infections, while lung, sinuses, and skin involvement was seen in different underlying conditions, like hematological disorders, solid malignancies, and chronic illnesses. Definitive diagnosis of *Fusarium* species requires isolation of the agent from infected sites, and culture identification from biopsy samples is especially important because of the histopathological similarities between *Fusarium* and other hyalohyphomycetes agents. Clinicians should be aware of local infections that may occur during follow-up of high-risk patients, and different samples are of importance in detection.

In vitro, *Fusarium* can be identified by the production of hyaline, crescent or banana-shaped, multicellular macroconidia. However, species identification is difficult and requires molecular methods [5, 18]. In this study, we used MLST for identification to the species level.

Globally, the FSSC is themost common group encountered in human infections and is present in approximately 50 % of patients [13]. Being responsible for about 20 % of infections, the FOSC ranks second [13]. In the present study, however, the FFSC was found to be the most frequently detected complex (51.5 %), followed by the FSSC (42.4 %), while the FOSC was isolated from only a single patient. The *F. andiyazi* strain isolated from one patient was the first isolation from a human case that we previously reported [32]. In a recent survey conducted in Europe (involving our region as well), the FFSC was also reported to be prevalent [31]. These data support the view that regional differences exist in the distribution of *Fusarium* species [10].

A link between species identity and course of infection has been observed in several studies. For example, the FFSC was prevalently isolated frominvasive and disseminated infections [8, 10, 17]. *F. proliferatum* and *F. verticillioides* are the most commonly encountered human opportunists in this complex. Also, in our study, *F. proliferatum* was the predominant species in locally invasive infections and along with *F. petroliphilum* in disseminated infections [33]. The FSSC has also been reported as the most commonly isolated species complex in superficial infections, mainly keratitis and onychomycosis, in America and Asia, while the FOSC ranks first in such infections in Europe [9, 34–36]. Although the number of cases was low in our study, the FSSC was responsible in 85.7 % of superficial infections. In the present study, flucytosine, fluconazole, itraconazole, anidulafungin, and caspofungin were ineffective against all isolates. Species-specific differences in the susceptibility of *Fusarium* isolates were apparent in the MICs to amphotericin B, voriconazole, and posaconazole. In agreement with previous reports [16, 37], amphotericin B was the most effective agent across all isolates. The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and the European Confederation of Medical Mycology (ECMM) recommend amphotericin B and voriconazole as the first optional agents in the treatment of cases with fusariosis [18]. However, in the present study, these compounds were not effective against some of the species, sometimes indicating high degrees of intraspecific variability (Table 4). Some studies have reported that the MIC values in *F. verticillioides* were high for amphotericin B and low for posaconazole [16, 37]. Similarly, we also observed activity of posaconazole against this species, but with a limited degree of confidence.

In conclusion, our study indicated that regional differences exist in the distribution of *Fusarium* species and that species-specific differences might also reflect in antifungal susceptibility patterns. As the observed numbers of fusariosis, particularly disseminated cases, are increasing, monitoring of local epidemiological data by determining fungal identity and antifungal susceptibility testing will definitely help clinicians develop appropriate therapies that have a high probability of successfully treating disseminated fusariosis.

Acknowledgments

Burcu Dalyan Cilo was supported by grants from the Scientific and Technological Research Council of Turkey and an FEMS fellowship via the affiliates Turkish Microbiological Society. Abdullah Mohammed Said Al-Hatmi was financially supported by the Ministry of Health, Muscat, Oman.

Conflict of interest

The authors declare to have no conflict of interest.

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CHAPTER 12

Chapter 12

Multidrug-resistant *Fusarium* keratitis: a clinico-mycological study of keratitis infections in Chennai, India

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Mycoses 2016; doi: 10.1111/myc.12578. (in press).

Summary

In this study, we aimed to present the first molecular epidemiological data from Chennai, India, analyse keratitis cases that have been monitored in a university hospital during 2 years, identify the responsible *Fusarium* species and determine antifungal susceptibilities. A total of 10 cases of keratitis were included in the study. *Fusarium* isolates were identified using the second largest subunit of the RNA polymerase gene (*RPB2*) and the translation elongation factor 1 alpha (*TEF1*). Antifungal susceptibility was tested by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) methodology. The aetiological agents belonged to *Fusarium solani* species complex (FSSC) (n = 9) and *Fusarium sambucinum* species complex (FSAMSC) (n = 1), and the identified species were *Fusarium keratoplasticum* (n = 7), *Fusarium falciforme* (n = 2) and *Fusarium sporotrichioides* (n = 1). All strains showed multidrug resistance to azoles and caspofungin but exhibited lower minimum inhibitory concentration (MIC) to natamycin and amphotericin B. *Fusarium keratoplasticum* and *Fusarium falciforme* belonging to the *Fusarium solani* species complex were the major aetiological agents of *Fusarium keratitis* in this study. Early presentation and 5% topical natamycin was associated with better patient outcome. Preventative measures and monitoring of local epidemiological data play an important role in clinical practice.

Keywords: *Fusarium*, trauma, keratomycosis, *RPB2*, *TEF1*, antifungal resistance

Introduction

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.[1] *Fusarium* species cause a broad spectrum of infections in humans, ranging from mildly superficial to fatally disseminated.[2] *Fusarium* keratitis is a serious ocular disorder that may lead to vision loss.[3] Because of its risk of permanent loss of vision, corneal infections are considered an ophthalmologic emergency.[4]

Awareness of keratitis caused by *Fusarium* with correct recognition of the fungal nature is increasing.[5] Traumatic *Fusarium* keratitis is observed especially among farmers and outdoor workers. However, the widespread use of contact lenses has dramatically increased the incidence of keratitis.[6] The traumatic episode may be minor, such as a small abrasion from a small foreign body, or tear insufficiency. The spectrum of etiological *Fusarium* species is dependent on geographic and climatic factors and may also change with populations living in rural or urban areas, and in western *versus* developing countries.[7] In the present study, we describe the molecular characterization and antifungal susceptibility testing of *Fusarium* species isolated from 10 patients with keratitis infections, observed at a tertiary Indian hospital diagnosed over a two-year period in South India.

Materials and Methods

Patients and fungal isolates

Fusarium keratitis cases that were diagnosed and treated at Sri Ramachandra Medical College and Research Institute over two years (July 2012 − July 2014) were selected for the study. Corneal scrapings were aseptically collected from keratitis patients presenting to the Department of Ophthalmology and inoculated onto two sets of blood and chocolate agar (HiMedia Laboratories, Mumbai, India) in 'C'-shaped streaks and were immediately sent to the Microbiology Laboratory and the Mycology Lab for incubation, one set each at 25 and 37 ^{0}C. Samples that grew *Fusarium* were cultured on Sabouraud Glucose Agar (SGA) with chloramphenicol and without cycloheximide (HiMedia Laboratories, Mumbai, India) at 25 ^{o}C. The isolated strains were stored in the Mycology Lab. Clinical details of the patients including age, sex, and relevant history was recorded. Patient identities were protected throughout the study. The Institutional Ethics Committee approval was obtained for the study (REF: IEC-NI/12/OCT/30/46).

Molecular identification

Preliminary identification down to genus level was carried out using morphology. Because of insufficient phenotypic characters for reaching species level, strains were sent to CBS-KNAW, Utrecht, The Netherlands for molecular identification. DNA extraction was performed using glass beads (Sigma G9143, Sigma-Aldrich Co, Missouri, USA) according to the protocol

described by Al-Hatmi *et al*.[8] Briefly, two gene regions were amplified: *TEF1* and *RPB2*. Primers for *TEF1* were EF1 and EF[9] and for *RPB2* these were RPB2-7cr and RPB2 -5f.[10] Both were amplified and sequenced following the methods published by Al-Hatmi *et al*.[11] For unambiguous molecular identification, a search for similarity was performed with the sequences of each gene using BLAST (Basic Local Alignment and Search Tool) of the NCBI database, the Fusarium-ID database and the *Fusarium* MLST database. Besides phylogenetic placements with species or the haplotypes within species, the sequences of the study isolates were checked with the above mentioned databases specifically for matches with type and reference strains.

Antifungal susceptibility testing

Antifungal susceptibility testing (AST) was performed according to M38-A2 broth microdilution method of Clinical and Laboratory Standards Institute (CLSI).[12] The six drugs tested were itraconazole (Sigma-Aldrich, Missouri, USA), voriconazole (Sigma-Aldrich, Missouri, USA), posaconazole (Sigma-Aldrich, Missouri, USA), Amphotericin B (HiMedia Laboratories, Mumbai, India), caspofungin diacetate (Sigma-Aldrich, Missouri, USA) and natamycin (Sun Pharma, Mumbai, India). *Paecilomyces variotii* CBS 132734 was used as the quality control strain for antifungal susceptibility testing.

Results

Ten cases of keratitis caused by *Fusarium* species were identified during this study period. Three additional patients with non-keratitis fusariosis, i.e. an invasive fusariosis in a patient with haematogenous malignancy, a pedal infection in a patient with diabetes and a Chronic obstructive pulmonary disease (COPD) case in a HIV-positive patient, were excluded from the study. All patients hailed from Tamilnadu state, India. The actively working adult age group was the most frequently affected (Table 1). The most common portal of entry was trauma among nine patients. The underlying condition of one patient who described an acute onset redness of eye after travelling on a dusty road was diabetic mellitus.

The majority of the agents (n=9) were found to belong to the FSSC. Within the FSSC, seven isolates were *F. keratoplasticum* and two were *F. falciforme*. The remaining isolate was *F. sporotrichoides.* All strains proved to be multi-drug resistant. The topical antifungal natamycin had the best activity, followed by amphotericin B and voriconazole which were found to have moderate activity against some of the isolates (Table 1), while none of the remaining drugs tested were found to be effective.

Table 1. Demographic data of patients, source of isolation, identification, GenBank accession and antifungal susceptibility results.

No	Age	Sex	Site	Associated factors	Identification		MIC/MEC (μgml^{-1}) after 48 hours of incubation						GenBank Accession	
					Morphological	Molecular	AMB	VOR	ITR	POS	NAT	CAS (MEC)	*TEF1*	*RPB2*
A01	37	M	Cornea	Trauma	FSSC	*F. keratoplasticum*	4	16	>32	16	2	16	KU711749	KU604346
A03	52	M	Cornea	Trauma	FSSC	*F. keratoplasticum*	4	8	>32	16	2	16	KU711750	KU604347
A04	56	M	Cornea	Trauma	FSSC	*F. keratoplasticum*	8	>32	>32	32	2	>16	KU711751	KU604350
A05	46	F	Cornea	Trauma	FSSC	*F. falciforme*	>32	16	>32	32	4	>16	KU711728	KU604365
A06	39	M	Cornea	Trauma	FSSC	*F. keratoplasticum*	8	>32	>32	32	2	>16	KU711752	KU604351
A07	49	M	Cornea	Trauma	FSSC	*F. keratoplasticum*	4	16	16	16	2	16	KU711755	KU604355
A08	55	M	Cornea	Trauma	FSSC	*F. keratoplasticum*	4	8	>32	32	2	16	KU711753	KU604348
A10	34	M	Cornea	Splint injury	FSSC	*F. keratoplasticum*	2	>32	>32	32	2	16	KU711754	KU604349
A22	38	F	Cornea	Trauma	FSSC	*F. falciforme*	4	>32	16	32	2	>16	KU711729	KU604370
A42	40	M	Cornea	DM type2	*Fusarium* spp.	*F. sporotrichioides*	8	16	>32	16	2	>16	KU711720	KU604382

DM – Diabetes Mellitus; AMB – Amphotericin B; VOR – Voriconazole; ITR – Itraconazole; POS – Posaconazole; CAS – Caspofungin; MIC – Minimum Inhibitory Concentration; MEC – Minimum Effective Concentration; FSSC- *Fusarium solani* species complex

Discussion

The importance of *Fusarium* as an opportunistic pathogen has been mounting during recent years due to an increasing incidence of ocular fusariosis in certain regions of the world.[13] In India, *Fusarium* species have been reported to be the most common causative agents of fungal keratitis.[3,14,15] There is an urgent need to expand our knowledge of these species in terms of epidemiology, methods of prevention, and treatment.

Among our patients, the male/female ratio was 4:1, and all of them belonged to the age group of working adults. The active male population with an outdoor occupation is more prone to accidental infection. In tropical developing countries such as India, males, especially field labourers and factory workers may be more at risk for fungal keratitis secondary to trauma at their workplace.[16] Recent reports suggest the emergence of keratitis by *F. sacchari*, a member of the *Fusarium fujikuroi* species complex (FFSC) among sugarcane farmers in north India.[17] Therefore, protective eye wear should be recommended for people at risk for these types of injuries. Among our cases of *Fusarium* keratitis, trauma by vegetation and splinter injury was found to be a consistent cause followed by diabetic mellitus.

Although preliminary identification at genus level gives healthcare workers enough to start empirical treatment, further characterization contributes to understanding epidemiology and spread of antifungal resistance. In the present study *TEF1* and *RPB2* genes differentiated between various *Fusarium* species. O'Donnell *et al.*[18] demonstrated the utility of *RPB2* and *TEF1* nucleotide variation for *Fusarium* speciation.

Our study reconfirmed that the FSSC is the most frequently involved group in human *Fusarium* keratitis. Within this complex, *F. keratoplasticum* and *F. falciforme* are especially significant human opportunists. *Fusarium* genotypes from human infections, particularly of the cornea (mycotic keratitis) are frequently identical to those of strains isolated from biofilms in plumbing systems.[19] *Fusarium keratoplasticum* is now recognized as a plumbing-inhabiting agent, causing plant diseases and also superficial infections.[20-22] In agreement with Hassan *et al.,*[3] our results showed that *F. keratoplasticum* was dominant in keratitis. Also *F. sporotrichioides* was isolated from corneal scrapings of one of our patients. It is a known producer of type-A trichothecene T-2 toxin.[23]

Limited data exist to correlate the clinical outcome of fusariosis with *in vitro* susceptibility testing. Most of the available data on human fusariosis concern retrospective analyses of case reports. In the current study, most of the drugs tested were inactive *in vitro* against *Fusarium,* including amphotericin B, confirming data of O'Day *et al.*[24] Unsatisfactory susceptibility profiles may be attributed to many factors, including the inoculum size and incubation period during susceptibility testing,[19] but in *Fusarium* the main problem is multi-resistance of isolates. We confirmed that itraconazole showed no *in vitro* activity against *Fusarium*. Natamycin exhibited the lowest MICs ranging 2-4 µg/ml. Among the FSSC members, *F. falciforme* (n=2)

and *F. keratoplasticum* (n=7) were inhibited by amphotericin B at the lowest concentration of 2 µg/ml and highest of 8 µg/ml, and one isolate of *F. sporotrichoides* was inhibited at 8 µg/ml amphotericin B. This study demonstrated that the *Fusarium* spp. had high MICs above published epidemiological cut off values (ECVs)[25], for itraconazole (16 µg/ml), voriconazole (8 µg/ml), and minimum effective Concentration (MEC) for caspofungin (16 µg/ml). Posaconazole also showed elevated MICs ranging 16-32 µg/ml (Table 1).

Since susceptibility of *Fusarium* species to antifungal agents is variable, no agent can be considered as the drug of choice, and testing individual strains involved in specific infections is recommended.[26] Given the generally resistant nature of *Fusarium* species to antifungal agents, a combination of compounds with moderate activity should be considered. At present, in some countries topical natamycin is used for fusarial keratitis.[27] Al-Hatmi *et al*.,[28] reported 70% synergistic reaction between natamycin and voriconazole in keratitis patients. For members of the FSSC, a high dose amphotericin B preparation is considered to be the treatment of choice and for other species amphotericin B and/or voriconazole can be administered. Voriconazole has an advantage over amphotericin B in that it can be used even in patients with kidney disease. Currently, voriconazole and natamycin are the only FDA approved drugs for the treatment of infections caused by *Fusarium* species. Although natamycin shows poor activity against *Aspergillus,*[27] it was shown to have excellent activity against *Fusarium* with lowest MIC among the six antifungals tested and all ten patients treated with 5% topical application at two-hourly intervals for the first four days and thereafter every six hours for three weeks had favourable outcomes and did not require keratoplasty.

Our study supports the fact that *F. keratoplasticum* and *F. falciforme* of the FSSC are the most common agents of human *Fusarium* keratitis. Preventing occupational exposure, early presentation and topical natamycin therapy are recommended for the best outcome. Monitoring of local epidemiological data is of importance in guiding the clinical follow up. In addition, identifying the etiological agent is important for intiating the proper treatment of keratitis cases caused by *Fusarium* species.

Acknowledgments

Ananya Tupaki-Sreepurna received financial assistance in conducting this study by the Indian Council of Medical Research through the ICMR-TSS Integrated MD-PhD Fellowship. This study was partially funded by the Ministry of Health, Oman. Formal Agreement no. 28/2014. A. M. S. A. received a PhD scholarship from the Ministry of Health, Oman.

Conflict of interest

Authors declare no conflict of interest.

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CHAPTER 13

Chapter 13

Global molecular epidemiology and genetic diversity of *Fusarium*, a significant emerging group of human opportunists from 1958-2015

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Emerging Microbes & Infections 2016; 5: e33; doi:10.1038/emi.2016.126 (in press).

Abstract

Fusarium is a rapidly emerging, multidrug resistant genus of fungal opportunists, first identified as such in 1958 and presently recognized with numerous cases of fusariosis yearly. The authors examined trends in global *Fusarium* distribution, clinical presentation and prevalence since 1958 with the assumption that their distributions in each region had remained unaltered. Phylogeny and epidemiology of 127 geographically diverse isolates representing 26 *Fusarium* species were evaluated by using partial sequences of the *RPB2* and *TEF1* genes and compared with AFLP fingerprinting data. Molecular data of *Fusarium* species were compared to archived data, which enabled interpretation of hundreds of cases published in the literature. Our findings indicate that fusariosis is distributed globally, with a focus in (sub)tropical areas. Considerable species diversity is observed; genotypic features did not reveal any clustering with either clinical data or environmental origins. This study suggests that infections with *Fusarium* species might be truly opportunistic. The three most common species are *F. falciforme* and *F. keratoplasticum* (members of *Fusarium solani* species complex) followed by *Fusarium oxysporum* (*Fusarium oxysporum* species complex).

Key words: *Fusarium*, fusariosis, genotyping, AFLP, molecular epidemiology

Introduction

Fusarium infections are a major challenge with respect to diagnosis and treatment, especially in neutropenic patients. Disseminated infections may be fatal and are a considerable source of increased costs of healthcare. A major area of concern is the intrinsic resistance to a broad range of antifungals[1] which is a characteristic for *Fusarium*. During the past decade, *F. solani* complex has received special interest because of increasing numbers of infections worldwide.[2] More than 300 cases of *Fusarium* keratitis were associated with contaminated contact lens cleaning solution, causing outbreaks between 2005 and 2007, where members of the *F. solani* species complex played a major role.[3] Furthermore, reservoirs of infectious *Fusarium* species in hospital environments, especially plumbing and water systems, have been reported.[4]

While human fusariosis has been recognized only in the late 1950's, and endemic areas are mostly located in tropical and subtropical countries,[5] their global significance came into focus only during the last three decades. Etiological agents differ in antifungal susceptibility,[6] virulence profiles, geographic distribution, ecological niches, life-cycle, host, and mycotoxin production.[7] Although agents of fusariosis are mostly environmental[8], the potential of nosocomial transmission has recently been raised,[9] especially with reference to the high mortality rate of ~90% in patients with prolonged severe neutropenia.[10]

The burden of disease has not been established, but numerous clinical case series and case reports provide an estimate of the magnitude of the problem. Most published studies focused on prevalence in single healthcare centers.[10-16] Nucci *et al*.[17] reported 233 cases from different hospitals on a global scale. Mohammed *et al*.[18] reported 26 cases from the U.S.A. and reviewed 97 cases from the literature and Horn *et al*.[12] described 65 cases from the North American Path Alliance registry. A major problem in comparative studies is the subdivision of classical species into series of molecular siblings, which renders older literature without sequence data uninterpretable. Despite the current clinical importance of the organism, phylogenetic relationships among species, varieties and geographical groups in *Fusarium* are at present elusive. Hence, re-interpretation of these data in the light of modern molecular phylogeny is compulsory.

Molecular phylogenetic studies have led to the description of many *Fusarium* species with clinical relevance. These include members of the *F. solani* species complex, viz. *F. falciforme*, *F. keratoplasticum*, *F. lichenicola F. petroliphilum*, *F. pseudensiforme*, *F. solani* (FSSC 5), also known as *Fusisporium solani* and *Fusarium* haplotype '6'. The *Fusarium oxysporum* species complex contains three lineages which are involved in fusariosis and which still have not been formally introduced as taxonomic species. The *F. fujikuroi* species complex includes *F. acutatum, F. ananatum, F. anthophilum, F. andiyazi, F. fujikuroi s.s., F. globosum, F. guttiforme, F. musae, F. napiforme, F. nygamai, F. verticillioides, F. proliferatum, F. ramigenum, F. sacchari, F. subglutinans, F. temperatum* and *F. thapsinum*. Although rare, species of other *Fusarium*

lineages are emerging as potential opportunistic pathogens, e.g. in the *F. incarnatum-equiseti* species complex (*F. incarnatum* and *F. equiseti*), the *F. dimerum* species complex (*F. dimerum*, *F. delphiniodes* and *F. penzigii*), the *F. chlamydosporum* species complex, the *F. sambucinum* species complex (*F. armeniacum*, *F. brachygibbosum*, *F. langsethiae* and *F. sporotrichioides*), and the *F. tricictum* species complex (*F. acuminatum* and *F. flocciferum*).[1]

Over the last decade the number of cases of fusarioisis has increased worldwide and there are few reports describing the molecular epidemiology, therefore, the aim of the present study is to introduce a hypothetical system that allows interpreting and using at least part of the literature where sequence data are lacking. Pre-molecular publications which included interpretable case reports and geographical information were collected. Subsequently, available *Fusarium* strains collected worldwide and deposited during the last century in the Centraalbureau voor Schimmelcultures-Koninklijke Nederlandse Akademie van Wetenschappen (CBS-KNAW) culture collection, Fungal Biodiversity Centre, Utrecht, The Netherlands) were sequenced and re-identified with current diagnostic technology which enables phylogenetic analysis of the human-pathogenic *Fusarium* species, and these data were compared to published materials and their distribution with the assumption that their distributions in each region had remained unaltered.

Materials and methods

Fungal strains

A total of 127 strains collected from clinical samples (*n*=74; 58.3%; collected between 1978 and 2015) and strains collected from the environment (*n*=53; 41.7%; collected between 1929 and 2015) were analyzed. All strains were maintained under the name '*Fusarium*' in the reference collection of (CBS-KNAW), Utrecht, The Netherlands. Data on geographic origins and sources of isolation are listed in Table 1. All available type strains were included. Stock cultures were maintained on slants of 2 % malt extract agar (MEA) at 24 °C. Strains were assigned as clinical subgroup and environment subgroup.

DNA extraction

DNA was extracted following the Quick Cetyl trimethylammonium bromide (CTAB) protocol. 1–10 mm^3 fungal material was transferred to two mL screw-capped tubes prefilled with 490 µL 2× CTAB-buffer and 6–10 acid-washed glass beads. 10 µL Proteinase K was added and mixed thoroughly on a MoBio vortex for 10 min. 500 µL Chloroform: isoamylalcohol (24:1) was added and shaken for 2 min after incubation for 60 min at 60 °C. Tubes were centrifuged for 10 min at 14,000 r.p.m. Supernatant was collected in a new Eppendorf tube. To ~400 µL DNA sample 2/3 vol (~270 µL) of ice-cold iso-propanol was added and centrifuged again at 14,000 r.p.m. for 10 min and the upper layer was dissolved in 1 mL ice-cold 70 % ethanol. Tubes were

centrifuged again at 14,000 r.p.m. for 2 min, air-dried and re-suspended in 50 µL TE-buffer. Quality of genomic DNA was verified by running 2–3 µL on a 0.8 % agarose gel. DNA was quantified with NanoDrop 2000 spectrophotometer (Thermo Fisher, Wilmington, DE, U.S.A.). Samples were stored at −20 °C until analysis.

Table 1. Isolates of *Fusarium* included in this study used for sequence analysis and amplified fragments-length polymorphism analysis, except for six *RPB2* and *TEF1* which were retrieved from GenBank

CBS No	Species name	Country	Source	GenBank accession no.	
				TEF1	*RPB2*
CBS 130548	*F. acutatum*	Iran	Onycomycosis (Human)	KR071756	KU604289
CBS 113964	*F. acutatum*	Egypt	Environmental	KR071759	KU604290
CBS 739.97	*F. acutatum*	India	Environmental	KR071757	KU604288
CBS 401.97	*F. acutatum*	India	Environmental	KR071755	KU604287
CBS 402.97	*F. acutatum*	India	Environmental	KR071754	KT154005
CBS 118517	*F. ananatum*	South Africa	Environmental	KR071761	KU604273
CBS 118518	*F. ananatum*	South Africa	Environmental	KU711690	KU604271
CBS 118516	*F. ananatum*	South Africa	Environmental	KR071760	KU604269
CBS 184.29	*F. ananatum*	Unknown	Environmental	KR071762	KU604272
CBS 256.93	*F. andiyazi*	Cuba	Environmental	KR071719	KU604231
CBS 119857	*F. andiyazi*	South Africa	Environmental	KP662901	KT154004
CBS 737.97	*F. anthophilum*	Germany	Environmental	KU711685	KU604277
CBS 222.76	*F. anthophilum*	Germany	Environmental	KR071766	KT154006
CBS 119858	*F. anthophilum*	USA	Environmental	KR071764	KU604275
CBS 119859	*F. anthophilum*	New Zealand	Environmental	KR071765	KU604279
CBS 961.87	*F. concolor*	South Africa	Environmental	KR071773	KU604556
CBS 676.94	*F. concolor*	South Africa	Environmental	KR071774	KU604237
CBS 111770	*F. concolor*	Spain	Keratitis (Human)	KU711719	KU604323
C26	*F. delphinoides*	India	Keratitis (Human)	KU711775	KU604380
C52	*F. dimerum*	India	Keratitis (Human)	KU711776	KU604381
CBS 135550	*F. equiseti*	Mexico	Keratitis (Human)	KU711721	KU604324
CBS 135552	*F. equiseti*	Mexico	Keratitis (Human)	KU711723	KU604325
CBS 135553	*F. equiseti*	Mexico	Keratitis (Human)	KU711722	KU604326
CBS 135532	*F. falciforme*	Mexico	Keratitis (Human)	KU711737	KU604356
CBS 135533	*F. falciforme*	Mexico	Keratitis (Human)	KU711738	KU604362
CBS 135521	*F. falciforme*	Mexico	Keratitis (Human)	KU711733	KU604357
CBS 135520	*F. falciforme*	Mexico	Keratitis (Human)	KU711732	KU604363
CBS 135526	*F. falciforme*	Mexico	Keratitis (Human)	KU711734	KU604366
CBS 135524	*F. falciforme*	Mexico	Keratitis (Human)	KU711730	KU604358
CBS 135525	*F. falciforme*	Mexico	Keratitis (Human)	KU711731	KU604359
CBS 135558	*F. falciforme*	Mexico	Keratitis (Human)	KU711736	KU604368
CBS 135559	*F. falciforme*	Mexico	Keratitis (Human)	KU711735	KU604369
CBS 135513	*F. falciforme*	Mexico	Onycomycosis (Human)	KU711724	KU604360
CBS 135512	*F. falciforme*	Mexico	Onycomycosis (Human)	KM401894	KM401892
C256	*F. falciforme*	India	Keratitis (Human)	KU711725	KU604361
CBS 135522	*F. falciforme*	Mexico	Keratitis (Human)	KU711726	KU604364
CBS 135523	*F. falciforme*	Mexico	Keratitis (Human)	KU711727	KU604367
CBS 125177	*F. ficicrescens*	Iran	Environmental	KP662898	KT154001
CBS 125178	*F. ficicrescens*	Iran	Environmental	KP662899	KT154002

Table. 1 (Continued)					
CBS 125181	*F. ficicrescens*	Iran	Environmental	KP662900	KT154003
CBS 449.95	*F. fujikuroi*	France	Environmental	KR071742	KU604259
CBS 257.52	*F. fujikuroi*	Japan	Environmental	KU711678	KU604257
CBS 262.54	*F. fujikuroi*	India	Environmental	KR071744	KU604256
CBS 221.76	*F. fujikuroi*	Taiwan	Environmental	KR071741	KU604255
CBS 130402	*F. fujikuroi*	USA	Human skin (Human)	KU711677	KU604261
CBS 121864	*F. fujikuroi*	USA	Environmental	KR071743	KU604258
CBS 119855	*F. fujikuroi*	USA	Environmental	KU711679	KU604260
CBS 454.97	*Fusarium spp*	Sudan	Environmental	KU711697	KU604266
CBS 483.94	*Fusarium spp*	Australia	Environmental	KU711698	KU604267
CBS 119850	*Fusarium spp*	Australia	Environmental	KU711699	KU604268
CBS 135528	*F. keratoplasticum*	Mexico	Keratitis (Human)	KU711743	KU604338
dh22044/F610	*F. keratoplasticum*	Netherlands	Keratitis (Human)	KU711744	KU604339
CBS 135527	*F. keratoplasticum*	Mexico	Keratitis (Human)	KU711742	KU604340
CBS 135531	*F. keratoplasticum*	Mexico	Eumycetoma (Human)	KU711741	KU604353
CBS 135530	*F. keratoplasticum*	Mexico	Eumycetoma (Human)	KU711740	KU604352
CBS 135529	*F. keratoplasticum*	Mexico	Keratitis (Human)	KU711739	KU604354
dh21918/F605	*F. keratoplasticum*	Netherlands	Nail infection (Human)	KU711746	KU604344
dh22043/F609	*F. keratoplasticum*	Netherlands	Foot infection (Human)	KU711747	KU604341
CBS 748.97	*F. napiforme*	Namibia	Environmental	KR071712	KU604233
CBS 674.94	*F. napiforme*	Australia	Environmental	KR071713	KU604239
CBS 135139	*F. napiforme*	India	Keratitis (Human)	KR071717	KU604234
CBS 135140	*F. napiforme*	India	Keratitis (Human)	KR071714	KU604235
dh21772/F602	*F. oxysporum*	Netherlands	Nail infection (Human)	KU711714	KU604318
dh22047/F611	*F. oxysporum*	Netherlands	Nail infection (Human)	KU711711	KU604314
CBS 135560	*F. oxysporum*	Mexico	Keratitis (Human)	KU711709	KU604317
CBS 135561	*F. oxysporum*	Mexico	Keratitis (Human)	KU711710	KU604316
CBS 463.91	*F. oxysporum*	Germany	Nail infections (Human)	KU711712	KU604315
CBS 135515	*F. petroliphilum*	Mexico	Keratitis (Human)	KU711760	KU604336
CBS 135518	*F. petroliphilum*	Mexico	Keratitis (Human)	KU711762	KU604334
CBS 135519	*F. petroliphilum*	Mexico	Keratitis (Human)	KU711765	KU604331
CBS 135535	*F. petroliphilum*	Mexico	Keratitis (Human)	KU711761	KU604335
CBS 135514	*F. petroliphilum*	Mexico	keratitis (Human)	KU711767	KU604330
CBS 187.34	*F. phyllophilum*	UK	Environmental	KU711680	KU604300
CBS 246.61	*F. phyllophilum*	Germany	Environmental	KU711681	KU604301
CBS 480.77	*F. proliferatum*	Netherlands	Environmental	KR071736	KU604245
CBS 182.32	*F. proliferatum*	USA	Environmental	KR071734	KU604246
CBS 183.29	*F. proliferatum*	Japan	Environmental	KR071735	KU604242
CBS 184.33	*F. proliferatum*	Guyana	Environmental	KR071737	KU604244
CBS 125014	*F. proliferatum*	USA	Deep infection (Human)	KR071738	KU604243
CBS 131391	*F. proliferatum*	Australia	Environmental	KR071732	KU604247
CBS 133030	*F. proliferatum*	Iran	Onycomycosis (Human)	KR071733	KU604248
CBS 135547	*F. proliferatum*	Mexico	Keratitis (Human)	KU711675	KU604254
CBS 135549	*F. proliferatum*	Mexico	Keratitis (Human)	KU711676	KU604253
CBS 116324	*F. proliferatum*	Spain	Keratitis (Human)	KR071731	KU604249
CBS 130179	*F. proliferatum*	USA	Deep infection (Human)	KR071739	KU604241
CBS 132761	*F. proliferatum*	France	Deep infection (Human)	KU711673	KU604250
CBS 132762	*F. proliferatum*	France	Deep infection (Human)	KU711674	KU604252

Table. 1 (Continued)					
CBS 132764	*F. proliferatum*	Unknown	Environmental	KU711672	KU604251
CBS 135554	*F. pseudensiforme*	Mexico	Eumycetoma (Human)	KU711769	KU604377
CBS 135555	*F. pseudensiforme*	Mexico	Eumycetoma (Human)	KU711770	KU604375
CBS 135557	*F. pseudensiforme*	Mexico	Keratitis (Human)	KU711771	KU604376
CBS 135142	*F. sacchari*	India	Corneal ulcer (Human)	KR071749	KU604304
CBS 135143	*F. sacchari*	India	Corneal ulcer (Human)	KR071748	KU604307
CBS 135144	*F. sacchari*	India	Corneal ulcer (Human)	KR071750	KU604305
CBS 135145	*F. sacchari*	India	Corneal ulcer (Human)	KR071751	KU604306
CBS 223.76	*F. sacchari*	India	Environmental	KU711669	KU604309
CBS 134.73	*F. sacchari*	Guyana	Environmental	KR071753	KU604303
CBS 131369	*F. sacchari*	Australia	Environmental	KR071752	KU604302
CBS 121683	*F. sacchari*	India	Endophthalmitis (Human)	KR071747	KU604308
CBS 135563	*F. solani* (FSSC5)	Mexico	Hyalohyphomycosis (Human)	KU711758	KU604372
CBS 135564	*F. solani* (FSSC5)	Mexico	Hyalohyphomycosis (Human)	KU711759	KU604373
CBS 135565	*F. solani* (FSSC5)	Mexico	Hyalohyphomycosis	KU711757	KU604371
CBS 119831	*F. subglutinans*	New Guinea	Environmental	KR071769	KU604281
CBS 747.97	*F. subglutinans*	USA	Environmental	KU711691	KU604280
CBS 135538	*F. temperatum*	Mexico	Pulmonary infection (Human)	KF956082	KU604283
CBS 135539	*F. temperatum*	Mexico	Pulmonary infection (Human)	KF956083	KU604286
CBS 135540	*F. temperatum*	Mexico	Keratitis (Human)	KF956084	KU604285
CBS 135541	*F. temperatum*	Mexico	Keratitis (Human)	KF956085	KU604284
CBS 776.96	*F. thapsinum*	USA	Environmental	KR071726	KU604294
CBS 733.97	*F. thapsinum*	South Africa	Environmental	KR071730	KU604299
CBS 130176	*F. thapsinum*	Italy	Human mycetoma (Human)	KR071727	KU604298
CBS 119833	*F. thapsinum*	USA	Environmental	KU711717	KU604297
CBS 109077	*F. thapsinum*	Ethiopia	Environmental	KR071728	KU604295
CBS 114579	*F. verticillioides*	Austria	Sputum (Human)	KU711696	KU604220
CBS 115135	*F. verticillioides*	Sweden	Deep infection (Human)	KR071710	KU604217
CBS 131390	*F. verticillioides*	Australia	Environmental	KR071711	KU604225
CBS 116665	*F. verticillioides*	Unknown	Environmental	KR071705	KU604221
CBS 135542	*F. verticillioides*	Mexico	Onychomycosis (Human)	KU711693	KU604227
CBS 135543	*F. verticillioides*	Mexico	Onychomycosis (Human)	KU711694	KU604228
CBS 135545	*F. verticillioides*	Mexico	Onychomycosis (Human)	KX584417	KU604229
CBS 576.78	*F. verticillioides*	Russia	Environmental	KR071703	KU604216
CBS 579.78	*F. verticillioides*	USA	Ulcer of left leg (Human)	KR071706	KU604223
CBS 122159	*F. verticillioides*	Spain	Environmental	KR071707	KU604224
CBS 123670	*F. verticillioides*	USA	Environmental	KR071708	KU604222
CBS 102699	*F. verticillioides*	Germany	Abdominal drainage (Human)	KR071704	KU604218
CBS 108922	*F. verticillioides*	Germany	Urine (Human)	KR071709	KU604219
CBS 131389	*F. verticillioides*	Australia	Environmental	KU711695	KU604226

DNA amplification and sequencing

The following two gene regions were amplified directly from the genomic DNA: the second largest subunit of RNA polymerase (*RPB2*; Reeb *et al*.[19]) and the translation elongation factor-1α (*TEF1α;* O'Donnell *et al.*[20]) were amplified and sequenced following the methods published by Saleh *et al*.[16] PCR reactions were performed in a volume of 12.5 µL containing 1.25 µL 10× PCR buffer, 7.5 µL water, 0.5 µL dNTP mix (2.5 mM), 0.25 µL of each primer (10 pmol), 0.05 µL Taq polymerase (5 U/µL), 0.7 µL DMSO, and 1 µL template DNA (100 ng/µL). Amplification was performed with an ABI Prism 2720 (Applied Biosystems, Foster City, CA, U.S.A.). Cycling conditions included one cycle of 5 min at 94 °C, ten cycles of 45 sec at 94 °C, 45 sec at 55 °C and 1.5 min at 72 °C, 30 cycles of 45 sec at 94 °C, 45 sec at 52 °C and 1.30 min at 72 °C and post elongation step of 6 min at 72 °C for *TEF1* (EF1, EF2) and Pre-denaturation for 3 minutes at 95 °C, five cycles of 45 sec at 95 °C, 45 sec at 58 °C and 2 min at 72 °C, five cycles of 45 sec at 95 °C, 45 sec at 56 °C and 2 min at 72 °C, 30 cycles at 45 sec at 95 °C, 45 sec at 52 °C and 2 min at 72 °C and post elongation step of 8 min at 72 °C for *RPB2* (5F2, 7cr). PCR products were visualized by electrophoresis on 1% (w/v) agarose gels. Sequencing PCR was performed as follows: 1 min at 95 °C, followed by 30 cycles consisting of 10 sec at 95 °C, 5 sec at 50 °C and 2 min 60 °C. Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequencing was done on an ABI 3730xL automatic sequencer (Applied Biosystems) with BigDye™ v3.1 terminator cycle sequencing kit (Applied Biosystems).

Identification

Strains were identified by BLAST in GenBank, *Fusarium* MLST (http://www.cbs.knaw.nl/fusarium/)[20] and the FUSARIUM-ID (http://isolate.fusariumdb.org/)[21] databases. Additionally, phylogenetic placements with species/haplotypes within species complexes were checked with available databases specific for *Fusarium*.

Phylogenetic analyses

Sequences of *TEF1* and *RPB2* were undertaken to extend the genetic characterization of 127 isolates of *Fusarium* species previously characterized in terms of morphological characteristics. Sequences were edited using SeqMan in the Lasergene package (DNAstar, Madison, WI, U.S.A.). A phylogenetic approach was used to investigate relationship between 65 strains of *Fusarium* species including type and reference strains. The sequences were aligned using MAFFT v. 7.127 (http://mafft.cbrc.jp), followed by manual adjustments with MEGA v. 6.2.[22] A combined alignment was constructed for *RPB2* and *TEF1* for both the reference and test strains. The best-fit model of evolution was determined by MEGA v. 6.2.[22] Bootstrapped Maximum Likelihood analysis was performed by using RAxMLVI-HPC v. 7.0.3[23] as implemented on

the Cipres portal (http://www.phylo.org/)[24] with non-parametric bootstrapping using 1,000 replicates. Detailed analyses of medically important were compared in related with their clinical cases. For instance, *F. solani* actually represents a complex (i.e., *F. solani* species complex).

AFLP

The *Fusarium* strains were subjected to amplified fragment length polymorphism (AFLP) genotyping by using a previously described method.[23] However, for the amplification of the DNA fragments the selective residues (underlined) of the HpyCH4IV-primer (5'-GATGAGTCCTGACTAATGAG-3') and MseI-primer (5'-Flu-GTAGACTGCGTACCCGTAC-3') (MseI-C selective primer: ere replaced, respectively). Amplicons were 20× diluted using ddH_2O; 1 µL of the diluted amplicon was then added to a mixture of 8.9 µL ddH_2O and 0.1 µL LIZ600 (Applied Biosystems) followed by a heating step for 1 min at 100 °C and cooling down to 4 °C. AFLP fragment analysis was carried out using an ABI3500xL Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Raw data were then inspected visually after importation into BioNumerics v7.5 (Applied Maths, St. Martens-Latem, Belgium) and analyzed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering using the Pearson correlation coefficient. The analysis was restricted to DNA fragments in the range of 40-400 bp. The final AFLP dendograms was based on the combination of sequencing and AFLP data of both dendrograms.

Meta-analysis

The authors analyzed the existing medical literature on human cases of fusariosis from 1958 till December 2015. The authors conducted a systematic literature search for which PubMed was searched with the terms '*Fusarium* and 'fusariosis', and both were also searched as MeSH words and free words. Studies were only included for those that reported data for individual cases, because data provided in aggregate often lacked specific information for individual cases. Only cases with either histologically or culture proven *Fusarium* infection were included. 388 case reports in about 265 published papers were collected on a worldwide basis. Numbers are approximate because some cases have been used in repeated publications. Only cases with either histologically or culture proven *Fusarium* infection were included.

Results

Types of articles

A total of 388 cases of fusariosis, from 1958 until December 2015, was used in the literature data analysis. Included articles were mostly single case reports, two patients' cases and series of cases of fusariosis. Reported cases of fusariosis were identified from all over the world, and particularly from tropical and subtropical countries with a large agrarian population, such as Brazil, China, Colombia, India and Mexico. Other areas with frequent fusariosis were Australia, South Africa, Turkey and the Americas. *Fusarium* infections have also been reported from different countries in Eastern and Western Europe.

Patient characteristics

An overview of cases of fusariosis published in the medical literature, which includes the great majority of cases published to date, is given in Table 2. The majority of patients were male (*n*=253; 65.2%) (mean 41 years; range 3 months – 83 years). Over a third of the patients (*n*=143; 36.9%) had various underlying conditions at the time when the *Fusarium* infection was diagnosed. Causes of immunosuppression were hematological diseases and hematologic malignancies (*n*=122; 31.4%) and cancer of solid organs (*n*=17; 4.8%). Other causes of immunosuppression were medication (*n*=140; 36%), which included antibiotic (*n*=34; 8.8%) and steroid treatment (*n*=10; 2.6%). Pathogen introduction was ranked as trauma (*n*=18; 4.6%), indwelling catheters (*n*=2; 0. 5%), nasogastric tubes (*n*=2; 0.5%) and dialysis (*n*=3; 0.77%). No metabolic disorders, such as diabetes, were recorded in association with infection.

Type of infections

Infections due to *Fusarium* were predominantly found to be superficial and subcutaneous (*n*=174; 44.8%), occurring on skin (*n*=62; 16%), eye (*n*=66; 17%) and nail (*n*=25; 6.4%). Deep infections involved bone, joint and lung (*n*=4; 1%), heart (*n*=3; 0.77%) and peritoneum (*n*=2; 0.5%). The total of invasive and disseminated cases was (*n*=109; 28%), some of which were associated with fungemia (*n*=25; 6.4%) or disseminated disease with brain abscesses (*n*=4; 1%) (Table 2).

Table 2. Characteristics of 388 patients with fusariosis, literature cases from 1958 till 2015.

Characteristic	**No. of patients**
Total	388
Age, y (range)	3 months−82 years
Sex, M:F:Unknown	253 (65.3%):125 (32.2%):10 (2.5%)
Underlying condition	
• Transplantation	
▪ liver	5 (1.2%)
▪ lung	4 (1%)
▪ bone morrow	29 (7.5%)
▪ multivisceral (stomach, duodenum, pancreas and intestine)	1 (0.25%)
	7 (1.8%)
▪ kidney	3 (0.77%)
▪ heart	4 (1%)
▪ stem cells	38 (9.8%)
• Trauma/burns	27 (7%)
• Foreign body:	18 (4.6%)
▪ contact lens	4 (1%)
▪ catheter	2 (0.5%)
▪ graft	3 (0.77%)
▪ nasogastric tube	3 (0.77%)
▪ dialysis	4 (1%)
• Cancer	
▪ hematologic	122 (31.4%)
▪ solid organ	17 (4.8%)
• Medication	
▪ antibiotics	140 (36%)
▪ steroids	34 (8.8%)
• No	20 (5%)
Site of infection	
• Superficial	
▪ skin	62 (16%)
▪ eye	66 (17%)
▪ nail	25 (6.44)
▪ bone	4 (1%)
▪ joint	4 (1%)
▪ lung	4 (1%)
▪ endocarditis	3 (0.77%)
▪ peritoneum	2 (0.5%)
▪ prinephric abscess	2 (0.5%)
• Disseminated	109 (28%)
▪ blood	25 (6.4%)
▪ brain	4 (1%)

Treatment

An overview of the reported treatment of cases of fusariosis is shown in Table 3. The most widely used antifungal agent was amphotericin B deoxycholate (*n*=198; 51%), followed by liposomal amphotericin B (*n*=45; 11.6%), voriconazole (*n*=42; 10.8%), 5-flucytosin (*n*=30; 7.7%), itraconazole (*n*=26; 6.7%), fluconazole (*n*=25; 6.4%) and ketoconazole (*n*=19; 4.9%).

Antifungal combinations were used in treating fusariosis given either as a two or a three drug combination. The most frequently used combination of two drugs was amphotericin B with voriconazole (*n*=24; 6%), followed by amphotericin B with 5-flucytosin (*n*=20; 5%), amphotericin B with ketoconazole (*n*=4; 1%) and amphotericin B with fluconazole (*n*=4; 1%). Other combinations were used in one or two cases. Triple combinations were used in 14 cases (*n*=14; 3.6%). Surgery with antifungal treatment was used in 80 cases (20.6%). Besides antifungal therapy and surgery, granulocyte transfusions or granulocyte-colony stimulating factor (G-CSF) transfusion was used. Only 7 isolates were associated with cases where no treatment was reported (Table 3). It was not possible to look at changes in treatment over time, although, the authors assume that azole treatments have increased while AmB has declined. With the current guidelines, liposomal amphotericin B (*n*=45; 11.6%) and voriconazole (*n*=42; 10.8%) are very close according to the data from reported cases.

Table 3. Treatment administered to 388 patients with fusariosis.

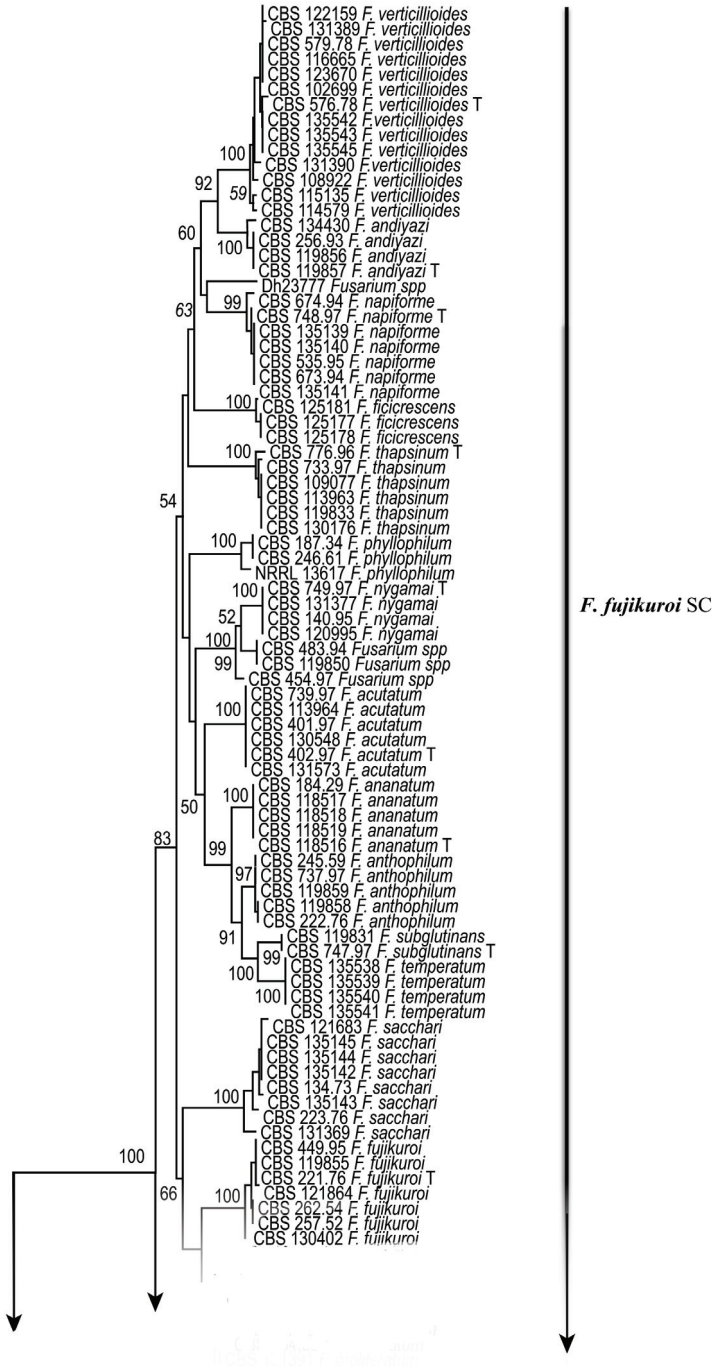
Treatment	
• Primary treatment (one drug)	
▪ Amphotericin B	198 (51%)
-deoxycholate	45 (11.6%)
-lipid/liposomal	42 (10.8)
▪ Voriconazole	30 (7.7%)
▪ Flucytosin	26 (6.7%)
▪ Itraconazole	25 (6.4%)
▪ Fluconazole	19 (4.9%)
▪ Ketoconazole	13 (3.4)
▪ Rifampicin	3 (0.77%)
▪ Posaconazole	-
▪ Terbinafine	2 (0.5%)
▪ Natamycin	80 (20.6%)
▪ Surgery	25 (6.4%)
▪ G-CSF	13 (3.4%)
▪ Granulocyte-transfusion	7 (1.8%)
▪ No therapy	
• Combinations (two drugs)	
▪ Amphotericin B/voriconazole	24 (6%)
▪ Amphotericin B/5-flucytosin	20 (5%)
▪ Amphotericin B/ketoconazole	4 (1%)
▪ Amphotericin B/fluconazole	4 (1%)
▪ Amphotericin B/posaconazol	2 (0.5%)
▪ Amphotericin B/itraconazole	2 (0.5%)
▪ Amphotericin B/caspofungin	1 (0.26%)
▪ Voriconazole/caspofungin	2 (0.5%)
▪ Voriconazole/anidulafungin	1 (0.26%)
▪ Voriconazole/terbinafine	1 (0.26%)
▪ Ketoconazole/terbinafine	1 (0.26%)
▪ Ketoconazole/rifampicin	1 (0.26%)
▪ Itraconazole/terbinafine	2 (0.5%)
▪ Itraconazole/terbinafine	1 (0.26%)
• Combinations (three drugs)	
▪ Amphotericin B/flucytosin/rifampicin	2 (0.5%)
▪ Amphotericin B/itraconazole/rifampicin	2 (0.5%)
▪ Amphotericin B/flucytosin/ketoconazole	2 (0.5%)
▪ Amphotericin B/fluconazole/voriconazole	2 (0.5%)
▪ Amphotericin B/fluconazole/itraconazole	1 (0.26%)
▪ Amphotericin B/itraconazole/voriconazole	1 (0.26%)
▪ Amphotericin B/flucytosin/itraconazole	1 (0.26%)
▪ Amphotericin B/fluconazole/rifampicin	1 (0.26%)
▪ Amphotericin B/voriconazole/micafungin	1 (0.26%)
▪ Amphotericin B/voriconazole/terbinafine	1 (0.26%)

Genetic analysis

127 *Fusarium* strains deposited in the CBS-KNAW collection were partially sequenced for *RPB2* and *TEF1*. The resulting two phylogenies yielded almost identical topologies, with similar resolution. Almost all strains of known species in all complexes of *Fusarium* formed independent clades in each tree. A concatenated tree (Fig. 1), including all major human pathogenic complexes of *Fusarium*, was based on 146 selected sequences. Lengths of generated sequence data were 795 bp and 507 bp for *RPB*2 and *TEF1,* respectively. Of the 1,302 nucleotides sequenced, 720 (55.1%) were constant, 551 (42.2%) were parsimony-informative, and 576 (44.1%) were variably and parsimony non-informative using MEGA v. 6.2.[22] The combined tree was subdivided into several species complexes, with high bootstrap values (Fig. 1). Seven clades represented human opportunists within the *F. solani* species complex. Thirteen groups represented opportunistic species in the *F. fujikuroi* species complex, with smaller human-associated clusters in the *F. oxysporum* species complex and to a lesser extent in the *F. chlamydosporum*, *F. polyphialidicum* (syn. *F. concolor*), *F. dimerum* and *F. incarnatum* species complexes. Strains CBS 454.97, CBS 483.94 and CBS 119850 had been identified morphologically as *F. napiforme* but formed a separate cluster different from the three strains including the type strain of *F. napiforme* (Fig. 1).

AFLP profiles contained approximately 50−60 fragments in the range of 40−400 bp. The AFLP dendrogram comprised seven main clusters at the species complex level, and additional subgroups within the main species clusters revealing genetic diversity within each species complex (Fig. 2). However, profiles did not vary significantly between *F. solani* species complex such as *F. falciforme*, *F. keratoplasticum*, *F. lichenicola F. petroliphilum*, *F. pseudensiforme*, whereas there was significant AFLP variation between isolates within the *F. fujikuroi* species complex with separate profiles for each species, as well as within other species complexes of *F. chlamydosporum*, *F. concolor*, *F. dimerum*, *F. incarnatum-equiseti* and *F. oxysporum*.

When comparing AFLP clusters with the distribution of DNA sequence lineages, the groups were largely concordant. Groups 1−7 matched with previous identifications using *RPB2* and *TEF1* sequences. The *Fusarium concolor* species complex had one clinical subgroup, the *F. dimerum* species complex two, and the *Fusarium fujikuroi* species complex consisted of 16 clinical subgroups (15 named subgroups and one unnamed molecular lineage). The *F. incarnatum-equiseti* species complex had a single clinical group, the *F. oxysporum* species complex was divided into two subgroups and the *F. solani* species complex comprised six named and one unnamed subgroups. The AFLP clusters and subclusters were almost identical to the sequencing identifications except for few strains within the *F. solani* species complex (Fig. 2). The AFLP clusters were based on sequencing and AFLP data generated from combinations of both dendrograms. In this study, similar clinical presentations of fusariosis were observed among the different AFLP species/genotypes and that there seems to be no relation between species and clinical presentation.


CBS 122159 F. verticillioides
CBS 131389 F. verticillioides
CBS 579.78 F. verticillioides
CBS 116665 F. verticillioides
CBS 123670 F. verticillioides
CBS 102699 F. verticillioides
CBS 576.78 F. verticillioides T
CBS 135542 F.verticillioides
CBS 135543 F. verticillioides
CBS 135545 F. verticillioides
CBS 131390 F.verticillioides
CBS 108922 F. verticillioides
CBS 115135 F. verticillioides
CBS 114579 F. verticillioides
CBS 134430 F. andiyazi
CBS 256.93 F. andiyazi
CBS 119856 F. andiyazi
CBS 119857 F. andiyazi T
Dh23777 Fusarium spp
CBS 674.94 F. napiforme
CBS 748.97 F. napiforme T
CBS 135139 F. napiforme
CBS 135140 F. napiforme
CBS 535.95 F. napiforme
CBS 673.94 F. napiforme
CBS 135141 F. napiforme
CBS 125181 F. ficicrescens
CBS 125177 F. ficicrescens
CBS 125178 F. ficicrescens
CBS 776.96 F. thapsinum T
CBS 733.97 F. thapsinum
CBS 109077 F. thapsinum
CBS 113963 F. thapsinum
CBS 119833 F. thapsinum
CBS 130176 F. thapsinum
CBS 187.34 F. phyllophilum
CBS 246.61 F. phyllophilum
NRRL 13617 F. phyllophilum
CBS 749.97 F. nygamai T
CBS 131377 F. nygamai
CBS 140.95 F. nygamai
CBS 120995 F. nygamai
CBS 483.94 Fusarium spp
CBS 119850 Fusarium spp
CBS 454.97 Fusarium spp
CBS 739.97 F. acutatum
CBS 113964 F. acutatum
CBS 401.97 F. acutatum
CBS 130548 F. acutatum
CBS 402.97 F. acutatum T
CBS 131573 F. acutatum
CBS 184.29 F. ananatum
CBS 118517 F. ananatum
CBS 118518 F. ananatum
CBS 118519 F. ananatum
CBS 118516 F. ananatum T
CBS 245.59 F. anthophilum
CBS 737.97 F. anthophilum
CBS 119859 F. anthophilum
CBS 119858 F. anthophilum
CBS 222.76 F. anthophilum
CBS 119831 F. subglutinans
CBS 747.97 F. subglutinans T
CBS 135538 F. temperatum
CBS 135539 F. temperatum
CBS 135540 F. temperatum
CBS 135541 F. temperatum
CBS 121683 F. sacchari
CBS 135145 F. sacchari
CBS 135144 F. sacchari
CBS 135142 F. sacchari
CBS 134.73 F. sacchari
CBS 135143 F. sacchari
CBS 223.76 F. sacchari
CBS 131369 F. sacchari
CBS 449.95 F. fujikuroi
CBS 119855 F. fujikuroi
CBS 221.76 F. fujikuroi T
CBS 121864 F. fujikuroi
CBS 262.54 F. fujikuroi
CBS 257.52 F. fujikuroi
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F. fujikuroi SC

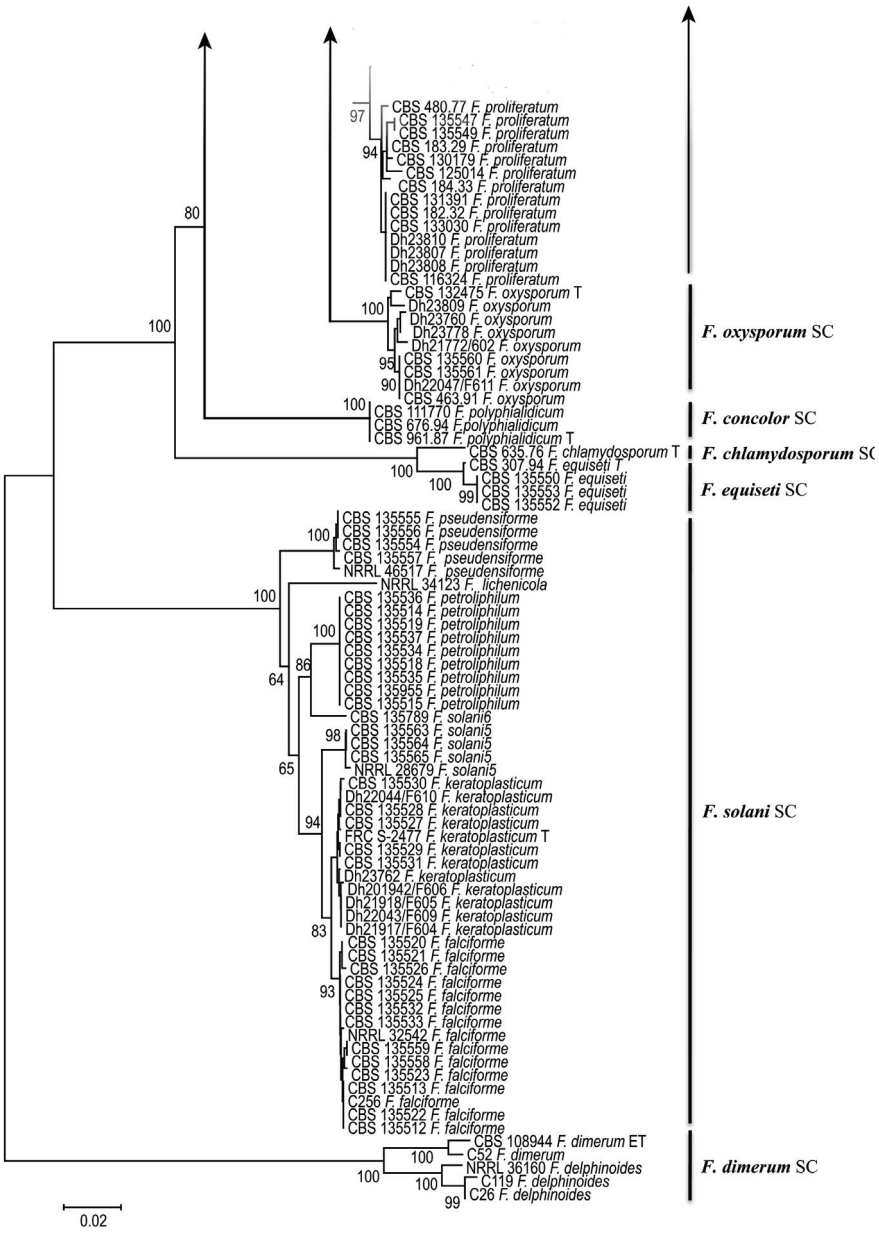

Figure 1. Phylogenetic tree resulting from RAxML analysis for the *RPB2* and *TEF1* genes. Total alignment length is 1,302 bp. Maximum Likelihood analysis was performed by using RAxML with non-parametric bootstrapping using 1 000 replicates. Numbers above branches are bootstrap support values ≥ 0.70 shown. As out group the epitype (ET) strain of *F. dimerum* CBS 108944.

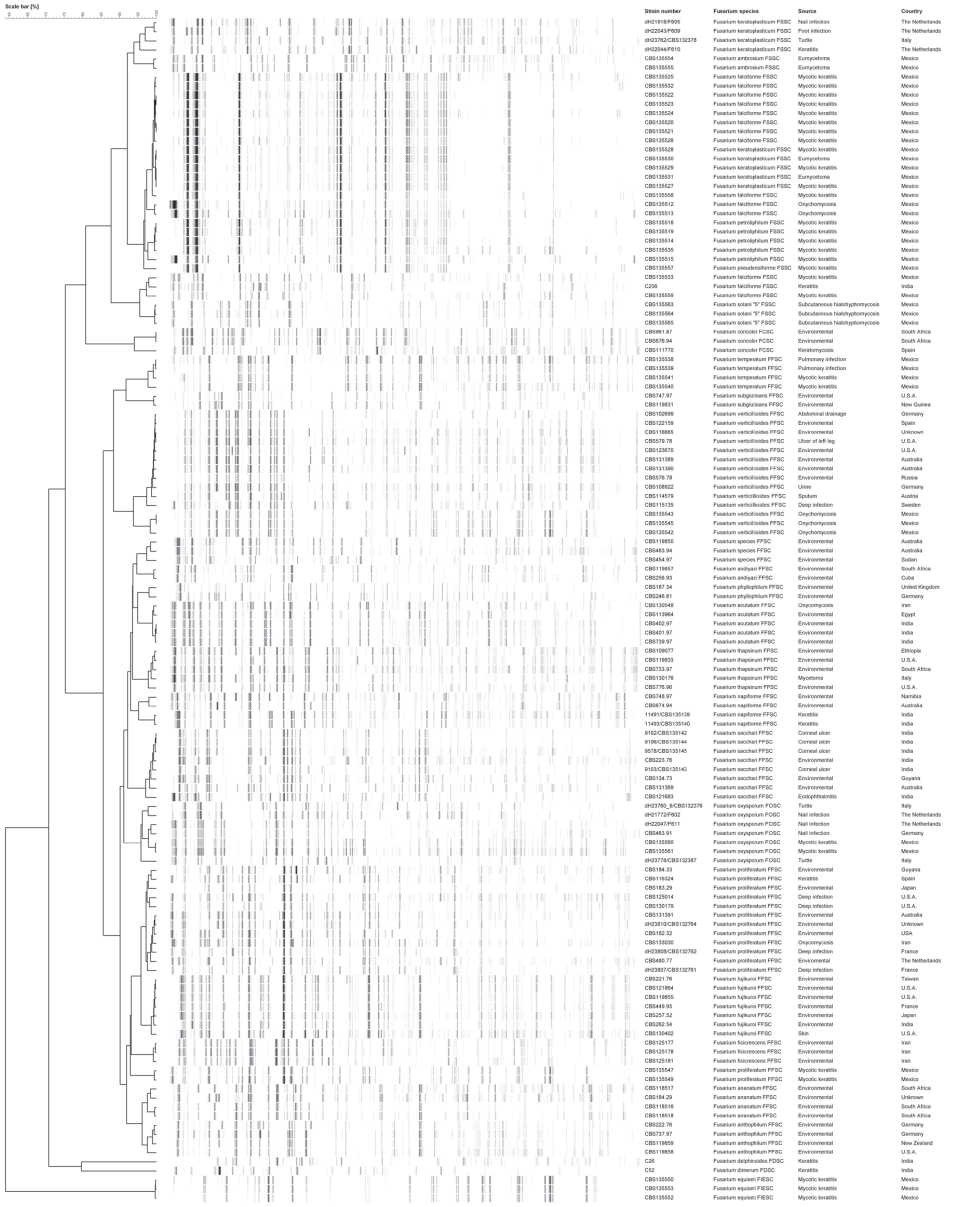

Figure 2. Clustering of amplified fragment-length polymorphism banding pattern of *Fusarium* spp. and combined with sequence analysis of (*RPB2* and *TEF1*), constructed by Bionumerics v7.5 (Applied Maths, Sint Martems-Latum, Belgium) and the dendrogram was generated by using the UPGMA algorithm.

Discussion

The molecular epidemiology of *Fusarium* was investigated on the basis of genetic data generated for *RPB2* and *TEF1* sequence analysis supplemented with AFLP fingerprint data. This study provides information on the locality, sources, species and geographic distribution in countries in which these fungi are common, such as Brazil, China, Colombia, India, Mexico and the U.S.A. One hundred and thirty *Fusarium* isolates including eight type strains deposited at the CBS-KNAW collection were included. Overall, there was good correlation between sequence analysis and AFLP genotypes. To analyze the genetic relationships among the AFLP and sequencing genotypes, we used phylogenetic algorithms which are commonly used to detect evidence of population subdivision and differentiation.[26]

The second largest subunit of RNA polymerase (*RPB2*) and *TEF1* showed high resolution with high species-level resolution, distinguishing 29 *Fusarium* species including the most common clinically important *Fusarium* species *F. falciforme*, *F. keratoplasticum*, *F. oxysporum*, *F. petroliphilum*, *F. proliferatum*, and *F. verticillioides*. Furthermore, these two loci were able to distinguish between other rare *Fusarium* species that cause a variety of infections (Table 1).

Although human opportunists were highlighted in many studies focusing on specific regions of the world and on specific type of infections[10-13, 27-34], the 127 *Fusarium* strains from the current study were collected from 26 countries in six continents and included clinical and environmental strains and isolates from cold blooded animals. Of these, Australia, Brazil, India, Mexico, and U.S.A. were among the top ten countries with the highest *Fusarium* infections based on clinical isolates in the CBS collection. Not surprisingly, 75 of the 127 patients from this study acquired their infection in one of these countries.

Previously, the majority of the clinically relevant *Fusarium* species were classified in two species complexes that in the past were referred to as single species, *F. oxysporum* and *F. solani*.[35] Approximately 80% of the human infections are caused by members of both species complexes[36], but a significant share of infections is caused by novel species complex members: *F. dimerum, F. fujikuroi* and *F. incarnatum-equiseti*. Within the *F. solani* complex, there are six recognized species and one unnamed lineage (FSSC6) clinically involved in fusariosis (Fig. 1). Of these species, *F. falciforme* (*n*=14/127 cases; 11%), was the dominant species in our study and mainly isolated from keratitis cases in Brazil, India and Mexico. Recently, Hassan *et al*.[13] showed that the majority of keratitis cases (*n*=46/65 cases; 70.7%) were *F. falciforme*. This species is emerging as one of the most virulent *Fusarium* species associated with fusariosis and keratitis.[15,36,37]

However, in the 2005–2006 mycotic keratitis outbreaks in Southeast Asia and North America, associated with contact lens cleaning solution, *F. petroliphilum* and *F. keratoplasticum* were the most involved species[36], consistent with the current study. AFLP genotypic variability

was higher in environmental species than in clinical species. A potential explanation is that not all environmental genotypes are sufficiently adapted to host tissue and are not selected, or perhaps a sampling effect is concerned. Zhang *et al*.[35] studied the *F. solani* species complex, specifically those species that cause infections in humans and plants, and concluded that clinical isolates often shared multi-locus haplotypes with isolates from different environmental sources including hospital locations. An increase of fusariosis among immunosuppressed patients was noted in the bone marrow transplant unit and also among patients with superficial infections in a hospital in Rio de Janeiro, Brazil.[38] These authors concluded that this increase might be due to airborne conidia circulating in this geographical region. Short *et al*.[36] concluded that there is no evidence that clinical isolates differ from those collected from other sources.

The large diversity of the *F. oxysporum* species complex is not completely resolved and it is not yet known whether the species have one or several phylogenetic origins and whether a single species or a species complex is concerned. From a traditional taxonomic point of view, *F. oxysporum* isolates have been differentiated from each other on the basis of pathogenicity as *formae speciales*, but this has been shown to be an unreliable approach.[8] The species delimitation was for the *F. oxysporum* species complex (FOSC) and at least 26 sequence types (ST) within the complex were involved in human infection.[39] Our FOSC clinical isolates were distributed over the complex although some clustering was found in the clade marked as ´sequence type 33´, based on *TEF1* alone and this sequences type is considered as the most common clade containing clinical *F. oxysporum* strains. The remaining species complexes *F. chlamydosporum, F. concolor, F. dimerum* and *F. incarnatum-equiseti* form separate clusters in the highly resolved sequence based ML tree (Fig. 1).

The *F. incarnatum-equiseti* species complex (FIESC) compromises twenty-eight phylogenetically distinct lineages[34] and only two are named and mainly involved in human infections (*F. incarnatum* and *F. equiseti*).[40]Although several members of the FIESC were included in the CDC *Fusarium* keratitis outbreak investigations, these species have not been observed yet to occur in epidemics or causing outbreaks. Concerning geography, 51 clinical isolates were recovered from the U.S.A., revealing that phylogenetically diverse human-opportunists and well represented in North America.[40] In our study, three clinical *F. equiseti* strains originated from Mexico and this might suggest that species of this complex are common in this region. The virulence of members of the *F. incarnatum-equiseti* species complex has been ascribed to their production of type A and B trichothecene mycotoxins.[39]

Fusarium dimerum and *F. delphinoides* belong to the *F. dimerum* species complex, and were both isolated from superficial and disseminated infections.[15] In our dataset, a supported clade of FDSC matching with AFLP data mainly contained strains from India and this might suggest regional prevalence. *Fusarium chlamydosporum* was reported from disseminated infections in patients with aplastic anemia and lymphocytic lymphoma from the U.S.A.[41,42]

CBS 111770 (*F. concolor*) is the only clinical strain in the *F. concolor* species complex; it was reported from a keratitis case from Spain.[42]

By comparing AFLP and MLST data *F. falciforme* and *F. keratoplasticum* appear to be widely distributed at least in Mexico, North America, Europe and India with dominancy in superficial infections including keratitis and onychomycosis. *Fusarium petroliphilum* is the second most diverse species and also is frequently involved in disseminated infections. *Fusarium solani sensu stricto,* '5', recently described as *Fusisporium* (*Fusarium*) *solani* (FSSC5)[7] contains strains such as CBS 135559, CBS 135564 and CBS 135565 which originate from Mexico and also shows significant occurrence in keratitis cases. This species was recently also reported from Asia (India and Qatar).[13,16] Given the large distances of identical strains occurring in many different countries, airborne distribution seems likely. However, the presence of *F. incarnatum, F. equiseti* and *F. chlamydosporum* in clinical samples from various infections in Northern America remains puzzling, but this perhaps can be explained by sampling effects.

As noticed before[44], the *F. fujikuroi* complex contains the highest number of species. In our study, 15 supported clades were recognized in all molecular analyses (Fig. 1, 2). Nearly all clades have various geographic distributions. Within the *F. fujikuroi* species complex, *F. proliferatum* and *F. verticillioides* were the dominant clinically-relevant species, have a global distribution and dominate in disseminated infections. *Fusarium sacchari* is the second most prevalent species and was often isolated from keratitis restricted to India. Although *F. nygamai* and *F. napiforme* are the most multi-drug resistant species within the *F. fujikuroi* complex[45], their presence in human infections is rare. *F. acutatum* was reported from nail infections from four cases in Qatar showing a low degree of variability and has been suggested to be clonal.[16] These results emphasize that *F. acutatum* is an emerging human opportunist which thus far was detected in Asia only. Sequence analysis of *RPB2* and *TEF1* and AFLP analyses showed that strains CBS 119850, CBS 483.94, and CBS 454.97 were nested within the *F. fujikuroi* complex, close to *F. nygamai* and *F. andiyazi* forming a well-supported monophyletic branch suggesting a novel species.

Deep fusariosis is rare in healthy individuals; a single brain infection has been reported.[46] Local infections may occur after direct inoculation or tissue breakdown by trauma or entrance of foreign bodies. Treatment of superficial infections is usually successful and requires surgery and removal of the foreign body, as well as antifungal therapy. The most important risk factors for severe fusariosis are prolonged neutropenia and T-cell immunodeficiency in patients suffering from a hematologic malignancy. *Fusarium* infections in the majority of these cases were due to neutropenia. Furthermore, in solid organ transplant recipients and cancer patients with neutropenia, infections due to *Fusarium* spp. increased and lead to disseminated infection. Patients develop painful skin lesions which vary from papules to nodules, with or without central necrosis.[47] In the majority of disseminated infections, secondary skin lesions lead to

diagnosis in > 50% of patients and preceded fungemia by approximately 5 days. [48] In contrast to aspergillosis, fusariosis frequently shows positive blood cultures and this is because *Fusarium* conidia are hydrophilic allowing dissemination.[47] Comparing fusariosis with mucormycosis[49], solid tumors and diabetes do not seem to be important risk factors. Only 17 (4.8%) cases were found in patients with solid tumors and 7 infections were reported in patients with diabetes mellitus. No underlying condition was observed in 20 (5%) cases.

Fusarium treatment depends on the site of infection. Surgery with antifungals was used in 80 cases (20.6%). Disseminated fusariosis in immunocompromised patients is usually treated with amphotericin B and voriconazole as first line therapy as is also suggested by recent guidelines.[50] In our literature review most antifungal therapy was amphotericin B deoxycholate, followed by liposomal amphotericin B and voriconazole. The most commonly used combination is amphotericin B/ voriconazole followed by amphotericin B/5-flucytosin. Triple combinations were used in 14 cases with different antifungals.

Major findings of the present study include: (i) human-associated fusaria were nested within 7 species complexes (i.e. *F. chlamydosporum, F. concolor, F. dimerum, F. fujikuroi, F. incarnatum-equiseti, F. oxysporum* and *F. solani*), (ii) The three most common species presented in both clinical and environmental groups are *F. falciforme* and *F. keratoplasticum* (members of *F. solani* species complex) followed by *F. oxysporum* (*F.oxysporum* species complex). (iii) most of the reported *Fusarium* species in this study were shared between patients and the environment and this might be due to the colonization of some patients with *Fusarium* isolates from the environment. Hence, there is genetic similarity between clinical and environmental isolates of the same *Fusarium* species, (iv) the species distributions show some evidence of geographical clustering among some of the species studied, although the present study is limited by an overrepresentation of isolates from Mexico and India.

Acknowledgments

This study was partially funded by the Ministry of Health, Oman (Formal Agreement no. 28/2014). Abdullah M. S. Al-Hatmi received a PhD scholarship from the Ministry of Health, Oman.

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Part III

In vitro antifungal susceptibility testing and combinations

- Specific antifungal susceptibility profiles of opportunists in the *Fusarium fujikuroi* complex
- Antifungal susceptibility and phylogeny of opportunistic members of the genus *Fusarium* causing human keratomycosis in south India
- *In vitro* resistance of clinical *Fusarium* species to amphotericin B and voriconazole using the EUCAST antifungal susceptibility method
- *In vitro* combinations of natamycin with voriconazole, itraconazole and micafungin against clinical *Fusarium* strains causing keratitis
- Comparative evaluation of Etest, EUCAST and CLSI methods for amphotericin B, voriconazole and posaconazole against clinically relevant *Fusarium* species

CHAPTER 14

Chapter 14

Specific antifungal susceptibility profiles of opportunists in the *Fusarium fujikuroi* complex

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Journal of Antimicrobial Chemotherapy 2015; 70: 1068–1071.

Abstract

Objectives: The aim of the present study was to evaluate and assess the *in vitro* activity of eight drugs, including the new azole isavuconazole, against 81 strains representing 13 species of the *Fusarium fujikuroi* species complex. **Methods**: A total of 81 *Fusarium* spp. isolates, within the *F. fujikuroi* species complex, were identified by molecular methods and tested according to CLSI M38-A2. Eight antifungal compounds, including the new azole isavuconazole, were tested. Isolates were selected to represent the widest variety of geographical regions and to include clinical as well as environmental strains. **Results**: Susceptibility profiles differed between and within species, with *Fusarium verticillioides* showing the lowest MICs and *Fusarium nygamai* the highest MICs. Amphotericin B was the most active drug, followed by voriconazole, posaconazole, isavuconazole and natamycin. The remaining antifungals (fluconazole, itraconazole and micafungin) showed poor activity with MIC/minimum effective concentration values of ≥32, ≥16 and 8 mg/L, respectively. **Conclusions:** Resistance patterns in the *F. fujikuroi* species complex are species specific and therefore identification down to species level is important for the choice of antifungal treatment.

Key words: molecular identification, multiresistant, triazole agents, isavuconazole

Introduction

Fusarium in general is resistant to the older azoles (e.g. itraconazole and fluconazole) and to echinocandins and has variable resistance to triazoles and amphotericin B.[1] Amphotericin B and voriconazole have been used for localized and disseminated fusariosis and have been recommended in a recent guideline.[2] Other drugs such as posaconazole and terbinafine have some activity against some *Fusarium* species.[3]

The few *in vitro* studies available have yielded variable results for antifungal susceptibility testing. Cuenca-Estrella et al.[4] concluded that susceptibility could be species or even strain dependent, similar to susceptibility profiles in *Scedosporium*.[5] The exceptionally large phylogenetic diversity in *Fusarium* interferes with species recognition and molecular verification of reported cases is often lacking.[6] For example, the *Fusarium fujikuroi* group comprises a large number of species, which sometimes can be distinguished by molecular parameters only. To date, 45 species have been described in the *F. fujikuroi* species complex (FFSC), 13 of which have been reported to cause human infection (*Fusarium acutatum*, *Fusarium ananatum*, *Fusarium andiyazi*, *F. fujikuroi*, *Fusarium guttiforme*, *Fusarium napiforme*, *Fusarium nygamai*, *Fusarium verticillioides*, *Fusarium proliferatum*, *Fusarium sacchari*, *Fusarium subglutinans*, *Fusarium temperatum* and *Fusarium thapsinum*).[7] FFSC is predominant in deep infections, while only few reports addressing susceptibility testing are available. Tortorano et al.[8] reported *F. verticillioides* to be the prevalent species (57%) in deepseated infections. Management of *Fusarium* infections is largely based on anecdotal data of retrospective studies without molecular characterization. Because susceptibility testing seems to differ between species, a definitive molecular identification to species level is important. Here, we report antifungal susceptibility profiles of 81 molecularly characterized strains of clinical and environmental isolates representing 13 rare species within the FFSC from a variety of geographical regions.

Materials and methods

Strains

Clinical (n=29) and environmental (n=52) isolates were obtained from worldwide sources supplemented with strains from the CBS reference collection, Utrecht, The Netherlands. The selected species, including type strains, were *F. acutatum* (n=7), *F. ananatum* (n=6), *F. andiyazi* (n=4), *F. anthophilum* (n=5), *F. fujikuroi* (n=7), *F. napiforme* (n=7), *F. nygamai* (n=7), *F. proliferatum* (n=9), *F. verticillioides* (n=10), *F. sacchari* (n=9), *F. subglutinans* (n=3), *F. temperatum* (n=1) and *F. thapsinum* (n=6). Species identification was confirmed by MLST consisting of partial sequencing of translation elongation factor-1 α (*TEF1*) and the RNA polymerase II largest subunit (*RPB2*).

Susceptibility testing

Antifungal susceptibility testing was performed as per CLSI document M38-A2 guidelines for non-dermatophyte species.[9] Briefly, the isolates were cultured on malt extract agar (Oxoid, UK) and incubated at 25 °C for 7 days prior to susceptibility testing. For assay preparation, the inocula were prepared by gently scraping the surface of the fungal colonies with a sterile cotton swab moistened with saline containing 0.05% Tween 20. The inoculum was adjusted spectrophotometrically at 530 nm to a percentage transmission in the range of 68%–70%, corresponding to 0.8–5.0×105 cfu/mL; this was further diluted 1:5 with RPMI and then this again was diluted 1:10 with RPMI medium. One hundred microliters of this suspension was added to each well (1: 2), giving a final concentration of 2.9×10^4 cfu/mL. Plates were incubated in duplo at 35 °C with the following drug concentration ranges: for amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands), itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer Central, Sandwich, UK), posaconazole (Merck, Whitehouse Station, NJ, USA) and isavuconazole (Basilea Pharmaceuticals, Basel, Switzerland) the concentration ranges were 0.016–16 mg/L; for fluconazole (Pfizer) and natamycin (DSM, Delft, The Netherlands) the concentration ranges were 0.062– 64 mg/L; and for micafungin (Astellas Pharma, Ibaraki, Japan) the concentration range was 0.008–8 mg/L. After 48 h of incubation, MICs and minimum effective concentrations (MECs) were determined visually with a mirror by comparing the growth in the wells containing the drug with the drug-free control. Quality control testing was performed on every new batch of MIC plates with the following reference strains *Aspergillus flavus* ATCC 204304, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258. The MICs for the reference strains were within the expected range.

Results and discussion

MIC ranges and geometric mean (GM) MICs of eight drugs per species are summarized in Table 1. For fluconazole, itraconazole and micafungin, all strains showed high MIC/MEC values of ≥32, ≥16 and >8 mg/L, respectively. In general, *Fusarium* isolates showed variable susceptibilities to amphotericin B, voriconazole, posaconazole and isavuconazole, with MICs ranging between 0.125 and 8 mg/L for amphotericin B, 1 and 8 mg/L for voriconazole (one strain had an MIC of 16 mg/L), 0.125 and.16 mg/L for posaconazole and 1 and ≥16 mg/L for isavuconazole. Posaconazole showed highest activity against *F. subglutinans*, *F. ananatum*, *F. verticillioides*, *F. anthophilum*, *F. fujikuroi* and *F. temperatum*. Amphotericin B had good activity (GM MIC ≤1.5 mg/L) against *F. subglutinans*, *F. ananatum*, *F. sacchari*, *F. andiyazi*, *F. anthophilum*, *F. fujikuroi*, *F. proliferatum* and *F. temperatum*. The MICs of voriconazole were lower for *F. subglutinans* and *F. temperatum* compared with other species.

Table 1. MIC ranges and GM MICs of amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, isavuconazole, natamycin and micafungin for the FFSC.

Species (n)	MIC (mg/L) [a]															
	Amphotericin B		Fluconazole		Itraconazole		Voriconazole		Posaconazole		Isavuconazole		Natamycin		Micafungin	
	MIC range	GM	MIC range	GM	MIC range	GM	MIC range	GM	MIC range	GM	MIC range	GM	MIC range	GM	MIC range	GM
***F. subglutinans* (3)**	0.5 - 1	0.66	64 - >64	64	>16	>16	1	1	0.125 – 0.25	0.20	2 -4	3.3	4	4	>8	>8
***F. ananatum* (6)**	0.125 - 2	0.70	>64	>64	>16	>16	1- 4	2.5	0.5 - 1	0.83	1-8	3.85	4	4	>8	>8
***F. verticillioides* (10)**	1 - 2	1.90	64 - >64	64	16 - >16	16	1- 4	1.3	0.25 - 1	0.42	1- 2	1.6	4	4	>8	>8
***F. sacchari* (9)**	1 - 4	1.5	>64	>64	>16	>16	1- 4	3	0.5 - >16	1.81	2 - 16	7.3	4	4	>8	>8
***F. napiforme* (7)**	1 - 8	3.57	>64	>64	>16	>64	1- 4	2	2 - >16	6	2 - 8	5.42	4	4	>8	>8
***F. andiyazi* (4)**	1 - 2	1.25	32 - >64	48	>64	16	1 - 4	2.25	0.25 - 4	1.68	1 - 8	3.75	4	4	>8	>8
***F. anthophilum* (5)**	0.25 - 1	0.70	64 - >64	64	16 - >16	16	1 - 4	1.8	0.25 – 0.5	0.45	1 - 8	4.2	4	4	>8	>8
***F. fujikuroi* (7)**	0.25 - 1	0.78	>64	>64	16 - >16	16	2 - 8	3.70	1 - 2	1.14	4 - 16	9.14	4	4	>8	>8
***F. proliferatum* (9)**	0.5 - 2	1.1	>64	>64	16 - >16	>16	2 - 8	4.22	1 - >16	2	4 - >16	9.3	4	4	>8	>8
***F.acutatum* (7)**	1 - 8	2.07	>64	>64	>16	>16	2 - 8	5.14	1 - 2	1.85	4 - > 16	14.8	4	4	>8	>8
***F. thapsinum* (6)**	1 - 2	1.6	>64	>64	>16	>16	1 - 4	2.6	2 - >16	11.6	8 - 16	10.6	4	4	>8	>8
***F. nygamai* (7)**	1 – 4	2	>64	>64	>16	>16	4 – 16	6.85	>16	>16	8 - >16	14.8	4	4	>8	>8
***F. temperatum* (1)**	0.25	0.25	>64	>64	>16	>16	1	1	0.25	0.25	1	1	4	4	>8	>8
Total (81)	0.125 - 8	1.6	64 - >64	64	16 - >16	16	1- 8	3.01	0.125 - >16	4.69	1 - >16	7.57	4	4	>8	>8

[a] Determined according to CLSI M38-A2, after 48 h of incubation at 35 °C.

We evaluated whether the MIC/MEC values correlated with different species using the Mann–Whitney–Wilcoxon test for skewed distribution. At a ≤0.05, amphotericin B had the lowest MIC for all isolates with a GM MIC of 1.6 mg/L, followed by voriconazole with a GM MIC of 3.01 mg/L. However, the latter drug was slightly less active against the environmental isolates (GM MIC 3.22 mg/L) than the clinical isolates (GM MIC of 2.65 mg/L; P<0.013). Isavuconazole had MICs of one dilution step higher than voriconazole and showed good activity against *F. verticillioides*, with MICs ranging from 1 to 2 mg/L.

Moderate activity of natamycin was detected against all *Fusarium* strains, with a GM MIC of 4 mg/L (Table 1). The one tested isolate of *F. temperatum* showed relatively low MICs of amphotericin B, voriconazole, posaconazole and isavuconazole (0.25, 1, 0.25 and 1 mg/L, respectively). Based on these *in vitro* data, the most resistant species was *F. nygamai* with GM MICs as follows: fluconazole, >64 mg/L; itraconazole, >16 mg/L; voriconazole, 6.85 mg/L; isavuconazole, 14.8 mg/L; posaconazole, >16 mg/L; and micafungin, >8 mg/L. In contrast, the GM MIC of amphotericin B was 2 mg/L. *F. thapsinum* was the second most resistant species.

Overall, our findings on *in vitro* activities of the main antifungal drugs are in agreement with previous, smaller studies.[10-12] Despite the relatively high MICs of fluconazole and itraconazole found in most studies, two cases reported favourable clinical outcomes with these drugs.[13] In our study, 97% of isolates had low MICs of amphotericin B and only two environmental strains had deviating MICs of 8 mg/L and this is comparable to previous studies. [3,14] The newer triazoles voriconazole and posaconazole show low, variable and species-dependent MICs for FFSC strains. All species tested except *F. nygamai* (MIC range 4–16 mg/L) were inhibited by voriconazole, one of the currently used drugs in the treatment of fusariosis. This result was in consonance with published reports.[14–16] However, *in vitro* susceptibilities of 54 *Fusarium* isolates showed that 6 out of 16 *F. proliferatum* isolates had high voriconazole MICs of >16 mg/L.[8] This could be attributed to the method used for determining susceptibility or might reflect hidden resistance among some *F. proliferatum* strains. Indeed, a recent clinical study relating MICs and *Fusarium* keratitis outcome recommended against using voriconazole as first-line therapy.[17]

The relatively low but variable MICs (ranging from 0.125 to >16 mg/L) of posaconazole found in this study suggests this drug is promising for the treatment of fusariosis caused by selected species within the FFSC, with the exception of *F. nygamai* and *F. thapsinum*. This agrees with a previous report on *F. thapsinum*.[3] Additionally, posaconazole also showed high MICs of >16 mg/L for two out of nine isolates of *F. sacchari*, two out of seven isolates of *F. napiforme* and two out of nine isolates of *F. proliferatum*. The results for *F. proliferatum* agreed to some extent with the data obtained by Tortorano et al.[8] in which 11 out of 16 isolates had MICs of >16 mg/L. A comparison of posaconazole and voriconazole shows that posaconazole has lower MICs (range 0.125–2 mg/L) than voriconazole and also than amphotericin B; this

especially holds true for *F. subglutinans*, *F. ananatum*, *F. verticillioides*, *F. anthophilum* and *F. acutatum*. Isavuconazole is a new azole for the treatment of invasive fungal infections and as yet few data are available in *Fusarium*. Our results for isavuconazole were variable (ranging from 1 to >16 mg/L) and mostly one dilution less active than voriconazole, except for *F. verticillioides*, which showed low MICs ranging from 1 to 2 mg/L. Our results for isavuconazole against *Fusarium* agreed with data obtained by Guinea et al.,[18] who used *Fusarium* strains identified by morphology only. Natamycin is used for treating keratitis caused by different moulds and our findings for *Fusarium* species agree with previous studies.[17,19]

F. nygamai and *F. thapsinum* had high MICs of all drugs other than amphotericin B and natamycin, emphasizing that identification and susceptibility testing of aetiological agents is essential for initiating therapy. The molecular mechanism that lies behind the recent emergence of drug resistance among *Fusarium* species is currently unknown, but increased and prolonged use of triazole fungicides in agriculture has raised concerns about the development of resistance in opportunistic filamentous fungi.[20]

In conclusion, amphotericin B appears highly active against most of the tested strains, followed by the triazole drugs voriconazole and posaconazole. Isavuconazole has generally only one dilution step higher MICs than voriconazole. Our data present antifungal susceptibility profiles that differ between and within *Fusarium* species.

Funding

This study was funded by the Ministry of Health, Oman. Formal Agreement no. 28/2014. A. M. S. A. received a PhD scholarship from the Ministry of Health, Oman.

Transparency declarations

J. F. M. has received grants from Astellas, Basilea and Merck, has been a consultant to Astellas, Basilea and Merck, and has received speaker's fees from Merck and Gilead. All others authors: none to declare**.**

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CHAPTER 15

Chapter 15

Antifungal susceptibility and phylogeny of opportunistic members of the genus *Fusarium* causing human keratomycosis in south India

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Medical Mycology 2016; 54: 287-294.

Abstract

Fusarium species are reported frequently as themost common causative agents of fungal keratitis in tropical countries such as India. Sixty-five fusaria isolated from patients were subjected to multilocus DNA sequencing to characterize the spectrum of the species associated with keratitis infections in India. Susceptibilities of these fusaria to ten antifungals were determined *in vitro* by the broth microdilution method. An impressive phylogenetic diversity of fusaria was reflected in susceptibilities differing at species level. Typing results revealed that the isolates were distributed among species in the species complexes (SCs) of *F. solani* (FSSC; n = 54), *F. oxysporum* (FOSC; n = 1), *F. fujikuroi* (FFSC; n = 3), and *F. dimerum* (FDSC; n = 7). Amphotericin B, voriconazole, and clotrimazole proved to be the most effective drugs, followed by econazole.

Key words: Keratitis, *Bisifusarium*, *Neocosmospora*, treatment options.

Introduction

Diseases affecting the cornea are a major cause of blindness worldwide and comprise a large variety of infectious and inflammatory eye diseases.[1] In tropical southern India, which locally has wet or dry climatic conditions and is semi-arid in certain regions, the majority of the people are engaged in agriculture. This profession carries a risk for corneal fungal infection from plant associated sources. Species of *Fusarium* are commonly occurring environmental fungi that potentially cause eye infections in humans, with keratitis being one of the most frequent clinical manifestations.[2,3,4,5]

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.[6,7] In 2013, Geiser[8] and many coauthors from clinical and phytopathological backgrounds have launched a plea for nomenclatural stability and the preservation of the name *Fusarium* for the clinically relevant species complexes. However, recently Lombard et al.[9] moved *F. solani* to the genus *Neocosmospora*, while the *F. dimerum* species complex was transferred to the new genus *Bisifusarium*. A counterproposal for *Fusicolla* as genus name for the *F. solani* complex is in preparation. To preserve the nomenclature stability, we adhere in this paper to *Fusarium* as best known descriptor for the etiological agents observed in the study.

Although culture techniques remain the cornerstone for the diagnosis of most cases of fungal keratitis,[2] misdiagnosis or lack of advanced investigative tools to accurately identify the causal agents may potentially lead an ophthalmologist to initiate inappropriate antifungal therapy.[10] In this context, it is becoming increasingly evident that efficient therapy of keratitis relies on precise identification of the fungal pathogens to the species level using advanced and reliable molecular methods. Furthermore, treatment is limited by the restricted number of prescribed antifungals for specific ophthalmic use and especially local (un)availability of the compounds themselves. Conventional treatment varies per country and depends on obtainability and registration of antifungal drugs. In India, *Fusarium* keratomycosis is often treated with topical natamycin, while for instance in the Netherlands natamycin is restricted for use as food preservative and hence amphotericin B and voriconazole are the drugs of choice there.

Antifungal susceptibilities of the different *Fusarium* SCs vary, and fusaria show remarkable resistance to most clinically applied antifungal drugs.[11,12,13] Therefore, the present study was undertaken with the following objectives: (i) to determine the species complex level of *Fusarium* isolates from human keratomycosis (South India) based on the partial sequences of the second-largest subunit of RNA polymerase II (*RPB2*) and translation elongation factor 1 α (*TEF1*); and (ii) to determine the *in vitro* susceptibilities of *Fusarium* isolates to ten antifungals.

Materials and methods

Isolates

A total of 1628 corneal scrapings of human keratomycosis patients attending Aravind Eye Hospital and Postgraduate Institute of Ophthalmology (Coimbatore, Tamilnadu, India) during the years 2012 and 2013 were processed for isolation of the causative agent as described before.[14] Initial identification of *Fusarium* isolates was based on colony morphology on potato dextrose agar (PDA - 250 g of potato slices, 15 g agar, 10 g dextrose, and 1000 ml distilled water) plates and microscopic feature in a lactophenol wet mount preparation.[15] Isolates that grew after subculturing twice on PDA were deposited in the reference collection of the CBS-KNAW Fungal Biodiversity Centre in Utrecht, The Netherlands.

DNA extraction

DNA extraction was performed using glass beads (Sigma G9143, Sigma-Aldrich Co, Missouri, USA) according to the protocol described by Al-Hatmi et al.[16]

DNA amplification and sequencing

Two gene regions were amplified directly from the extracted genomic DNA for multilocus sequencing. The primer pairs for *TEF1* and RNA polymerase II were EF1 and EF217 and *RPB2*-7cr and *RPB2* -5f, respectively.[18] Polymerase chain reaction (PCR) products were visualized and checked by electrophoresis on a 1% (w/v) agarose gel. Amplicons were purified using exoSAP. The ABI PrismH Big DyeTM Terminator v. 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) was used for sequencing PCR. Sequencing reaction was performed as follows: 1 min at 95 ^{o}C, followed by 30 cycles consisting of 10 sec at 95 ^{o}C, 5 sec at 50 ^{o}C, and 2 min 60 ^{o}C. Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden), and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, USA) with ABI PRISM BigDyeTM terminator cycle sequencing kit. A BLAST search of *TEF1* and *RPB2* sequences against the database FUSARIUM-ID (http://isolate.fusariumdb.org), the *Fusarium* MLST (http://www.cbs.knaw.nl/fusarium) and GenBank databases (www.ncbi.nlm.nih.gov) were used as an initial step to identify isolates to species and/or species complex. The *TEF1* and *RPB2* nucleotide sequences for all the isolates were deposited in GenBank.

Phylogenetic analyses

Sequences were edited using SeqMan in the Lasergene package (DNAstar, Madison, USA). A phylogenetic approach was used to investigate relationship between 65 strains of *Fusarium* species including type and reference strains. The sequences were aligned using MAFFT v. 7.127 (http://mafft.cbrc.jp/alignment/software/), followed by manual adjustments with MEGA

v. 6.[19] A combined alignment was constructed for *TEF1* and *RPB2* for both the reference and test strains. The best-fit model of evolution was determined by jModelTest v. 0.1.1.[20] A Maximum Likelihood tree was constructed by using RAxML (7.6.6)[21] with 1000 bootstrap replicates and edited with MEGA v. 6 software 6.[19]

***In vitro* susceptibility testing**

The following clinically used and commercially available antifungals were employed in the antifungal susceptibility tests: amphotericin B (AMB), nystatin (NYT), ketoconazole (KTZ), fluconazole (FLZ), and miconazole (MCZ) [HiMedia,Mumbai, India]; voriconazole (VRZ), econazole (ECZ) and clotrimazole (CLZ) [Aurolab, Madurai, India]; natamycin (NTM) and itraconazole (ITZ) [Sigma-Aldrich, St. Louis, MO, USA]. RPMI 1640 medium was used for preparing the drug solutions and the final drug concentrations ranged from 64 to 0.125 μg ml−1. Stocks were stored at −20 °C until needed. Inoculum preparation and determination of the minimum inhibitory concentrations (MICs) were done following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) M38-A2 document.[22] Experiments were performed in triplicate. *Aspergillus flavus* ATCC 204304 was included in the tests as a quality control.

Results

Identification of *Fusarium* isolates and phylogeny of 1628 specimens, a total of 624 (38.3%) were culture positive for different microbial pathogens and 417 (66.8% of 624) keratitis cases were found to be caused by fungal isolates, out of which 196 isolates (47% of 417) were confirmed as *Fusarium* spp. A total of 65 *Fusarium* isolates were chosen for further analyses. Upon molecular identification based on *TEF1* and *RPB2* partial genes analysis, the majority of *Fusarium* isolates were found to be members of FSSC: *F. falciforme* (n = 45) followed by *F. keratoplasticum* (n = 8), and *F. lichenicola* (n = 1).We also identified members of other species complexes: *F. oxysporum* (n =1) in FOSC, *F. nygamai* (n = 1) and *F. sacchari* (n = 2) in FFSC, as well as *F. delphinoides* (n = 6) and *F. dimerum* (n = 1) in FDSC.

In order to obtain understanding of the phylogenetic positions of species found in this study, they were compared to reference sequences from the GenBank and CBS databases (n = 9). A phylogenetic tree (Fig. 1) was constructed with a total of 74 sequences for two genes, with *F. dimerum* (synonym *Bisifusarium dimerum*) as outgroup as other studies confirm this group as themost basal lineage.[8] The combined phylogenetic analysis of *TEF1* and *RPB2* (1215-bp alignment) of 74 strains could be aligned confidently over the entire genus *Fusarium*. The tree topologies of these two loci appeared to be strictly concordant and effective for genotyping all species concerned. The generated tree separated into four clades. Clade 1 included all members of FSSC, clade 2 represented FOSC, clade 3 the FFSC, while clade 4 the FDSC. The final result of the identification process was that 54 isolates were confirmed as members of FSSC, one

isolate belonged to FOSC, three isolates to FFSC and seven to FDSC.

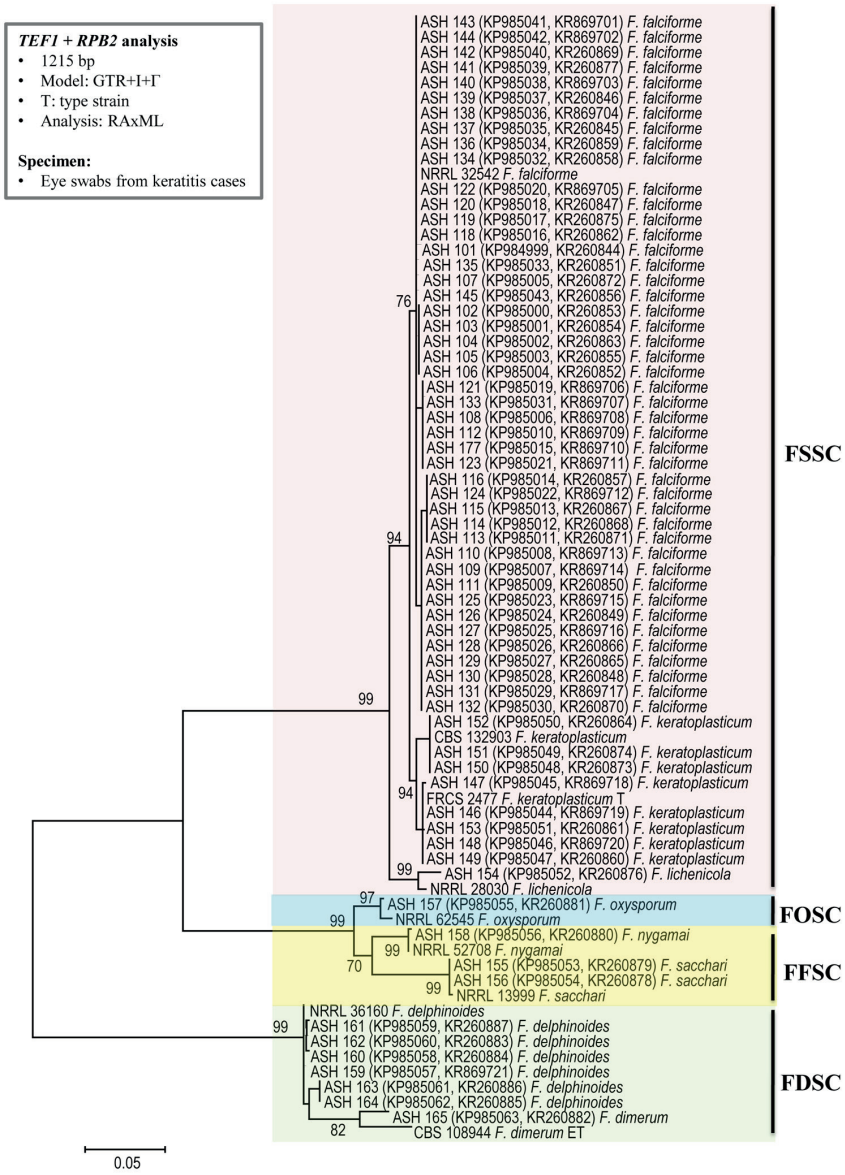

Fig 1. Phylogenetic tree resulting from RAxML analysis for the *TEF1* and *RPB2* genes (values over 70% for maximum likelihood are shown). List of the 65 isolates examined in this study including their GenBank accession numbers, CBS number and others. FDSC or *Fusarium dimerum* species complex corresponds to the genus *Bisifusarium*, while FSSC or *Fusarium solani* species complex corresponds to *Neocosmospora* according to Lombard et al.[9] As outgroup the FDSC group has been used including the epitype (ET) strain of *F. dimerum* CBS 108944.

Antifungal susceptibility

Ten polyene and azole antifungal compounds used before to treat keratomycoses were tested in our study: Polyenes AMB and NTM are both commonly used to treat fungal keratitis, while NYT is somewhat less potent and hence less commonly used clinically. AMB is used both for treatment of yeasts and filamentous fungi, while NTM proves to have a broader spectrum against filamentous fungi and has a better penetration of the intact corneal epithelium. The other studied antifungal drugs are all azoles.

The lowest MIC of 0.5 μg ml^{-1} AMB and VRZ was recorded for four (*F. falciforme* strains ASH 134, ASH 139, ASH 144) and one (*F. falciforme* strain ASH 142) FSSC isolates, respectively. NTM and FLZ inhibited most of the FSSC isolates (38, 70%) at a concentration of 16 μg ml^{-1}. The FOSC member (n = 1) was inhibited at 1 μg ml^{-1} of AMB. For FFSC isolates, 4 μg ml^{-1} of CLZ followed by AMB and VRZ was found to be effective. Most of the FDSC members were inhibited at 4 μg ml^{-1} of CLZ (n = 5) and 8 μg ml^{-1} of NYT (n = 6) (Table 1). Approximately 80% of the *Fusarium* isolates were susceptible to concentrations of $\leq$ 4 μg ml^{-1} of AMB (84%), VRZ (80%), CLZ (78%).Nearly half of the isolates were susceptible to concentrations $\leq$ 4 μg ml^{-1} of ECZ (53%), and MCZ (43%), while lesser number of isolates showed susceptibility to similar levels of KTZ (29%), ITZ (13%) and NTM (1.5%).

Table 1. Distribution of the tested *Fusarium* species complexes according to their minimum inhibitory concentrations (MICs).

Species complex[1]	Antifungal agent	MIC (µgml^{-1})									
		0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**
FSSC (n=54)	Amphotericin B			4	8	12	20	10			
	Natamycin						1	12	38	3	
	Nystatin							37	17		
	Voriconazole			1	1	11	28	13			
	Econazole					7	20	27			
	Clotrimazole				1	10	31	11	1		
	Ketoconazole				1	2	11	14	26		
	Itraconazole					1	6	6	17	24	
	Miconazole				1	3	19	31			
	Fluconazole							16	38		
FOSC (n=1)	Amphotericin B				1						
	Natamycin								1		
	Nystatin								1		
	Voriconazole						1				
	Econazole						1				
	Clotrimazole							1			
	Ketoconazole							1			
	Itraconazole							1			
	Miconazole							1			
	Fluconazole								1		

Table 1. (Continued)											
FFSC (n=3)	Amphotericin B				1		2				
	Natamycin							1	2		
	Nystatin							2	1		
	Voriconazole			1			2				
	Econazole						1	2			
	Clotrimazole						3				
	Ketoconazole						1	1	1		
	Itraconazole							2		1	
	Miconazole							3			
	Fluconazole							1	2		
FDSC (n=7)	Amphotericin B				1	6					
	Natamycin							1	6		
	Nystatin							6	1		
	Voriconazole				2	3	2				
	Econazole			1	3	1	1	1			
	Clotrimazole					1	5	1			
	Ketoconazole			1	1	1	1	2	1		
	Itraconazole			1	1			2	2	1	
	Miconazole			1	3		1	2			
	Fluconazole							3	4		

[1]*F. solani* species complex (FSSC); *F. oxysporum* species complex (FOSC); *F. fujikuroi* species complex (FFSC); *F. dimerum* species complex (FDSC) isolates.

Discussion

Fungal keratitis occurs more frequently in dry and warm environments, especially people with outdoor professions, such as agricultural workers are prone to this infection.[23] In India, *Fusarium*[24] and *Aspergillus*[25] species are reported to be the most common causative agents of fungal keratitis. In this study, *Fusarium* species obtained after processing corneal scrapings from human keratomycosis cases were investigated. Molecular identification was performed for 65 clinical *Fusarium* isolates using *TEF1* and *RPB2*. Both genes possessed enough polymorphism to serve as excellent markers with 99–100% accuracy for identification of *Fusarium* at the species level with sequences deposited in GenBank, the *Fusarium*-ID and the *Fusarium* MLST databases.

The largest part of the identified strains (n = 54) proved to belong to FFSC. Of these, 45 were identified as *F. falciforme* and 8 as *F. keratoplasticum*, suggesting a significant pathogenic potential of these species and/or a common prevalence in the environment.[26,27] As shown by the molecular analysis of combined sequence data of *TEF1* regions and *RPB2* (Fig. 1), *F. lichenicola* formed a distinct group. This species was first described as *Cylindrocarpon* but using phylogenetic data, Summerbell and Schroers[28] showed a relationship with *F. keratoplasticum* and *F. falciforme* (separated at 94% bootstrap support). Support values above 75% were found between other species in *Fusarium*. The present study confirms the genetic diversity of clinical strains in the FSSC, as reported in earlier studies.[29,30] In addition to the FSSC in our phylogenetic analysis, some strains were nested within known phylogenetic species of FOSC, FFSC, and FDSC. Homa et al.[24] reported similar distributions; most *Fusarium* isolates from human keratomycosis are proven to be members of FSSC (n = 53), while occasional isolates belonging to FDSC (n = 6), FFSC (n = 6), FOSC (n = 3), and *F. incarnatum- equiseti* SC (n = 2) were also encountered.

Species delimitation within FOSC has been studied intensively by O'Donnell et al.[31] We observed only a single FOSC infection in our study, suggesting that these species occur only at low frequency in India. Seventy three *Fusarium* species have been reported to cause human infection, 15 of which belonged to FFSC including *F. temperatum* and *F. musae*.[32] In this study we report two species belonging to the FFSC complex, *F. nygamai* and *F. sacchari*. According to Guarro et al.[6] three recognized members of FDSC *viz*., *F. dimerum, F. penzigii,* and *F. delphinoides* and two unnamed lineages are also involved in human infections: the current study yielded one isolate of *F. dimerum* and 6 of *F. delphinoides* belonging to FDSC.

Antifungal susceptibilities of clinical isolates were analysed and the results were comparable to those reported in the current literature. In the present study 82% of FSSC isolates were inhibited by $\leq 4\ \mu g\ ml^{-1}$ of AMB followed by CLZ (78%) and VRZ (76%). ITZ was found to be the most ineffective antifungal agent, which corresponds with the findings of Homa et

al.[24], Xie et al.[33], and Al-Hatmi et al.[12]. In contrast to our findings, O'Day et al.[34] stated that AMB was ineffective against *Fusarium*. Among the FSSC members, *F. falciforme* (n=3) and *F. keratoplasticum* (n = 1) were inhibited at the lowest concentration of 0.5 μg ml^{-1} of AMB and one isolate of *F. falciforme* was inhibited at 0.5 μg ml^{-1} of VRZ. Of FOSC and FFSC, only one and three clinical isolates, respectively, were available in this study. Due to the small sample size and high variability of MIC data it was not possible to make an overall statement about the susceptibility profile of these three species. However, one isolate of *F. sacchari* had a MIC of 0.5 μg ml^{-1} of VRZ and 1 μg ml^{-1} of AMB was effective against *F. oxysporum*. Only 1.5% of all isolates tested in the current study were susceptible (MIC $\leq$ 4 μg ml^{-1}) to NTM. Contrary to this, Xie et al.[33] stated that 94.2% and 91.3% of FSSC and FOSC members, respectively, were susceptible to NTM. Lalitha et al.[35] and Xuguang et al.[36] also reported good activity of NTM against *Fusarium*. However, in the present study, 95.38% of all the isolates tested were susceptible to the NTM concentration $\leq$ 16 μg ml^{-1}. Topical use of NTM is common practice in India but also approved for ophthalmic use in the United States. The discrepancy between the high *in vitro* MICs and clinical success may be explained by the effective corneal penetration of the antifungal and the resulting higher local concentration compared with many other drugs.

AMB was found to be the most effective drug against FDSC isolates. Among six isolates of *F. delphinoides*, one had low MIC (0.5 μg ml^{-1}) values for ECZ, KTZ, ITZ, and MCZ. In the present study, most of the FSSC members (n = 24) were inhibited at 32 μg ml^{-1} of ITZ. Lalitha et al.[35] and Alfonso et al.[37] reported that the MIC_{90} of ITZ for *Fusarium* sp. was > 8 μg ml^{-1} and 16 μg ml^{-1}, respectively. A total of 53% and 78% of *Fusarium* strains tested had low MICs of $\leq$ 4 μg ml^{-1} of ECZ and CLZ, respectively. The results correlated with those of Shobana et al.[38] Overall, 84%, 80% and 78% of the tested fusaria were inhibited at $\leq$ 4 μg ml^{-1} of AMB, VRZ, and CLZ, respectively. Twenty-six isolates of FSSC had MICs of 16 μg ml^{-1} of KTZ. Pujol et al.[39] and Xie et al.[33] reported higher MICs of KTZ in *Fusarium*. In our study, 69% of the isolates were inhibited at 8 μg ml^{-1} and 16 μg ml^{-1} of NYT and FLZ, respectively. A total of 37 isolates had 8 μg ml^{-1} MIC for MCZ.

It is concluded that the majority of *Fusarium* isolates tested belong to FSSC. Based on the observed MIC data in the present study, we report that AMB, VRZ, and CLZ at $\leq$ 4 μgml^{-1} and FLZ and NTM $\leq$ 16 μgml^{-1} are potential antifungal agents for the treatment of human keratomycosis caused by *Fusarium*. However, application of the drugs and their penetration into the affected area should be taken into account as well.

Acknowledgements

This study was partially funded by the Ministry of Health, Oman (Formal Agreement no. 28/2014). Abdullah M. S. Al-Hatmi received a PhD scholarship from the Ministry of Health, Oman. The study was also supported by the Indian National Science Academy and the Hungarian Academy of Sciences within the frames of the Indo– Hungarian bilateral exchange program (INSA-HAS Project 2013–2015/189). Csaba Vágvölgyi thanks the visiting professor program, Deanship of Scientific Research at King Saud University, Riyadh. The Indian research group was supported by the University Grants Commission (UGC), Bahadur Shah Zafar Marg, New Delhi-110 002, India [F. No. 42 469/2013 (SR)].

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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CHAPTER 16

Chapter 16

In vitro resistance of clinical *Fusarium* species to amphotericin B and voriconazole using the EUCAST antifungal susceptibility method

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Diagnostic Microbiology and Infectious Diseases 2016; 85: 438–443.

Abstract

Susceptibility testing using the EUCAST-AFST method against 39 clinical *Fusarium* strains consecutively collected from local and invasive infections during the last 10 years assessed the *in vitro* activities of amphotericin B (AmB) and triazole antifungal agents. In addition, the susceptibility pattern of 12 reference strains fromthe CBS-KNAW Fungal Biodiversity Centre (CBS) was evaluated. In particular *Fusarium petroliphilum* and *F. solani* sensu lato were involved in disseminated infections and known for treatment failure. AmB displayed the lowest MICs followed by voriconazole VRC, posaconazole (POC). Itraconazole (ITC) showed high MIC values, displaying *in vitro* resistance. Clinical isolates were significantly ($P < 0.05$) more resistant to AmB, VRC, and POC, than the CBS reference isolates probably due to previous exposure to antifungal therapy. Resistant profiles to AmB and VRC, which are the currently recommended agents in the guidelines for treatments, and a late diagnosis, may be associated with high mortality rate in immunocompromised patients. The present antifungal susceptibility profiles showed that species- and strain-specific differences in antifungal susceptibility exist within *Fusarium* and that susceptibility testing is important and may improve the prognosis of these infections.

Key words: *Fusarium*, EUCAST antifungal susceptibility, Amphotericin B, Triazoles, Resistance

Introduction

Fusarium species are among the most resistant fungi. Hence infections are refractory against treatment with conventional antifungal agents (Guarro, 2013). The optimal treatment for patients with severe fusariosis infection remains unclear. ESCMID and ECMM joint guidelines suggest that early therapy with voriconazole (VRC) or lipid based amphotericin B (AmB) in conjunction with surgical intervention is of utmost importance to prevent dissemination (Tortorano et al., 2014a). However, mortality rates of 50–75% in disseminated infections may arise, especially in immunosuppresed patients (Muhammed et al., 2013; Nucci et al., 2004). Localized fusariosis frequently manifests as keratitis and onychomycosis in immunocompetent individuals (Godoy et al., 2004; Zaias et al., 2014), whereas dissemination occurs in patients with prolonged neutropenia, immune deficiency, and especially hematological malignancies (Muhammed et al., 2013; Nucci et al., 2003, 2004).

Fusarium pathogens typically show broad *in vitro* resistance to antifungal agents with a high variability being present within each species (Alastruey-Izquierdo et al., 2008; Al-Hatmi et al., 2015; Araujo et al., 2015; Azor et al., 2007; Tortorano et al., 2008). In general, members of the *Fusarium solani* species complex (FSSC) are most commonly observed in all clinical infections and show highest minimum inhibitory concentrations (MICs) against various antifungal drugs. AmB is the most active drug followed by VRC (Azor et al., 2007). The same trend was reported for the members of the *Fusarium fujikoroi* species complex (FFSC) responsible for approximately 1/3rd of the disseminated infections and with *F. verticillioides* displaying the lowest and *F. nygamai* the highest MICs (Al-Hatmi et al., 2015). Although we lack clinical break points for *Fusarium* spp. and antifungal agents, CLSI epidemiological cutoff values (ECVs) were established for members of the more common *Fusarium* species complexes (Espinel-Ingroff et al., 2015). Most of the available MIC data on AmB and triazole have been reported for a variety of *Fusarium* species, using the CLSI based methods. (Alastruey-Izquierdo et al., 2008; Espinel-Ingroff et al., 2008; O'Donnell et al., 2008; Tortorano et al., 2014b). Susceptibility testing may represent a tool for the selection of an appropriate therapy. Here, we report clinical presentations and the EUCAST susceptibility profiles of 39 clinical isolates collected from local and invasive infections, to amphotericin B and three triazoles.

Materials and methods

Patients

Thirty nine *Fusarium* spp. strains were isolated from immunocompromised and immunocompetent patientswith various clinical diseases who presented at Hamad Hospital, Doha, Qatar. Underlying conditions, clinical specimens, fungal etiology and *in vitro* susceptibility data are reported in Table 1. Pathogens were isolated from nails (n = 17), skin (n = 5), cornea (n = 6), blood (n = 3), wounds (n = 3), skin tissues (n = 3) and burn injuries (n

= 2). In only one patient *Fusarium* spp. was detected from two body sites (i.e. blood and skin tissue). The Hamad Medical Corporation research and ethics committee (reference number RC/104044/2015) granted approval to conduct this study.

Pathogen isolation and identification

Fusarium spp.were isolated and identified bymorphology according to standard laboratory procedures. Thus, clinical specimens were cultured on either Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, MI) plus chloramphenicol (SDAC), SDA without antibiotics, or brain heart infusion plus 40 U/ml streptomycin and 20 U/ml penicillin. Blood cultures were performed using the instrumented blood culture system BD BACTEC™ (BD Diagnostic Systems, USA). If appropriate, direct microscopy was done using Blankophore P fluorescent stain to confirm the fungal etiologic agent in the clinical specimens. Culture plates were incubated at 26 °C and 37 °C and were observed daily for growth up to 10 days. Strains were sub-cultured to ascertain their macroscopic andmicroscopic morphology. Isolates were sent to the CBS-KNAW, Fungal Biodiversity Center, Utrecht, The Netherlands and subcultured on oatmeal agar (OA; home-made at CBS) and incubated for 5 days at 28 °C. They were deposited under accession number in the reference collection of the CBS-KNAW. All strains were previously identified (Salah et al., 2015) using two-loci, namely the second largest subunit of the RNA polymerase gene (*RPB2*) and the translation elongation factor 1 alpha (*TEF1*) thatwere amplified directly from genomic DNA. For the partial sequence of *TEF1*, primers EF1 and EF2 (O'Donnell et al., 1998) were used with modification of the PCR program according to Davari et al. (Davari et al., 2013). For *RPB2* the primers used were 7cr and 5 f2 (Reeb et al., 2004). Nucleotide sequence accession numbers for the isolates are KR 679913-KR674025 (Salah et al., 2015).

Antifungal susceptibility testing

Antifungal susceptibility tests were performed for 39 clinical and 12 reference CBS strains (i.e. 10 environmental and two clinical strains) (Table 2). Minimal inhibitory concentrations (MICs) for AmB, and three triazoles were determined following the standard reference method for susceptibility testing of conidium-formingmolds as recommended by EUCAST-AFST, and all plates were incubated for 48 hr at 37 °C (Rodriguez-Tudela et al., 2008). The MICs for the quality control strains *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were all within the reference ranges. Presently, clinical breakpoints for *Fusarium* spp. and various antifungal drugs are not available, thus the breakpoint of ≤l μg/ml was used to define susceptibility to AmB, voriconazole (VRC), posaconazole (PSC), and itraconazole (ITC) (Alastruey-Izquierdo et al., 2008).

Table 1. *Fusarium* species, patients underlying conditions that associated with site of infections and *in vitro* resistance to antifungal agents.

Patient No.	Underlying condition	Organism	Site of infection	Susceptibility
1	DM	*F. keratoplasticum*	Skin	R
2	None	*F. keratoplasticum*	Nail	R
3	None	*F. keratoplasticum*	Nail	R
4	None	*F. keratoplasticum*	Nail	R
5	None	*F. keratoplasticum*	Skin	R
6	None	*F. keratoplasticum*	Nail	R
7	None	*F. keratoplasticum*	Nail	AmB
8	DM	*F. keratoplasticum*	Nail	R
9	DM	*F. keratoplasticum*	Nail	AmB
10	DM	*F. keratoplasticum*	Skin	AmB
11	None	*F. keratoplasticum*	Skin	R
12	None	*F. keratoplasticum*	Wound	R
13	None	*F. falciforme*	Cornea	AmB
14	None	*F. falciforme*	Nail	AmB,VRC,POC
15	DM	*F. falciforme*	Tissue	R
16	None	*F. falciforme*	Cornea	AmB
17	None	*F. falciforme*	Cornea	AmB
18	None	*F. falciforme*	Cornea	R
19	None	*F. falciforme*	Cornea	AmB
20	DM, postoperative infection	*F. falciforme*	Skin	AmB
21	None	*F. falciforme*	Nail	R
22	None	*Fusisporium solani*	Nail	AmB
23	DM	*Fusisporium solani*	Corenea	AmB
24	Renal transplant	*Fusisporium solani*	Nail	R
25	None	*F. lichenicola*	Wound	AmB
26	None	*F. lichenicola*	Nail	AmB,VRC
27	Autoimmune disease	*F. petroliphilum*	Blood	R
28	AML	*F. petroliphilum*	Blood	R
29	DM	*F. petroliphilum*	Nail	R
30	Burn	*F. solani* sensu lato	Skin	AmB
31	T-cell lymphoma	*F. solani* sensu lato	Blood	AmB
32	None	*F. oxysporum*	Nail	R
33	None	*F. proliferatum*	wound	AmB,VRC,PSC
34	AML	*F. proliferatum*	Cellulitis	AmB,VRC
35	None	*F. acutatum*	Nail	R
36	None	*F. acutatum*	Nail	AmB
37	DM	*F. acutatum*	Nail	VRC,PSC
38	Burn	*F. delphinoides*	Burn	AmB, VRC
39	Burn	*F. cf. dimerum*	Burn	AmB, VRC

DM; diabetes mellitus, AML; acute myeloid leukemia, R; resistance to all antifungal agents, AmB; amphotericin B susceptible at ≤ 1, VRC; voriconazole; PSC; posaconazole.

Table 2. Origin and date of isolation/accession of CBS *Fusarium* reference isolates.

Accession number	Organism	Location	Substrate /host	Isolation/ year
CBS 187.34	*F. sambucinum*	UK	Zostera marina	1934
CBS 139.40	*F. verticillioides*	Pisa, Italy	Phyllocactus hybridus	1955
CBS 253.50	*F. tricinctum*	Pyhajärvi, Finland	Hordeum sativum grain	1938
CBS 258.52	*F. xylarioides*	Ivory Coast e	Coffe trunk	1951
CBS 258.54	*F. annulatum*	New Caledonia	Oryza sativa	1959
CBS 246.61	*F. proliferatum*	Germany	Sansevieria dooneri	1955
CBS 635.76	*F. chlamydosporum*	New Zealand	Cynodon lemfuensis	1973
CBS 307.94	*F. equiseti*	Germany	soil	1994
CBS 125.95	*F. sambucinum*	Spain	Beta vulgaris	1968
CBS 737.97	*F. anthophilum*	Germany	Hippeastrum	unknown
CBS 108944	*F. dimerum*	Netherlands	blood from patient	2000
CBS 135806	*F. keratoplasticum*	India	cornea, 50 y old man	2012

Clinical cases and *in vitro* resistance definitions

Diseases and resistance were defined as follows: invasive *Fusarium* infection (fusariosis) infection with at least one positive blood culture or the isolation of the same strain from two or more body sites. Neutropenia was defined as an ANC <500 cells/µL, while counts of ≤100 cells/µL were considered as "profound neutropenia" (Freifeld et al., 2011). Localized diseases, *Fusarium* skin, nail, cornea and wound infections without deep tissue involvement. Proven *Fusarium* infection required the visualization of fungal elements by direct microscopy of the clinical specimens, the isolation of the fungal etiology in culture, and compatibility with infectious disease processes (De Pauw et al., 2008). MIC values >l µg/ml may indicate of resistance to AmB, VRC, PSC, and ITC (Alastruey-Izquierdo et al., 2008).

Statistical analysis

The difference between actual MIC values of the clinical and CBS reference isolates were determined and tested using Wilcoxon rank sum test (Royston, 1982). The MICs for the antifungals (AmB and VRC) were also compared using Wilcoxon signed rank test.

Results

Patients

During the last ten years, 39 cases of *Fusarium* infections were obtained; 22 patients (56.4%) showed no apparent risk factors and these included patients with local infections of nails, skin and cornea. Seventeen patients (43.6%) showed classical predispositions, such as suffering from diabetes mellitus (n=9, 23%) or hematological malignancy (n=3) and other disorders, see Table 1. Three patients were identified with invasive and proven fusariosis, see Table 3. The underlying condition of patient 1 was autoimmune disease "Bullous pemphigoid" with profound

neutropenia (≤100 cells/μL). This patient received fluconazole as empirical therapy and died at the day of fungal diagnosis. Patient 2 suffered from acute myeloid leukemia and *Fusarium* fungemia; liposomal amphotericin B plus granulocyte macrophage colony stimulating factor (GM-CSF) was sufficient to render blood cultures negative with relief of neutropenia. Patient 3, a 24 year female with T-cell lymphoma suffered from severe fungal infection with necrotic papular skin lesions; skin biopsies displayed the presence of *Fusarium* hyphae and conidia. The latter patient died despite treatment with a combination of AmB, VRC and GM-CSF. Etiologic agents belonged to the *Fusarium solani* species complex (FSSC) with *F. petroliphilum* (n = 2) and *F. solani* sensu lato (n = 1) being involved. Patients with localized infections were treated with various antifungal agents, had several surgical interventions and recovered at least from their infection, as far as it was feasible to record this data.

Table 3. Invasive infections caused by *Fusariun solani* species complex (FSSC)

No.	Underlying condition	Age/ Sex	Neutropenia	Organism	Treatment	*In vitro* susceptibility	Outcome
1	Autoimmune disease*	80/F	Neutropenia <100 cells/ μL	*F. petroliphilum*	FLCZ	Resistant to all drugs	Died at the day of diagnosis
2	AML	37/M	<500 cells/ μL	*F. petroliphilum*	AmB, (GM-CSF)	Resistant to all drugs	Restored
3	T-cell lymphoma	24/F	Neutropenia <100 cells/ μL	*F. solani* sensu lato	AmB, VOC(GM-CSF)	AmB at MIC 1 μg/ml	Died

* Bullous Pemphigoid; AML, acute myeloid leukemia

Susceptibility profiles

Geometric means (GMs) and MIC ranges of four antifungal agents are shown in Table 4. Clinical strains displayed resistance to AmB (n = 17/39; 43.5%), while resistance to VRC was (n = 32/39; 82%). All AmB resistant strains were found to be also resistant to VRC. The susceptibility to AmB and at least one of the triazoles were very low (12.8%). Clinical strains that were less susceptible to other azoles displayed high resistance to POC (n = 36/39; 92.3%) and 100% resistance to ITC. AmB was the most active agent against *Fusarium* spp., its overall GM MIC was 1 μg/ml. Twenty two clinical strains were susceptible to AmB; AmB MICs ≥2 μg/ml were documented for *F. keratoplasticum* (9 of 12), *F. falciforme* (2 of 9), *F. solani* sensu stricto (1 of 3), *F. oxysporum*, and *F. acutatum* (2 of 3).

Overall, all the evaluated azoles showed high MICs for most clinical and reference *Fusarium* species. (Table 4). ITC showed GM MICs of 4 μg/ml. VRC was the most active azole with 17.9% of the clinical isolates showing MICs ≤1 μg/ml. In addition, VRC showed broad

activity covering *F. proliferatum*, *F. falciforme*, *F. delphinoides*, *F. lichenicola*, *F. petroliphilum*, *F. acutatum*, and *F. cf. dimerum*. VRC was more active against CBS reference strains with 8/12 (66.6%) showing MIC ≤1 µg/ ml. POC was less active against clinical *Fusarium* spp. than VRC with 7.7% of the strains displaying MICs ≤1 µg/ml and with an overall GM MIC of 7.7 µg/ml. Overall AmB had the lowest MIC for all reference CBS isolates, followed by VRC. However, the latter drug was slightly less active against the clinical than reference CBS isolates. AmB for clinical isolates displayed higher GM MIC values (1.07) than CBS strains (0.86) and the AmB actual MIC mean ± SD was (1.33 ± 0.62) and (0.88 ± 0.47) for clinical and CBS reference strains respectively (p-value = 0.04). The same trend was shown for VRC (GM = 2.36 Vs 1.29) and (actual MIC mean ± SD; 11.2 ± 6.8 Vs 3.8 ± 5.8; pvalue = 0.001) and POC (GM = 2.81 Vs 1.61) and (actual MIC mean ± SD; 13.5 ± 5.4 Vs 6.4 ± 7.1; p-value = 0.001) (Table 5). Wilcoxon signed rank test showed that clinical strains were more susceptible to AmB than VRC (P=0.001).

Table 4. MIC ranges and geometric mean of antifungal agents for the clinical and CBS reference *Fusarium* strains.

***Fusarium* species (n)**	**MIC/MEC [µg/ml; range (GM)]**			
Clinical strains (n=39)	**AMB**	**VRC**	**PSC**	**ITC**
F. keratoplasticum(12)	1-2 (1.70)	2-16 (11.62)	8-16(15.1)	4 (4)
F. falciforme (9)	0.5-2 (0.93)	1-16 (6.96)	1-16 (9.84)	4 (4)
F. solani sensu stricto (3)	1-2 (1.25)	16(>16)	16 (>16)	4 (4)
F. lichenicola (2)	0.5 (0.5)	1-16(4)	16 (>16)	4 (4)
F. petroliphilum (3)	1-2 (1.25)	16 (>16)	16 (>16)	4 (4)
F. solani sensu lato (2)	1 (1)	16 (>16)	16 (>16)	4 (4)
F. oxysporum (1)	2 (2)	2 (2)	2 (2)	4 (4)
F. proliferatum (2)	0.5-1 (0.70)	0.5-1 (0.70)	1-2 (1.41)	4 (4)
F. acutatum (3)	1-2 (1.58)	1-16(3.17)	1-16 (3.17)	4 (4)
F. delphinoides (1)	0.5 (0.5)	0.5 (0.5)	8 (8)	4 (4)
Fusarium. cf. *dimerum* (1)	0.5 (0.5)	0.5 (0.5)	8 (8)	4 (4)
CBS reference strains (n=12)				
F. sambucinum CBS 187.34	1 (1)	1 (1)	1 (1)	4 (4)
F. verticillioides CBS 139.40	1 (1)	1 (1)	1(1)	4 (4)
F. tricinctum CBS 253.50	1 (1)	16 (16)	16 (16)	4 (4)
F. xylarioides CBS 258.52	0.062 (0.062)	1 (1)	4 (4)	4 (4)
F. annulatum CBS 258.54	1 (1)	1 (1)	1(1)	4 (4)
F. phyllophilum CBS 246.61	1 (1)	1 (1)	2 (2)	4 (4)
F. chlamydosporum CBS 635.76	0.5(0.5)	1 (1)	2 (2)	4 (4)
F. sambucinum CBS 125.95	0.5(0.5)	0.5 (0.5)	1 (1)	4 (4)
F. anthopilum CBS 737.97	0.5(0.5)	1 (1)	0.5(0.5)	4 (4)
F. dimerum CBS 108944	1 (1)	2 (2)	16 (16)	4 (4)
F. keratoplasticum CBS 135806	2 (2)	4(4)	16 (16)	4 (4)
Total (n=51)	0.062-2 (1)	0.5-16 (5.06)	0.5-16(7.7)	4 (4)

*GM, geometric mean; AMB, amphotericin B; FC, VRC, voriconazole; PSC, posaconazole; ITC, itraconazole.

Table 5. MIC ranges and geometric mean of antifungal agents for the clinical and CBS reference *Fusarium* strains

Antifungal agent	Clinical	CBS	P-value
Amphotericin B			
• Geometric mean (GM)	1.07	0.86	
• GM ratio (95% CI)	1.25 (1.04,1.49)		
• Mean ± SD (median, range)	1.33±0.62 (1,0.5 - 2)	0.8±0.47 (1,0.06-2)	0.040*
Voriconazole			
• Geometric mean (GM)	2.36	1.29	
• GM ratio (95% CI)	1,83 (1.3,2.59)		
• Mean ± SD (median, range)	11.2±6.8 (16,0.5-16)	3.8±5.8 (1,0.5-16)	0.001*
Posaconazole			
• Geometric mean (GM)	2.81	1.61	
• GM ratio (95% CI)	1.75 (1.3,2.34)		
• Mean ± SD (median, range)	13.5±5.4 (16,1 - 16)	6.4±7.1 (2,0.5- 16)	0.001*

CI: Confidence interval.* Wilcoxon rank sum test.

Discussion

Fusarium spp. are opportunistic, life threatening and emerging pathogens that may affect patients with hematological diseases and neutropenia (Nucci et al., 2014), and that are the most common cause of fungemia coupled with metastatic skin lesions, which is the most frequent presentation of disseminated fusariosis (Bodey et al., 2002; Muhammed et al., 2013; Nucci and Anaissie, 2007). It is worthwhile mentioning that patients with disseminated fusariosis had low survival rates of 33% (Horn et al., 2014).

Early diagnosis of diseasewith an accurate identification of *Fusarium* to the species level might be helpful to guide appropriate antifungal therapy (Tortorano et al., 2014a). Often clinically important, morphologically similar *Fusarium* species are identified to genus or species complex level only, and further genotypic characterization to species level is usually not done routinely in clinical laboratories. Conventional morphological identification of filamentous fungi to species level is no more available in most laboratories. In order to accomplish an easier and more accurate identification of clinical isolates molecular methods were developed for the identification of *Fusarium* species (O'Donnell et al., 2008).

Finding the optimal treatment strategy is a challenge, because *Fusarium* spp. shows high MICs to antifungal agents, partly most of the agents are inappropriate for treatment. Reversal of immunosuppression (Nucci et al., 2013) as well as treatment with VRC or lipid-based AmB

is highly recommended (Tortorano et al., 2014a). Clinical success rates range from 42-45% with VRC treatment (Lortholary et al., 2010; Perfect et al., 2003); and five patients out of 12 (41.6%) survived after treatment with VRC alone or in combination with other agents (Stempel et al., 2015).The role of any *in vitro* data for AmB are controversial, since MICs for *Fusarium* spp. range from 1 to 8 µg/mL for most species and may not be related to clinical outcome (Al-Hatmi et al., 2015; Pereira et al., 2013). Our study shows that AmB is the most active against clinical and CBS reference strains with MICs ranging from 0.062-2 µg/mL, which is relatively low when compared to values provided in other reports (Al-Hatmi et al., 2015; Pereira et al., 2013). Two patients with disseminated infection received therapy with AmB. In one patient treatment failed, but the other improved.We are aware of the fact that many factors influence the outcome of an infection e.g. drug doses, treatment duration, and drug serum levels which are all important parameters. Hence, we are not able to draw a clear correlation of *in vitro* with *in vivo* obtained data. An indication of the potential correlation between MICs for *Fusarium* spp. and response to treatment was only found in a recent report where CLSI based MICs for seven *Fusarium* isolates and the clinical response was documented for patients with invasive fusariosis (Stempel et al., 2015). Furthermore, VRC, which is the drug recommended for the treatment of invasive fusariosis, shows variable and species dependent susceptibility patterns. *F. proliferatum*, *F. delphinoides*, and *Fusarium* cf. *dimerum* as well as CBS reference strains showed significantly (P-value = 0.001) low MIC values for VRC (0.062-1 µg/mL) when compared to the clinically obtained pathogens. This is probably due to previous exposure to antifungal therapy in the clinical settings, as most of the CBS reference strains were isolated fromthe environment and collected in the era of pre-antifungal use. Interestingly, the two CBS strains of human origin displayed high MIC values to antifungal agents tested. Several out of the 16 *F. proliferatum* strains investigated displayed high MICs against VRC (n = 6) and POC (n = 11), with values N16 µg/mL, respectively (Tortorano et al., 2014b). The most resistant clinical species belonged to FSSC with *F. keratoplasticum*, *Fusisporium* (*Fusarium*) *solani*, and *F. falciforme* being the main agents of cornea infections; *F. petroliphilum* and *F. solani* sensu lato were the main agents of invasive infections in this study with VRC MICs ranging from 1 to N16 µg/mL, respectively. Our data are similar to the previously reported values for VRC (1–8 µg/mL) and POC (0.25- N 16 µg/mL) (Al-Hatmi et al., 2015; Azor et al., 2007; Tortorano et al., 2008). In contrast, members of *F. verticillioides* and *F. fujikuroi* showed lower MICs for POC (Alastruey-Izquierdo et al., 2008; Al-Hatmi et al., 2015) with ECVs of 2 µg/mL for *F. verticillioides* (Espinel-Ingroff et al., 2015). No data exist on the clinical use of posaconazole to treat infections with other *Fusarium* spp. Despite high MICs reported in most studies, few clinical trials reported successful outcomein patients with fusariosis and POC therapy, but the species involved in these studies were not molecularly determined (Raad et al., 2006). In other studies, VRC or POC monotherapy at day 30 may be linked to the improved survival rates

(Horn et al., 2014). However, POC prophylaxis (Bose et al., 2011) was associated with fungal breakthrough infections suggesting emergence of resistant strains. Values of ITC, demonstrated lack of activity against any of the isolates tested, which is in agreement with previous data (Alastruey-Izquierdo et al., 2008; Azor et al., 2008).

A late diagnosis and delay of adequate therapy can contribute to the mortality of patient few days after therapy is initiated. The efficacy of AmB and VRC for treating invasive fusariosis is still controversial as the percentage of patients cured in the different clinical trials is low (Guarro, 2013), and in our case it was not successful as shown in patient #3 (Table 3) due to the persistence of neutropenia. The prognosis also depends on the species causing the infection. For instance infections by *F. verticillioides* seem to have the best prognosis (Guarro, 2013).

In conclusion, AmB is the most active agent against *Fusarium* species; clinical *Fusarium* species displayed significant higher MIC values than the reference CBS strains; particularly the AmB and VRC, which are the currently recommended agents in the guidelines for treatments. Overall, AmB showed significantly lower MICs than VRC. The present antifungal susceptibility profiles showed that species- and strain specific differences in antifungal susceptibility exist within *Fusarium* and that susceptibility testing is important and may improve the prognosis of these infections.

Conflict of interest: Authors declare no conflict of interest

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CHAPTER 17

Chapter 17

In vitro combinations of natamycin with voriconazole, itraconazole and micafungin against clinical *Fusarium* strains causing keratitis

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Journal of Antimicrobial Chemotherapy 2016; 71: 953-955.

Abstract

Objectives: *Fusarium* species cause a broad spectrum of infections, from superficial to disseminated disease. Because *Fusarium* species are intrinsically resistant to most antifungal drugs, new approaches are needed. The aim of the present study was to evaluate the *in vitro* combination of natamycin with currently used antifungal drugs. **Methods**: The *in vitro* interactions of combinations between natamycin and voriconazole, itraconazole and micafungin applied to 20 clinical *Fusarium* strains (members of *Fusarium falciforme*, *Fusarium napiforme*, *Fusarium petroliphilum*, *Fusarium proliferatum*, *Fusarium pseudensiforme* and *Fusarium sacchari*) were evaluated using a chequerboard microdilution method. The MICs of all drugs alone and in combination were determined visually after 48 h and interactions were assessed using fractional inhibitory concentration index (FICI) analysis. **Results**: MICs of voriconazole and natamycin alone were 4 to >16 and 4–8 mg/L, respectively. Values were reduced 3.5–10-fold to 0.02–0.5 mg/L and 0.5–5-fold to 0.13–2 mg/L in combination, for the currently used antifungals and natamycin, respectively, demonstrating additive to synergistic interactions. The combinations natamycin/voriconazole, natamycin/itraconazole and natamycin/micafungin were synergistic (FICI ≤0.5) for 70%, 15% and 5% of the strains, respectively. No antagonism was found. **Conclusions:** The combination of natamycin with voriconazole was strongly synergistic at clinically achievable serum concentrations.

Key words: *Fusarium*, keratitis, synergy, natamycin, voriconazole, itraconazole

Introduction

Keratitis is among the difficult-to-treat fungal infections and inappropriate therapy may lead to a severe decrease of vision or ultimately ocular phthisis.[1] Under (sub)tropical climatic conditions, especially in countries such as India, China and Brazil, *Fusarium* species are prevalent accounting for 36%-67% of all cases of keratitis[2] including traumatic eye infection among agricultural workers.[3] Management of *Fusarium* keratitis is challenging due to limited information on antifungal susceptibilities of the aetiological agents, the limited number of clinical trials assessing antifungal susceptibility with clinical outcome and intrinsic resistance to most antifungal drugs.[4] Although treatment protocols for keratitis have changed over the last decade, with a decrease in the use of amphotericin B and an increase in natamycin and voriconazole therapy, available antifungal agents remain inadequate and the best drug to treat a specific *Fusarium* keratitis has not been determined.[5]

Natamycin has been reported to have a broad spectrum of activity against various fungi including *Fusarium*.[6] Natamycin (5%) and/or topical amphotericin B (0.5%) are the first-line treatments of fungal keratitis in some countries, but treatment failures have been reported due to poor penetration through the corneal layers and toxicity of amphotericin B.[4] Monotherapy in general proved to be insufficiently effective[7] and therefore assessment of efficiency of combination therapy may be of interest. Since clinical trials evaluating the efficacy of topical 1% voriconazole versus 5% natamycin first-line treatment in fungal keratitis have recently been completed[8,9] and since *Fusarium* species are intrinsically resistant to most antifungals,[6] we evaluated the *in vitro* interactions of natamycin with voriconazole, itraconazole and micafungin against different *Fusarium* species isolated from patients with keratitis in order to determine potentially useful combinations in the treatment of this infection.

Materials and methods

Strains

Twenty clinical strains of *Fusarium* species [7 *Fusarium falciforme* (CBS 135520, CBS 135521, CBS 135524, CBS 135526, CBS 135528, CBS 135532 and CBS 135558), 4 *Fusarium petroliphilum* (CBS 135514, CBS 135518, CBS 135534 and CBS 135536), 2 *Fusarium proliferatum* (CBS 116324 and CBS 135548), 2 *Fusarium pseudensiforme* (CBS 135554 and CBS 135556), 3 *Fusarium napiforme* (CBS 135139, CBS 135140 and CBS 135141) and 2 *Fusarium sacchari* (CBS 135143 and CBS 135145)] were obtained from corneal ulcers and used in this study. Strains were subcultured on Sabouraud glucose agar (SGA) with 0.02% chloramphenicol for 5–7 days at 35–37 ˚C. Molecular identification was performed using MLST consisting of partial sequencing of translation elongation factor 1α (*TEF1*) and the RNA polymerase II gene (*RPB2*).

***In vitro* combination testing (chequerboard method)**

MICs were determined by broth microdilution according to CLSI document M38-A2 guidelines for non-dermatophyte species.[10] Briefly, for the assay preparation, conidial suspensions were harvested after strains were subcultured on SGA at 35–37 ˚C for 5–7 days and were suspended in normal saline containing 0.025% Tween 20. Inocula were then adjusted spectrophotometrically to 68%-70% transmission at a wavelength of 530 nm and diluted 10-fold to yield a final inoculum of 1×10^4 to 5×10^4 cfu/mL. Itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer Central, Sandwich, UK), natamycin (DSM, Delft, The Netherlands) and micafungin (Astellas Pharma, Ibaraki, Japan) were dissolved in DMSO and 2-fold serial dilutions ranging from 0.031 to 16 mg/L itraconazole and voriconazole, from 0.062 to 64 mg/L natamycin and from 0.008 to 8 mg/L micafungin were prepared in 96-well flat-bottomed microplates and stored at -70 ˚C. After inoculation, plates were incubated for 48 h at 37 ˚C and MICs were determined visually with a mirror as the lowest drug concentration with complete inhibition of growth compared with the growth in the drug free control. For micafungin, the minimum effective concentration (MEC) was determined as the lowest concentration of drug that leads to the growth of small, rounded, compact hyphal forms compared with the hyphal growth visualized in the growth control well. Quality control (QC) was performed on every new batch of plates with the following reference strains: *Aspergillus* flavus ATCC 204304, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258.

Drug interactions were assessed by a two-dimensional 8×12 broth microdilution chequerboard method for the combinations natamycin/voriconazole, natamycin/itraconazole and natamycin/micafungin using the following range of concentrations: 0.031–16 mg/L for voriconazole and itraconazole, 0.008–8 mg/L for micafungin and 0.062–64 mg/L for natamycin. Growth in each well was graded after 48 h incubation at 37 ˚C on a scale of MIC-0 to MIC-4 as follows: MIC-4 indicated no reduction in growth; MIC-3 indicated a 25% reduction; MIC-2 indicated a 50% reduction; MIC-1 indicated a 75% reduction; and MIC-0 indicated an optically clear well. The fractional inhibitory concentration index (FICI) was used to classify drug interactions as described by Meletiadis et al.[11] For each dataset, the minimum (ΣFICImin) and maximum (ΣFICImax) were calculated. The interaction was defined as synergistic if the ΣFICImin was ≤0.5 using MIC-2 (i.e. 50% growth), additive if ΣFICImin was >0.5–4 and antagonistic if ΣFICImax was >4.0.[11] For the FICI analysis, we used the MIC-2 endpoint of each of the two antifungals assayed, since this endpoint was previously found to correlate with the MEC of echinocandins and to be more sensitive in detecting pharmacodynamic interactions.[12,13]

Results and discussion

All *Fusarium* strains grew well after 48 h of incubation at 37 ˚C. In each batch of broth microdilution tests, the MICs/MECs for the QC strains were within the reference ranges. The MICs/MECs of itraconazole and micafungin for all the strains were >16 and >8 mg/L, respectively. For natamycin and voriconazole, the median (range) MIC was 4 (4–8) and 4 (4 to >16) mg/L, respectively. The results revealed that none of the above-mentioned drugs was effective when tested alone against *Fusarium* strains. The results of the chequerboard analysis (FICI) for the combinations natamycin/voriconazole, natamycin/itraconazole and natamycin/micafungin against *Fusarium* strains are summarized in Table 1, with median (range) MICs of drugs alone and in combination.

The combination natamycin plus voriconazole was synergistic for 14 (70%) *Fusarium* strains (2 *F. proliferatum*, 2 *F. napiformе*, 4 *F. petroliphilum*, 2 *F. pseudensiforme* and 4 *F. falciforme*) and additive for the remaining strains. The median (range) ΣFICImin was 0.2 (0.05–0.5). The MIC of voriconazole plus natamycin was reduced by median (range) 3.5 (1–8) and 5 (1–5) 2-fold dilutions down to 0.5 (0.03–4) and 0.13 (0.13–2) mg/L, respectively, when combined. The effects of natamycin plus itraconazole combinations were synergistic for three (15%) *Fusarium* strains (two *F. proliferatum* and one *F. petroliphilum*). Additive interactions were found for the other strains. The MICs of itraconazole and natamycin were reduced by median (range) 10 (3–10) and 1 (0–2) 2-fold dilutions down to 0.03 (0.03–4) and 2 (1–4) mg/L, respectively, when combined. On the other hand, the combination natamycin plus micafungin demonstrated synergismfor one *Fusarium* strain (*F. proliferatum*) (5%). For the remaining strains, additive interactions were found. The MICs of micafungin and natamycin were reduced by median (range) 5 (1.5–5) and 0.5 (0.5–1) 2-fold dilutions down to 0.02 (0.02–2) and 2 (2–2) mg/L, respectively, when combined. No antagonism was observed for any combination.

Natamycin and voriconazole are currently the mainstays of treatment for *Fusarium* keratitis.[14] Voriconazole has been widely used for treating fungal keratitis because it has better penetration and is considered to be superior to natamycin. However, Prajna et al.[8] recently reported equal or inferior efficacy of 1% voriconazole in a clinical trial when compared with 5% natamycin eye drops. In cases of *Fusarium* keratitis, natamycin is known to be significantly better than voriconazole.[9,15] In the present study, poor susceptibility of *Fusarium* was obtained for all drugs tested alone, as previously described by other authors.[6,16] Several studies have reported data on the efficacy of combination therapy of azoles and echinocandins against *Fusarium* and have shown a synergistic or additive interaction against *Fusarium* species.[17,18]

Table 1. Median (range) MIC (mg/L) and FICI values for all *Fusarium* strains as determined by two-agent broth microdilution chequerboard method at 48 h of incubation at 37 °C according to CLSI M38-A2.

Combination (AF+NAT)	***MIC_{AF}***	***MIC_{NAT}***	***FICI***	***$MIC_{AF(NAT)}$***	***$MIC_{NAT(AF)}$***	***%Syn***	***MIC 2-fold reduction***	
							AF	**NAT**
Voriconazole + Natamycin	4(4->16)	4(4-8)	0.2(0.05-0.5)	0.5(0.03-4)	0.13(0.13-2)	70%	3.5(1-8)	5(1-5)
Itraconazole + Natamycin	>16(>16->16)	4(4-8)	0.5(0.25-1)	0.03(0.03-4)	2(1-4)	15%	10(3-10)	1(0-2)
Micafungin + Natamycin	>8(>8->8)	4(4-8)	0.5(0.25-0.63)	0.02(0.02-2)	2(2-2)	5%	5(1.5-5)	0.5(0.5-1)

AF, antifungal; NAT, natamycin; VRC, voriconazole; ITC, itraconazole; MCF, micafungin; [a]FICI corresponds to ΣFICImin since all ΣFICImaxs were <4.

Our study evaluates the interaction between natamycin and voriconazole. This combination was synergistic for the majority of strains (70%), whereas for the remaining strains additive interactions were found. This synergistic interaction between these two drugs might have great clinical significance for the management of *Fusarium* keratitis. The achievable maximum concentration of voriconazole is ~3.30 mg/L after 1 h of topical administration.[9] No antagonism was observed.

The combination of natamycin and itraconazole was synergistic for 15% of *Fusarium* strains tested, while additive interactions were found for the remaining strains. No antagonism was registered. This result agreed with a previous study where synergy was observed for itraconazole-based combinations (itraconazole+terbinafine and itraconazole+flucytosine) for <25% of the strains tested.[17] *In vivo*, topical natamycin (5%) was shown to be superior to topical itraconazole (1%) in the management of fungal keratitis including fungal keratitis caused by *Fusarium*.[19] Clinical data from *in vitro* studies on the combination of natamycin and micafungin against *Fusarium* have not been published. However, in this study, the combination of natamycin and micafungin was synergistic against 5% of *Fusarium* strains. Natamycin in combination with micafungin was additive for the remaining strains. No antagonism was found.

In conclusion, the combination of natamycin plus voriconazole was synergistic against most *Fusarium* strains, significantly reducing the concentrations required to inhibit fungal growth. Further work is warranted to assess if the *in vitro* activity is translated to an *in vivo* model or clinical trials.

Funding

This study was funded by the Ministry of Health, Oman. Formal Agreement no. 28/2014. A. M. S. A.-H. received a PhD scholarship from the Ministry of Health, Oman.

Transparency declarations

J. F. M. has received grants from Astellas, Basilea and Merck, has been a consultant to Astellas, Basilea and Merck, and has received speaker's fees from Merck and Gilead. All others authors: none to declare.

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CHAPTER 18

Chapter 18

Comparative evaluation of Etest, EUCAST and CLSI methods for amphotericin B, voriconazole and posaconazole against clinically relevant *Fusarium* species

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Antimicrobial Agents and Chemotherapy . 2016;61: e01671-16. https://doi.org/10.1128/AAC.01671-16. (*in press*)

Abstract

We compared EUCAST and CLSI methods *versus* Etest for antifungal susceptibility testing of 20 clinically relevant *Fusarium* species against amphotericin B, posaconazole and voriconazole. The median Etest amphotericin B and posaconazole MICs were 1 dilution higher than the median EUCAST and the CLSI MICs. The essential agreement (within ±1/±2 dilutions) was (60/90%, 80/95%, 70/85%) between the Etest and the EUCAST and (80/95%, 75/95%, 45/100%) between the Etest and the CLSI for amphotericin B, voriconazole and posaconazole, respectively. The categorical agreement was >85%. Etest can be used for antifungal susceptibility testing of *Fusarium*.

Key words: *Fusarium*, antifungal susceptibility, comparison, CLSI, EUCAST, Etest amphotericin B, posaconazole, voriconazole, *TEF1, RPB2*.

Opportunistic infections due to *Fusarium* are increasingly reported due to the rising numbers of immunocompromised patients, although immunocompetent individuals can also be infected (1). The most common opportunists are members of the *F. solani* species complex (SC) followed by *F. oxysporum* SC and *F. fujikuroi* SC (2). First line antifungal treatment options are amphotericin B (AMB) and voriconazole (VRC) (3), while posaconazole (POS) has been recommended as salvage therapy. Posaconazole shows some *in vitro* activity against *Fusarium* spp., depending on the species (3,4). Although *Fusarium* spp. are resistant to many antifungals, we previously reported that this resistance was species-specific and species identification is essential for early antifungal treatment (3,5).

Reference methods for antifungal susceptibility testing (AFST) and breakpoints (BPs) for *Candida* spp and *Aspergillus* spp. have been developed (6). However, species-specific BPs have not yet been established for *Fusarium* spp. Recently, Espinel-Ingroff *et al.* (7) established the epidemiological cutoff values (ECVs) for *Fusarium* spp. in order to differentiate wild-type from non wild-type isolates. Although a reproducible method for AFST of *Fusarium* spp. has been described by the Clinical and Laboratory Standards Institute (CLSI) (8), and by European Committee for Antimicrobial Susceptibility Testing (EUCAST) (9), both are based on broth microdilution (BMD) and are time consuming. Furthermore, the limited AFST data and the lack of comparison of the two reference BMD methods for *Fusarium* spp. prompted us to study the agreement between the CLSI and EUCAST methods for testing AMB and triazoles against *Fusarium*. In addition, the Etest has been suggested as an alternative approach for AFST of AMB or triazoles for non-*Aspergillus* molds in the clinical laboratory (10), but data on *Fusarium* are limited. The objective of this study was to assess whether Etest could accurately measure the MICs of AMB, VRC and POS for clinical *Fusarium* isolates in comparison with the CLSI and EUCAST methods.

Twenty clinical *Fusarium* isolates, belonging to the following species: *F. acutatum*, *F. chlamydosporum*, *F. delphinoides*, *F. dimerum*, *F. equiseti*, *F. fujikuroi*, *F. incarnatum*, *F. keratoplasticum*, *F. lichenicola*, *F. napifome, F. oxysporum, F. petroliphilum, F. proliferatum, F. sacchari, F. solani* 'FSSC5', *F. solani* 'FSSC6', *F. subglutinans, F. temperatum, F. thapsinum* and *F. verticilliodes* were subcultured on Sabouraud glucose agar (SGA, Difco) with 0.02% chloramphenicol for 5 to 7 days at 35 °C to 37 °C. Molecular identification was performed using translation elongation factor-1α (*TEF1*) and the RNA polymerase (*RPB2*) as previously described (11). Strains were identified using GenBank (BLAST), the *Fusarium* MLST and the *Fusarium* ID databases.

Susceptibility testing was carried using three different methods; M38-A2 BMD as described in the CLSI document M38-A2 (8), EUCAST E.Def 9.3 (9), and Etest manufacturer's guide (bioMérieux SA, Marcy-l'Étoile, France). Drug concentrations ranges in BMD for AMB (Bristol-Myers Squibb, Woerden, the Netherlands), VRC (Pfizer Central, Sandwich, U.K.) and

POS (Merck, Whitehouse Station, U.S.A.) were 0.016−32 µg/ml. For the CLSI M38-A2 and the EUCAST E.Def 9.3, minimum inhibitory concentrations (MICs) of AMB, VRC and POS were determined with an inverted magnifying mirror after 48 h at 35 °C as the lowest drug concentration with complete inhibition of growth. For the Etest, the inoculum concentration was adjusted to 0.5 McFarland standard (equivalent to 1–5 × 10^6 cfu/mL). Then, 0.5 mL of this suspension was inoculated onto plates containing RPMI 1640 agar with 2% glucose using a cotton swab. After a period of 15 minutes, the Etest strips were applied and incubated for 48 h at 35 °C. The reading of the Etest MICs was performed as described and illustrated in the Etest guide and the MIC was determined as the concentration at the intercept of elliptical complete inhibition zone using four ATCC strains (*Aspergillus flavus* ATCC 204304, *A. flavus* ATCC 204305, *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258) as quality controls. To directly compare the Etest MICs with the EUCAST and the CLSI MICs, the Etest MICs were converted to the nearest highest two-fold dilution value that matched the CLSI and EUCAST two-fold dilution scheme. MICs were transformed to log2 values and differences were assessed statistically with paired t test. The median and the range of the log2 MIC differences between the methods were calculated. Microbiologically significant differences between the methods were assessed calculating the agreement within 1 and 2 two-fold dilutions. Finally, clinically significant differences were assessed by calculating categorical agreement among the three methods using previously determined ECVs for *Fusarium* spp. namely 4 µg/ml for amphotericin B, 4 µg/ml for voriconazole and 2 µg/ml for posaconazole.

Table 1 summarizes the *in vitro* susceptibilities of 20 isolates of *Fusarium* spp. to AMB, VRC and POS. The data are presented as MICs, MIC ranges, median MIC and MIC_{90}. CLSI MIC results of AMB spanned a narrow range of 1 to 4 µg/ml. Overall, AMB and VRC showed the most potent activity with all three methods. The median, (range) and MIC_{90} with Etest, EUCAST and CLSI was 2(0.25->32)16, 1(0.25-8)8 and 1(0.5-4)4 µg/ml for AMB and 2(0.25->32)4, 2(0.5-16)4 and 2(0.5->16)4 µg/ml for VRC, respectively. POS exhibited high MICs against *F. chlamydosporum*, *F. dimerum*, *F. incarnatum*, *F. napifome*, *F. oxysporum*, *F. proliferatum*, *F. thapsinum* and all the reported species within *F. solani* species complexes, with median, (range) and MIC_{90} of 32(0.25->32)32 µg/ml for Etest, 16(0.5->16)16 µg/ml for EUCAST method and 8(0.25->16)16 µg/ml for CLSI method.

Table 1. MIC (µg/ml), median, range and MICs 90% obtained by antifungal testing of amphotericin B, voriconazole and posaconazole for 20 *Fusarium* species as determined by the Etest, EUCAST and CLSI methods at 48 h of incubation.

CBS No	Species	Method	MIC (µg/ml) of:		
			AMB	VRC	POS
130548	*F. acutatum*	Etest	4	0.5	0.25
		EUCAST	2	0.5	0.5
		CLSI	1	1	2
635.76	*F. chlamydosporum*	Etest	1	4	>32
		EUCAST	0.5	2	16
		CLSI	1	2	>16
120712	*F. delphinoides*	Etest	0.5	4	4
		EUCAST	0.5	4	2
		CLSI	1	4	2
108944	*F. dimerum*	Etest	0.25	4	>32
		EUCAST	1	2	16
		CLSI	0.5	2	>16
307.94	*F. equiseti*	Etest	1	0.25	0.25
		EUCAST	0.25	1	1
		CLSI	0.5	0.5	1
221.76	*F. fujikuroi*	Etest	2	0.5	0.25
		EUCAST	1	0.5	0.5
		CLSI	0.5	2	1
132894	*F. incarnatum*	Etest	1	4	>32
		EUCAST	0.5	4	16
		CLSI	1	4	>16
135806	*F. keratoplasticum*	Etest	16	0.25	4
		EUCAST	8	1	8
		CLSI	4	2	8
138750	*F. lichenicola*	Etest	1	2	>32
		EUCAST	0.5	4	16
		CLSI	0.5	2	>16
135140	*F. napifome*	Etest	>16	0.5	>32
		EUCAST	8	4	>16
		CLSI	4	2	8
137206	*F. oxysporum*	Etest	2	4	>32
		EUCAST	2	2	16
		CLSI	1	2	>16
135955	*F. petroliphilum*	Etest	0.5	4	>32
		EUCAST	0.5	8	16
		CLSI	1	4	>16
133030	*F. proliferatum*	Etest	2	4	>32
		EUCAST	1	4	16
		CLSI	0.5	4	>16

Table 1. (Continued)					
135145	*F. sacchari*	Etest	1	0.25	1
		EUCAST	1	1	1
		CLSI	1	0.5	1
135785	*F. solani* 'FSSC5'	Etest	2	4	>32
		EUCAST	2	8	16
		CLSI	2	4	>16
135798	*F. solani* 'FSSC6'	Etest	>32	>32	>32
		EUCAST	8	16	16
		CLSI	4	>16	>16
136481	*F. subglutinans*	Etest	2	1	0.5
		EUCAST	1	1	1
		CLSI	0.5	1	0.25
135540	*F. temperatum*	Etest	2	2	4
		EUCAST	1	1	2
		CLSI	0.5	0.5	0.5
130176	*F. thapsinum*	Etest	4	2	>32
		EUCAST	4	2	16
		CLSI	2	2	>16
579.78	*F. verticilliodes*	Etest	1	2	1
		EUCAST	1	1	1
		CLSI	2	1	0.25
All	**All**[a]	Etest	2(0.25->32)16	2(0.25->32)4	32(0.25->32)32
		EUCAST	1(0.25-8)8	2(0.5-16)4	16(0.5->16)16
		CLSI	1(0.5-4)4	2(0.5->16)4	8(0.25->16)16

[a]median (range)/MIC90 for all isolates; amphotericin: AMB; voriconazole: VRC; posaconazole: POS

Table 2 shows the analysis of the EA between 48 h CLSI, EUCAST and Etest MICs for each drug tested. The levels of agreement (within ±2 dilutions) between the results of the CLSI and the EUCAST method were 100% for AMB, VRC and POS. The levels of agreement (within ±2 dilutions) between the results of the EUCAST method and the Etest were 100 % for POS and 95% for both AMB and VRC. The agreement between the CLSI and the Etest was 90% for AMB, 95% for VRC and 85% for POS. AMB and POS MIC values with the Etest tended to be significantly higher (p=0.007-0.097) (median dilution difference 1-1.5) than the two reference methods, indicating that Etest MIC distribution is shifted to the right compared to the EUCAST and the CLSI MIC distributions. However, the most of the differences remained within ±2 dilutions. The comparison of MICs obtained with the three methods resulted in a considerable Pearson correlation coefficient (PCC) varying from 0.71 to 0.97. The correlation among Etest vs. CLSI and Etest vs. EUCAST methodologies to POS was higher (0.89 and 0.97) than AMB (0.71 and 0.86) and VRC (0.77 and 0.76) (Table 2). Moreover, the PCCs were statistically significant (P <0.0001), indicating a good correlation between the MICs obtained by the CLSI

or EUCAST and the Etest. The categorical agreement (CA) for all three methods and drugs was >85% with slightly higher levels of agreement found between the Etest and the EUCAST (90-100%) than the CLSI (85-95%).

Table 2. Comparison among the three methods for antifungal susceptibility testing of *Fusarium* spp.

Methods	Drug	Median (range) difference	Agreement		Paired t test p value	Pearson r[a]	CA[b]
			±1 dil.	±2 dil.			
CLSI vs. EUCAST	AMB	-1(-1-1)	100%	100%	0.234	0.78	85%
	VOR	0(-1-2)	95%	100%	1.000	0.81	90%
	POS	1(-2-2)	75%	100%	0.383	0.89	100%
EUCAST vs. Etest	AMB	-1(-3-2)	80%	95%	0.007	0.86	100%
	VOR	0(-2-3)	75%	95%	0.494	0.76	95%
	POS	-1.5(-2-2)	45%	100%	0.013	0.97	90%
CLSI vs. Etest	AMB	-1(-4-1)	60%	90%	0.008	0.71	85%
	VOR	0(-2-3)	80%	95%	0.479	0.77	95%
	POS	-1(-3-3)	70%	85%	0.097	0.89	90%

[a] $p<0.0001$ for all comparisons; [b]CA: Categorical agreement

Our results indicated a good agreement between the three methods. The EA and CA between the Etest and the two reference methods were high for all three drugs (≥ 85%). The MIC results for AMB spanned a range of 0.25 to 8 μg/ml with the CLSI and the EUCAST method and in a wider range of 0.25->32 with the Etest. Wide MIC ranges were also found with POS and VRC and all three methodologies indicating considerable inter-species variations. High MICs (>4 μg/ml) were found for AMB with *F. keratoplasticum*, *F. napifome* and *F. solani* 'FSSC6', for VRC with *F. petrophilum* and *F. solani* 'FSSC6' and for POS with most species except *F. acutatum*, *F. delphinoides, F. equiseti, F. fujikuroi, F. sacchari, F. subglutinans, F. temperatum* and *F. verticilliodes*. Our results were comparable to those of other studies using BMD testing (1,3,12-17). POS displayed high MIC results, with values ranging from 0.25 to over 32 μg/ml against *Fusarium* species with little difference when BMD and Etest methods were compared, which is in agreement with previously reported data (3,18).

Although, POS showed high MICs for most isolates in the present study, case reports have demonstrated successful treatment of fusariosis with this drug. In one case report, *F. solani* did not response to treatment with natamycin and AMB but responded to POS with a MIC 1 μg/ml, and the patient recovered completely (19). Tu et al. (20), describes 3 cases of keratitis due to *F. solani* successfully treated with POS. In another publication, HIV positive patient had onychomycosis due to *F. falciforme*, was successfully treated with POS with MIC 0.5 μg/ml (21). In addition, *Fusarium* peritonitis and keratitis were treated with POS (22, 23). Thus, POS may be effective against isolates with low MICs (≤1 μg/ml).

Recently, Espinel-Ingroff et al. (7) used the CLSI methodology to determine the epidemiological cutoff values (ECVs) for two *Fusarium* species complexes, namely *F. oxysporum* and *F. solani*, and *F. verticillioides* of the *fujikuroi* SC for AMB, ITC, VRC, POS (7). However, no ECVs for rare *Fusarium* species were determined in that study. Comparison of CLSI and EUCAST *versus* Etest was performed for filamentous fungi including few strains of *Fusarium* (10, 24-27). Two studies compared CLSI and Etest; Lamotha *et al*. (10), studied 34 clinical *Fusarium* isolates with 94% agreement for AMB and 100% for VRC and POS respectively whereas, Debourgogne *et al*. (28) reported lower overall agreement in FSSC only with 73% for AMB and 92% for VRC. Our study included molecularly identified *Fusarium* spp. and we also found high levels of agreement.

In conclusion, Etest overall resulted in 1 dilution higher MICs than the reference methods with most differences being within 2 dilutions which may lead to errors if same breakpoints will be applied. However, the categorical agreement was high (>85%) using previously published ECVs. Etest can be used for routine susceptibility testing of amphotericin B, voriconazole and posaconazole for *Fusarium* species. Further work is warranted in order to establish clinical breakpoints for *Fusarium*.

Funding

This study was partially funded by the Ministry of Health, Oman (Formal Agreement no. 28/2014). Abdullah M. S. Al-Hatmi received a PhD scholarship from the Ministry of Health, Oman.

Competing interests

JFM received grants from Astellas, Basilea, and Merck. He has been a consultant to Astellas, Basilea and Merck and received speaker's fees from Merck, United Medical and Gilead Sciences. All other authors declare that they have no conflict interests.

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Part IV

Summary and general discussion

- **Genral discussion**
- **Future perspectives**
- **Summary (In English)**
- **Samenvatting (Summary in Dutch)**
- **Acknowledgments**
- **Curriculum vitae**
- **List of publications**

CHAPTER 19

Chapter 19

General discussion and summary

Introduction

Over the past years, the numbers of human infections with *Fusarium* species have been increasing worldwide, both in immunocompromised[1] and in immunocompetent patients.[2] These infections are classified in three main groups: (i) superficial infections of skin (e.g. tinea pedis), nails (e.g. onychomycosis) and the cornea (e.g. keratitis), (ii) deep mycoses and (iii) disseminated infections.[3] Disseminated infections by *Fusarium* mostly occur in immunocompromised patients with hematologic disorders including neutropenia.[4]

Recent studies have shown that the majority of cases are caused by three species complexes i.e. in order of frequency the *F. solani* species complex (SC) followed by the *F. oxysporum* SC and the *F. fujikuroi* SC (2), the remaining species being highly exceptional.[5] A thorough knowledge of the identity of the causal agents is a basic requirement for sustainable disease management. Correct *Fusarium* species identification has important implications for the treatment of infections because antifungal resistance is species-specific. In this work, the accent has been placed on molecular, protein-based and antifungal susceptibility testing methods to answer taxonomic, phylogenetic, diagnostic and therapeutic questions in *Fusarium*.

The overall aim of this research project was to gain better understanding of the genus *Fusarium* in humans by studying (i) diversity and phylogeny of clinically relevant *Fusarium* species, (ii) by defining and selecting a good barcode marker for *Fusarium* diagnostics, (iii) by developing diagnostic tools for identification of *Fusarium* species, (iv) by investigating molecular genotyping to understand epidemiologic relationships and potential routes of transmission, and (v) by providing *in vitro* susceptibility data of a worldwide collection of *Fusarium* strains. Furthermore, given the interest of combination therapy for treating invasive and superficial fusariosis, assessment of *in vitro* interaction of antifungal drugs for different *Fusarium* species was studied.

This thesis consists of three parts: part I contains the Chapters 2, 3 and 4 that focus on taxonomy and barcoding of agents of fusariosis. Part II includes Chapters 5, 6, 7, 8, 9, 10, 11, 12 and 13 that focus on diagnosis, human infection and molecular epidemiology. Part III contains Chapters 14, 15, 16, 17 and 18 which focus on *in vitro* antifungal susceptibility testing of compounds singly and in combination. Part IV consists of summary and general discussion.

Part I: Taxonomy and barcoding of agents of fusariosis

Since the introduction of the genus, more than 200 years ago by Link,[6] mycologists have identified species in *Fusarium* by using morphological characters e. g. hyphae, phialides, conidiogenous cells, macroconidia, microconidia, chlamydospores, texture, colour, pigment, cultural characteristics, host associations and mating.[7] Although traditional taxonomic systems for *Fusarium* have been proposed based on these highly variable morphological characters, interspecific differences exist between some *Fusarium* species. Expertise in taxonomy is required for accurate and reliable identification. However, species delineation based on morphology in *Fusarium* is unsettled, and as a consequence taxonomists disagree on the number of species in the genus. This debate has been ongoing for years.[8] In addition to the morphological species concept, the biological species concept and the phylogenetic species concept are available in fungal diversity studies.[9]

O'Donnell et al.[10] introduced the term 'species complex' in the genus *Fusarium*, as an alternative to the subgeneric 'sections' that are currently in use in genera like *Aspergillus*.[11] The problem in *Fusarium* was that the phenotypic sections did not match with current phylogeny. The molecular species complexes in *Fusarium* are monophyletic, and hence they can be viewed as taxonomic entities.[12] With the introduction of DNA-based methods, *Fusarium* taxonomy has gone through major modifications. Recently, many studies,[13-21] have applied the phylogenetic species concept to study the taxonomy of *Fusarium* and authors have agreed to some extent with the species complex taxonomic system.[10]

However, the phylogenetic position of a large number of clinical species is still abandoned and not possible sometimes to obtain from very old specimens and lack of ex-type cultures. The best option in our opinion is to epitypify or neotypify them as Schroers et al.[22] recently did with *Fusisporium* (*Fusarium*) *solani*; the species was assigned to a common phylogenetic species in the *F. solani* species complex. By epitypification, the name of the species can be stabilized with a freshly collected living culture, and the phylogenetic position can be settled. There are ongoing efforts to describe new species with typification to stabilize nomenclature for future studies.

Phylogenetic determinations based on gene sequences not only permitted *Fusarium* identities to be clarified, but also provided a basis for species distinctions within the genus. Although amplification and sequencing success rates of ITS and LSU is high, these genes could not discriminate all *Fusarium* species.[20] ITS sequences are identical in many *Fusarium* complexes and they do not tend to evolve at a rate correlated with speciation. In contrast, protein-coding genes usually have higher information content and tend to evolve at higher rate than the more commonly used ribosomal genes.[23] Using multi-gene analysis, viz. ITS, LSU, *ACT*, *BT2 RPB1*, *RPB2* and *TEF1*, and applying species recognition by genealogical concordance concepts, *Fusarium* species were classified into twenty two species complexes

and several of them contain human opportunistic pathogens.[24] Identification to the species level is also essential for treatment of patients because antifungal susceptibilities may differ between closely related species.[25]

It should be noted that the nomenclature of *Fusarium* has not been without controversy, particularly with the recent movement of *solani* and *dimerum* complexes to two genera, *Neocosmospora* and *Bisifusarium,* respectively,[26] while another group of researchers renamed the *Fusarium solani* complex as *Fusisporium solani*.[22] A number of clinical investigators raised concerns particular about the change from *Fusarium* to *Neocosmospora* or *Fusisporium*, which may cause confusion among medical mycologists. However, the formal guidelines recommended by the fungal community combined with scientific and biological evidence was a reason for many researchers to preserve the name *Fusarium* for all clinically relevant species complexes.[27] Medical mycologists advocate keeping well-known names of clinically relevant fungi as stable as possible.[28]

In Chapter 1, ***General introduction and outline of the thesis,*** molecular diversity, phylogeny, barcoding, clinical aspects, epidemiology, application of rapid diagnostic tools, *in vitro* susceptibility testing and *in vitro* drug interaction for *Fusarium* are discussed and it is important to explore alternative approaches in these fields. Therefore, the research described in this thesis is aimed to provide some experimental evidence that will help to guide physicians in the diagnosis and treatment of patients with severe *Fusarium* infections.

In Chapter 2, ***Evaluation of two novel barcodes for species recognition of opportunistic pathogens in Fusarium*****,** two newly characterized genes were used as molecular markers for *Fusarium* species genotyping, i.e. topoisomerase I (*TOP1*) and phosphoglycerate kinase (*PGK*). *PGK* and *TOP1* have the potential of secondary barcode for *Fusarium* species and show sufficient polymorphism to precisely recognize all tested species in *Fusarium*. *PGK* and *TOP1* can be used as a single barcode marker or in combination with other routinely used markers such as *TEF1*. By analyzing of 144 *Fusarium* strains belonging to 52 species, *TOP1* and *PKG* provided concordance of molecular data with *TEF1*. The currently accepted *Fusarium* species were well-supported in phylogenetic trees of both new barcodes.

In Chapter 3, ***DNA barcoding, MALDI-TOF, and AFLP data support Fusarium ficicrescens as a distinct species within the Fusarium fujikuroi species complex,*** applying multilocus sequence analysis (MLSA) of *BT2*, *RPB2* and *TEF1*, and *TEF3* and following the concept of genealogical concordance phylogenetic species recognition, a novel taxon, *Fusarium ficicrescens* was introduced which was represented by strains collected from figs fruits in Iran and previously misidentified as *F. andiyazi*. In addition, MALDI-TOF, and AFLP data support *Fusarium ficicrescens* as a distinct species within the *F. fujikuori* complex.

In Chapter 4, ***Phylogenetic diversity of human pathogenic Fusarium and emergence of uncommon virulent species*****,** a total of 44 *Fusarium* isolates were collected from 43 (38

immunocompetent and 5 immunocompromised) patients at Hamad Hospital, Doha, Qatar from July 2003 to June 2014. Initial identification of the isolates as *Fusarium* species was done by conventional techniques. They were further characterized using two-locus sequencing of *RPB2* and *TEF1*. The identified species belonged to four species complexes (SC); the most common SC was *Fusarium solani* (FSSC) (75%), followed by *Fusarium oxysporum* (FOSC) (4.5%), *Fusarium fujikuroi* (FFSC) (13.6%), and *Fusarium dimerum* (FDSC) (6.8%). Our findings suggested that *F. acutatum* is an endemic species in the Middle East, with members of the *F. solani* species complex predominantly causing cornea, nail and bloodstream infections. Therefore, fast and more accurate molecular diagnostic tests to species level may contribute to an earlier and more precise diagnosis and subsequently optimized treatment.

Part II: Diagnosis, human infection and molecular epidemiology

Diagnosis

Given the changes in *Fusarium* taxonomy and the emergence of the opportunistic species with multi-drug resistance, accurate identification of the *Fusarium* species is a key to improved treatment. However, the current diagnostic techniques should be strengthened with the development of new methods. The use of non-specific diagnostic tools may have serious consequences of ineffective therapy. Prior to the application of molecular methods, identification of *Fusarium* as infectious agent was mainly based on culture, morphology, histopathology, radiology and serology.[29] These traditional diagnostic methods are still considered as gold standards, but have low sensitivity and specificity in diagnosing *Fusarium* infectious.[30] The need for diagnostic tools has led to an explosion of non-culture diagnostic approaches, including sequencing and Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). Sequence analysis comprises the following partial genes either as a single marker or MLSA analysis; *RPB1, RPB2*, *TEF1* and the β-tubulin gene.[20]

In this thesis, further diagnostic efforts included MALDI-TOF MS, a technique based on peptide profiles which is increasingly used in medical mycology.[31] MALDI-TOF MS for rapid identification of fifteen *Fusarium* species was developed in Chapter 5, ***Rapid identification of clinical members of Fusarium fujikuroi complex using MALDITOF-MS***. A species-specific spectra database was developed for clinical strains of *Fusarium fujikuroi* species complex (FFSC), namely *F. acutatum*, *F. ananatum, F. andiyazi, F. anthophilum, F. fujikuroi, F. napiforme, F. nygamai, F. proliferatum, F. verticillioides, F. sacchari, F. subglutinans, F. temperatum,* and *F. thapsinum*. Compared to DNA sequencing, MALDI TOF MS showed shorter turnaround time and lower costs. In addition, using log score 2.0 as a cutoff, 93.3% were correctly identified down to the species level, whereas the remaining isolates (with scores ≥1.7 but <2.0) were within the correct species complex. Culture methods take up to one week

and sequencing takes up to 48 h, depending on the species and the availability of the sequencing system used. In contrast, MALDI-TOF MS can be performed with minimal amounts of young colonies and takes only a few minutes, with comparable levels of resolution. These advantages make MALDI-TOF MS suitable for quick and reliable identification of *Fusarium* species in clinical laboratories which will consequently improve the diagnosis. MALDI-TOF MS has the potential to become an important tool for the routine identification of pathogenic *Fusarium* species, provided the availability of an extended database. Hence, the method might replace conventional diagnostics.

In Chapter 6, ***The concept of ecthyma gangrenosum illustrated by a Fusarium oxysporum infection in an immunocompetent individual*****,** we presented a case of Ecthyma Gangrenosum (EG) due to *F. oxysporum* based on histopathological, culture and molecular data. At present EG is known to be caused by bacteria such as *Pseudomonas aeruginosa* or related in immunocompromised patients. In this chapter we extended the concept of EG by using a clinical definition rather than by causative agent. Fungi including *Fusarium* are able to provoke the same clinical features. Given the fact that the etiologic agent can be of bacterial and of fungal nature, it is essential that the pathogen is identified. For identifying the causative agents down to the species level, *BT*, *TEF1* and *RPB2* were used to give a higher resolution. In order to distinguish between sibling species, the *TEF1* gene region is especially recommended but *BT* can be used also.

Human infection

Fusarium plays an important role in several clinical manifestations. *Fusarium* species cause a broad spectrum of infections in humans including superficial infections, such as keratitis and onychomycosis, as well as locally invasive and disseminated infections.[24] The incidence of invasive fusariosis has grown dramatically in recent years due to the increase in the number of hematological patients as well as patients undergoing bone marrow or solid organ transplantation.[32] The ability to survive and proliferate at 37 °C is an essential virulence attribute of pathogenic microorganisms.[33] Opportunistic species of *Fusarium* are able to tolerate at least human body temperatures.[34] The capacity of some species of *Fusarium* to grow in human tissue and proliferate in patients with immune deficiency supports the suggestion of opportunism of this genus in the development of the disease.

In Chapter 7, ***Fusarium ramigenum, a novel human opportunist in a patient with common variable immunodeficiency and cellular immune defects***, we presented a case of a long history of invasive pulmonary fusariosis in a patient with cellular immune defects. With extensive morphological and phylogenetic analysis of *BT2* and *TEF1* the cause of the infection was identified as a novel *Fusarium* opportunist, *F. ramigenum*. This report demonstrated that an opportunistic invasive fungal infection may indicate an underlying cellular immune impairment

of the host. After long-term treatment with voriconazole (6 months) and immunoglobulin substitution, the patient recovered from this opportunistic infection. *Fusarium ramigenum* was first described in 1998 from inedible wild Capri figs in California, U.S.A.[35]

In Chapter 8, ***Keratitis by Fusarium temperatum, a novel opportunist***, some of the published cases of fusariosis have been diagnosed on the basis of clinical, pathological, morphological and molecular data. Our case report from Mexico, in a worker who acquired the infection from plant material of maize, extends the significance of the genus *Fusarium* as agents of keratitis and underscores the utility of molecular verification of these emerging fungi in the human host. *Fusarium temperatum* was first described in 1998 from maize in Belgium.[36] A remarkable feature in *Fusarium* is the apparent combination of plant pathogenicity and the ability to cause infections in humans.[37]

In Chapter 9, ***Fusarium species causing eumycetoma: Report of two cases and comprehensive review of the literature*** introduces some potential human opportunists which have never been reported before or were wrongly identified. However, in this chapter we found that not only *Fusarium solani* species complex (FSSC), but also other species in the genus can cause human mycetoma. In the present study we utilized *TEF1* sequencing to identify *Fusarium* strains that were recovered from cases of human eumycetoma. In addition, an extensive review of reported *Fusarium* eumycetoma cases in the medical literature was performed. Agents in FSSC include the three closely related species *F. keratoplasticum*, *F. pseudensiforme*, and *Fusarium* cf. *solani*. In addition, *F. thapsinum,* a member of the *F. fujikuroi* complex also appeared to be involved in mycetoma. Two case reports were described in this chapter. In the first case, we isolated *F. keratoplasticum* from a patient's biopsy, while the second case was caused by a novel opportunist reported for the first time from a human infection. Although sequencing of partial *TEF1* is not routinely done in most clinical laboratories, we reinforce that it is important to get a correct identification of *Fusarium* species. Antifungal susceptibility tests should be performed to optimize medical treatment.

In Chapter 10, ***Proximal subungual onychomycosis caused by Fusarium falciforme successfully cured with posaconazole***, we report a case of onychomycosis by *F. falciforme* in a HIV-positive patient from Mexico. The lesion started 9 months earlier with a history of proximal leuconychia of the first toenails of both feet. The lesion was successfully cured with posaconazole, which may provide an alternative treatment for patients presenting with onychomycosis.

Molecular epidemiology

By typing *Fusarium* isolates the understanding of the genetic and epidemiological relationships between environmental and clinical isolates can be improved. Understanding pathogen distribution and relatedness is essential for determining the epidemiology of nosocomial

infections and helping in the design of control measures.[38] The genetic diversity in patients' samples with *Fusarium* species can be investigated to understand colonization, dissemination routes, epidemics and outbreaks.[39] For example, a *Fusarium* keratitis outbreak in the U.S.A., where 66 cases were caused by *Fusarium solani* species complex due to improper contact lens wear,[40] speciation of the isolates was performed using sequencing data which is considered as one of the gold standard techniques in molecular epidemiology. Similarly, multilocus sequence analysis (MLSA) identified up to 10 different species of *Fusarium* involved in a keratitis epidemic.[41] To obtain a higher resolution of the phylogenetic relationships of species within a genus or genera within a family, MLSA is currently a widely used method. In MLSA studies, partial sequences of genes coding for proteins with conserved functions are used to identify microorganisms and to generate phylogenetic trees. Furthermore, MLSA has become an accepted and widely used method in microbial taxonomy.[42] In this part of the thesis, we used amplified fragment length polymorphism (AFLP) and MLSA to investigate genetic diversity of clinical and environmental *Fusarium* isolates.

In Chapter 11, ***Emergence of fusariosis in a university hospital in Turkey during a 20-year period*****,** the first epidemiological data from Turkey of clinical *Fusarium* isolates collected during the last decades was conducted and the responsible *Fusarium* species were identified. *Fusarium* infections have increased and emerged during the last fifty years, particularly in immunocompromised patients, and epidemiological studies on clinical isolates with determination of their antifungal susceptibilities are important. In this study, a total of 47 cases of fusariosis were included, identified by multilocus sequence analysis (MLSA) and their antifungal susceptibilities (AFST) were tested according to the CLSI method. Of the *Fusarium* infections, 23.4% were superficial, 44.7% were locally invasive, and 31.9% were disseminated. A significant increase was observed over the years. Our study indicated that regional differences exist in the distribution of *Fusarium* species and that species-specific differences might also reflect in AFST patterns. As the observed numbers of *Fusarium* infections, particularly disseminated cases, are increasing, monitoring of local epidemiological data will help clinicians to develop appropriate therapies for successful treatment of particularly disseminated fusariosis.

In Chapter 12, ***Multidrug-resistant Fusarium keratitis: a clinico-mycological study of keratitis infections in Chennai, India***, we investigate the genetic diversity and molecular epidemiology of *Fusarium* isolates isolated from Chennai, India, from keratitis cases that had been monitored in a university hospital during a two-year period. Separation of genotypes eventually acting as units of evolution was investigated using two partial gene sequences, i.e. *TEF1* and *RPB2*; 10 *Fusarium* isolates were included. The etiological agents belonged to *F. solani* species complex (90%) and *F. sambucinum* species complex (10%); the identified species were *F. keratoplasticum* (n=7), *F. falciforme* (n=2), and *F. sporotrichioides* (n=1). We found that regional differences exist in the distribution of *Fusarium* species and monitoring

of local epidemiological data are of importance to clinical practice. All species were clearly differentiated on the basis of their *TEF1* and *RPB2* sequences.

In Chapter 13, ***Global molecular epidemiology and genetic diversity of Fusarium, a significant emerging group of human opportunists, 1958-2015***, genotyping by AFLP analysis, a molecular fingerprinting technique that was first introduced by Vos et al.,[43] is elaborated. The AFLP protocol includes four steps: (1) genomic DNA is cut with two restriction enzymes, one with average cutting frequency and a second with high cutting frequency, (2) Synthetic double-stranded DNA fragments are ligated to the obtained sticky ends to serve as primer binding sites in a successive PCR reaction, (3) pre-selective amplification of genomic fragments containing an adapter at the end and (4) selective amplification using primers with selective base extensions. Variations between different isolates originate by differences in the number and location of restriction enzymes recognition sites in the genome. A highly informative complex DNA pattern with bands of 40−400 bp is usually obtained.

Our investigation concerned an analysis of *Fusarium* strains isolated from a worldwide selection of clinical and environmental isolates from reference collections comprising isolates from human cases. The set of isolates was also compared with MLSA analysis. AFLP data showed that *Fusarium* species can be distinguished at the species complex level, and additional subgroups within the main species clusters revealed genetic diversity within each species complex. The AFLP clusters and subclusters were almost identical to the sequencing identifications. Our findings indicate that fusariosis is distributed globally, with a focus in (sub) tropical areas. Considerable species diversity is observed, with no differences between clinical and environmental isolates. This suggests that infections with *Fusarium* species are truly opportunistic. The three most common species are *F. falciforme* and *F. keratoplasticum* (members of *Fusarium solani* species complex) followed by *F. oxysporum* (*Fusarium oxysporum* species complex). AFLP has high reproducibility, resolution, and sensitivity at the whole genome level compared to other techniques by amplifying between 50 and 100 fragments at one time.[44]

Part III: *In vitro* antifungal susceptibility testing and drug combinations

Fusarium species are intrinsically resistant to most currently used antifungal agents.[24] Mechanisms of resistance have not been studied well in *Fusarium* species. Since the immune system of immunomcompromised patients may be suppressed for long periods, antifungal therapy is employed to prevent and control invasive infections.[45] However, the relevance of *in vitro* data remains unclear since no documentation is available on its correlation with the *in vivo* outcome and therefore also no clinical breakpoints have been defined for infections with *Fusarium* species. After having established the taxonomy of *Fusarium* species, as well as accurate diagnostic methods for their early recognition in the clinical laboratory, the next step in patient management is appropriate therapy. Amphotericin B and voriconazole are recommended

drugs to treat infections caused by *Fusarium* according to European guidelines.[46] Amphotericin B has *in vitro* activity against all *Fusarium* species, but also has the most severe toxic side effects for the patient, i.e. nephrotoxicity.[47] Therefore, as a replacement of amphotericin, it is generally recommended to use voriconazole, which, similar to other azoles, blocks synthesis of ergosterol, a major component of the fungal cell membrane.[24] However, the susceptibilities of rare and common *Fusarium* species to different drugs of different antifungal classes have not been established. Therefore, the need for standardized and clinically relevant antifungal susceptibility testing (AFST) of *Fusarium* has become obvious. This is particularly significant because of the increase of opportunistic *Fusarium* infections and the number of available antifungal drugs to correlate the *in vitro* results with *in vivo* activity, possibly predicting the outcome of therapy.

Combination therapy might be an alternative chemotherapeutic approach for management of infections caused by *Fusarium,* given the limited *in vivo* efficacy of antifungal monotherapy. Strains resistant to monotherapy are regularly encountered. In order to improve antifungal therapy, we tried to obtain more insight into the *in vitro* antifungal susceptibility of clinically related *Fusarium* species against various antifungal agents, tested according to Clinical and Laboratory Standards Institute (CLSI),[48] and the European Committee for Antimicrobial Susceptibility Testing (EUCAST),[49] reference methods for broth dilution antifungal susceptibility testing of filamentous fungi. In addition, we also evaluated the *in vitro* combination of natamycin with currently used antifungal drugs in treating keratitis due to *Fusarium* species.[50] Finally, we compare EUCAST and CLSI methods *versus* Etest for antifungal susceptibility testing of clinically relevant *Fusarium* species against amphotericin B, posaconazole and voriconazole.

In Chapter 14, ***Specific antifungal susceptibility profiles of opportunists in the Fusarium fujikuroi complex***, we evaluated the *in vitro* antifungal susceptibility of thirteen *Fusarium* species to eight antifungal agents by CLSI. High MICs/MECs were found with fluconazole, itraconazole, micafungin and natamycin, while amphotericin B was the most active drug followed by voriconazole, posaconazole and isavuconazole. These MICs were comparable to what has been reported for *Fusarium* species.[51] However, some species investigated, *F. nygamai* and *F. napiforme* in particular, showed very high MICs to these antifungals, while *F. verticillioides* showed much lower MICs. Our data presents antifungal susceptibility profiles which differ between and within *Fusarium* species. Selection of the proper antifungal is therefore crucial and accurate identification of *Fusarium* down to species level is predictive and can provide a first clue for the susceptibility. To the best of our knowledge this was the first report evaluating the above mentioned drugs including the new azole isavuconazole against a wide panel of *Fusarium* species within FFSC.

In Chapter 15, ***Antifungal susceptibility and phylogeny of opportunistic members of***

the genus Fusarium causing human keratomycosis in south India, the *in vitro* susceptibility of amphotericin B, nystatin, ketoconazole, fluconazole, miconazole, voriconazole, econazole, clotrimazole, natamycin and itraconazole against 65 clinical *Fusarium* strains was determined by CLSI method. Isolates were collected from patients and subjected to multilocus DNA sequencing to characterize the spectrum of the species associated with keratitis infections in India; the majority of *Fusarium* isolates tested belonged to FSSC. Amphotericin B, voriconazole, and clotrimazole with MICs ≤4 μg/ml proved to be the most effective drugs, followed by econazole. Large differences were noted between strains and the MICs were highly variable.

In Chapter 16, ***In vitro resistance of clinical Fusarium species to amphotericin B and voriconazole using the EUCAST antifungal susceptibility method, antifungal***, susceptibility of 39 clinical *Fusarium* strains collected from local and invasive infections during the last 10 years from Qatar were tested against amphotericin B and triazole (itraconazole, posaconazole and voriconazole) antifungal agents using EUCAST AFST method. The majority of strains demonstrated high MICs for itraconazole, indicating poor activity of this drug against *Fusarium* species. Our results also agreed with previous studies[24,25,50-54] that amphotericin B and voriconazole proved to be the most effective drugs *in vitro*, followed by posaconazole against *Fusarium* species. Overall, amphotericin showed significantly lower MICs than voriconazole.

In Chapter 17, ***In vitro combinations of natamycin with voriconazole, itraconazole and micafungin against clinical Fusarium strains causing keratitis*****,** we investigated whether combining these antifungals may result in synergy against *Fusarium in vitro* and whether such a combination may be potentially useful to improve the treatment outcome in the patients with keratitis. The *in vitro* interactions of combinations between natamycin and voriconazole, itraconazole, and micafungin applied to 20 clinical *Fusarium* isolates (members of *F. falciforme*, *F. napiforme*, *F. petroliphilum*, *F. proliferatum*, *F. pseudensiforme* and *F. sacchari* were evaluated using a checkerboard microdilution method. The results were analysed based on the FIC index model. The combination natamycin plus voriconazole was synergistic for 14 (70%) *Fusarium* strains (2 *F. proliferatum*, 2 *F. napiforme*, 4 *F. petroliphilum*, 2 *F. pseudensiforme* and 4 *F. falciforme*). The median (range) FIC_{min} was 0.2 (0.02 to 0.5). The effects of natamycin plus itraconazole combinations were synergistic for 3 (15%) *Fusarium* strains (2 *F. proliferatum,* 1 *F. petroliphilum*). Additive interactions were found for the other strains (FIC_{min} 0.5−1). On the other hand, the combination natamycin plus micafungin demonstrated synergism for one *Fusarium* strain (*F. proliferatum*) (5%). For the remaining strains additive interactions were found. No antagonism was observed for any combination.

In Chapter 18, ***Comparative evaluation of Etest, EUCAST and CLSI methods for amphotericin B, voriconazole and posaconazole against clinically relevant Fusarium species***, the performance of the Etest for amphotericin B, voriconazole and posaconazole susceptibility testing of 20 isolates of *Fusarium* was assessed in comparison with microdilution methods.

CLSI and EUCAST employed RPMI 1640 broth medium, and MICs were read after incubation for 48 h at 35 °C. Etest was performed as recommended by the manufacturer with RPMI 1640 medium with 2% glucose and incubated for 48 h at 35 °C. The Etest amphotericin B and posaconazole MICs were 1 dilution higher than the EUCAST and the CLSI MICs. The Etest was in better agreement with the EUCAST compared to the CLSI method. The essential agreement (within ±1/±2 dilutions) was higher between the Etest and EUCAST (60/90%, 80/95%, 70/85%) than CLSI (80/95%, 75/95%, 45/100%) for amphotericin B, voriconazole and posaconazole, respectively. Etest can be used for routine susceptibility testing of amphotericin B, voriconazole and posaconazole for *Fusarium* species.

Summary

In humans, *Fusarium* is the second most common life-threating filamentous fungal pathogen after *Aspergillus*, especially among immunocompromised patients. *Fusarium* species have recently been recognized as important and emerging opportunists and several *Fusarium* species have been described as novel human pathogens. The present thesis generated data on taxonomy, barcoding, diagnosis, clinical aspects, molecular epidemiology, *in vitro* antifungal susceptibility testing and combinations for agents of fusariosis. The results described in the thesis provide insights into major aspects of human fusariosis. The molecular and phylogenetic studies on the causative agents have broadened our knowledge on the diversity of the *Fusarium* species involved. Contribution towards improving the taxonomy of the genus *Fusarium* with accent on clinically relevant species was one of the main aims of this thesis. This was performed by using multi-gene analysis of *BT*, *TEF1*, *TOPO1*, *PGK*, *RPB2* and ITS. Our results also confirmed that *Fusarium* is monophyletic.

Phylogenetic analysis based on data obtained by different molecular approaches shows that human pathogens belong to 10 species complexes and that infection types may correlate with immune-status of patients. Infections may also occur in immunocompetent individuals. At this moment 74 taxonomic species have been suggested to cause human infections, judging from their isolation from clinical samples, and this number is expanding. The *F. solani* SC is the clinically most important *Fusarium* species complex, accounting for 40−60% of the reported cases of fusariosis, followed by the *F. oxysporum* SC and the *F. fujikuroi* SC.

Phylogenetic determinations based on gene sequences not only permitted the *Fusarium* identity to be clarified, but also provided a basis for species distinctions within the genus. The definitive identification tools are molecular methods which in general are time consuming and cost effective. To overcome these limitations MALDI-TOF MS is applied. The DNA sequence validated *Fusarium* strains were then subjected to MALDI-TOF MS in order to identify clinical *Fusarium* isolates. MALDI-TOF MS approach can be performed with minimal amounts of sample and takes only 15−30 minutes and are cheap. MALDI TOF MS is an important tool and is recommended for the identification of opportunistic *Fusarium* species, provided that sufficient databases are available.

Studies of local and global epidemiology of *Fusarium* species have been hampered by the lack of rapid, discriminatory and exchangeable typing methods. *Fusarium* species are frequently isolated from human infections. To investigate the epidemiological relation between environmental and clinical isolates, molecular typing techniques have been applied. AFLP fingerprinting has successfully been applied in this study with a large numbers of clinical and environmental strains. Our findings indicate that AFLP analysis and MLSA analysis provide high resolution data allowing discrimination between *Fusarium* species and genotypes. Both AFLP and MLSA typing are excellent techniques for analyzing the relatedness of *Fusarium*

species. However, each of the two techniques has advantages and disadvantages. Therefore, the choice should be based primarily on the specific goal for analyzing collections of isolates.

Treatment of fusariosis is a major challenge. After having established the taxonomy of *Fusarium* species with accurate diagnostic methods, the next step is appropriate therapy. *In vitro* antifungal testing was performed to investigate the differences in susceptibility between *Fusarium* species, showing the importance of a correct species or species complex identification for treatment. Based on the data generated in this thesis, amphotericin B and voriconazole were the antimycotics with the best overall *in vitro* activity, followed by posaconazole. Therefore, fusariosis remains difficult to treat, and new drugs with improved safety and efficacy are needed. In addition, susceptibilities of common as well as rare *Fusarium* species to drugs of different antifungal classes were established during this study. Antifungals belonging to the echinocandins have high MICs of >16 μg/ml against the majority of clinically related *Fusarium* species.

Considering the poor outcome obtained with monotherapy, attempts have been made to determine whether combinations of drugs lead to improved efficacy. Therefore, the *in vitro* combination activity of natamycin alone and in combination with voriconazole was determined for *Fusarium* keratitis. The results were analyzed based on the FIC index and showed that MICs of these compounds alone were >4 and 4−8 mg/L, respectively and that the combinations tested displayed (70%) *in vitro* synergistic effects against a significant number of isolates. However, there still is a lack of evidence to support treatment choices for disseminated fusariosis caused by multi-drug resistant *Fusarium*.

Future perspectives

At present, our experience with *Fusarium* taxonomy is that phylogenetic species concepts based on multilocus DNA datasets are generally unbiased and warrant a higher weight than classical concepts. In practice most researchers usually delimit species with the application of the phylogenetic concept and simply recognize distinct clades in the phylogenetic tree based on concatenated alignment as species without carefully examining species boundaries. In the future, we can expect *Fusarium* taxonomy to be the subject of more debate, especially with some recent modifications and transfer of two species complexes into different genera. We speculate that many differences between taxonomic entities in *Fusarium* have been overestimated, as they were based on small sample size and without careful examination of multiple characters. An important subject remains speciation in *Fusarium,* which can only be studied with larger sample sizes and a polyphasic approach. More molecular barcodes should be identified using whole genome sequencing, yielding additional genes that are as informative as *TEF1*, *RPB1 RPB2*, *TOPO1* and *PGK* to further improve *Fusarium* identification.

Opportunistic *Fusarium* pathogens have become a major concern to public health, mainly in immunocompromised patients. Therefore, further work on their ecology and evolution is of crucial importance, and more knowledge on primary niches, ecological preferences and factors involved in pathogenicity are needed to fully understand the ecology of *Fusarium*. Whole genome sequencing approaches and large-scale population studies will further clarify the complex interaction between the host's immune system and the invasive abilities of various cryptic species within *Fusarium*. Another clinically interesting research area is to determine whether *Fusarium* mycotoxins can be produced during human infection.

The need for standardized and clinically relevant antifungal susceptibility testing (AFST) of *Fusarium* became obvious as a results of an increase in the rate of infections and the number of available antifungal drugs to correlate the *in vitro* results with *in vivo* activity predicting the outcome of treatment. *In vitro* combination of antifungals is an area of AFST where more focus is needed, given the clinical implication of the results obtained from combination studies. In addition, experimental animal models of invasive and superficial fusariosis should be used to explore pharmacokinetic and pharmacodynamic properties of antifungals and to determine the dose-response relationship of antifungals.

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CHAPTER 20

Chapter 20

Appendix including: samenvatting, acknowledgments, curriculum vitae and list of publications

Nederlandse samenvatting

Na *Aspergillus* staan *Fusarium* infecties op de tweede plaats als meest voorkomende en levensbedreigende schimmel-infecties bij patiënten met een verlaagde afweer. *Fusarium* species zijn pas kort geleden erkend als één van de belangrijkste, sterk toenemende opportunistische schimmelgroepen. Verschillende *Fusarium* soorten zijn zelfs alleen maar bekend van de humane gastheer. In dit proefschrift zijn data verzameld op het gebied van de taxonomie, barcoding, herkenning, klinische aspecten, moleculaire epidemiologie, en *in vitro* antifungale gevoeligheid. De in dit proefschrift beschreven resultaten geven inzicht in een aantal belangrijke aspecten van humane fusariose. Moleculaire en fylogenetische studies van de verwekkers hebben geleid tot verbreding van de kennis over de diversiteit van pathogene fusaria. Meer in het algemeen zijn bijdragen geleverd aan een verbeterde taxonomie van het geslacht *Fusarium*, met nadruk op klinisch relevante soorten; dit was één van de hoofddoelstellingen van dit proefschrift. Het onderzoek werd uitgevoerd met behulp van genetische analyse van *BT, TEF1, TOPO1, PGK, rPB2* en ITS. Onze resultaten bevestigden dat het geslacht *Fusarium* monofyletisch is.

Uit de resultaten van onze fylogenetische analyses, gebaseerd op verschillende typen moleculaire data, blijkt dat er 10 soorten en soortscomplexen zijn die op de mens voorkomen, deels correlerend met de immuniteit van de patiënt. Infecties kunnen ook optreden bij immunocompetente personen. Tot op heden zijn 74 lineages (mogelijk taxonomische soorten) herkend die humane infecties zouden kunnen veroorzaken, te oordelen naar hun isolatie uit klinische monsters, en dit aantal is groeiende. Het *F. solani* SC is het klinisch meest belangrijke *Fusarium* species complex, goed voor 40–60% van de gemelde gevallen van fusariose, gevolgd door het *F. oxysporum* SC en het *F. fujikuroi* SC.

Fylogenetisch onderzoek met behulp van gensequenties helpt niet alleen om *Fusarium* te identificeren, maar verschaft ook een basis voor soorts-omgrenzing binnen het genus. Moleculaire methoden zijn in het algemeen tijdrovend en relatief kostbaar; deze nadelen zijn met MALDI-TOF MS grotendeels verholpen. *Fusarium* soorten zoals geclassificeerd op soortsniveau met DNA werden vervolgens onderzocht met MALDI-TOF MS om snellere diagnostiek te ontwikkelen die relevante data levert voor de clinicus – dat wil zeggen binnen een dag. MALDI-TOF MS kan worden uitgevoerd met minimale hoeveelheden monster, duurt slechts 15–30 minuten en is per test goedkoop. MALDI-TOF MS is een belangrijke ontwikkeling in de diagnostiek en zal worden aanbevolen voor de identificatie van opportunistische *Fusarium* species, zodra voldoende databases beschikbaar zijn.

Studies van lokale en globale epidemiologie van *Fusarium* soorten zijn moeilijk uitvoerbaar door een gebrek aan een snelle en reproduceerbare typeringsmethoden die tussen laboratoria kunnen worden gedeeld. Om de epidemiologische relatie tussen klinische isolaten en stammen uit de omgeving, de infectiebron, te onderzoeken, hebben we voor dit genus nieuwe typeringstechnieken gebruikt. AFLP fingerprinting is met succes toegepast op een groot

aantal klinische en omgevingsstammen. AFLP en MLSA analyses gaven een hoge resolutie, waarmee *Fusarium* soorten en genotypes konden worden onderscheiden. Ook individueel bleken AFLP en MLSA uitstekende technieken voor het analyseren van verwantschappen tussen *Fusarium* soorten. Echter, beide technieken hebben voor- en nadelen. Afhankelijk van het specifieke doel van de analyse wordt een techniek op de verzamelde isolaten toegepast.

Behandeling van fusariose blijft een grote uitdaging. Nu de taxonomie van veel *Fusarium* species is opgehelderd en accurate diagnostische methoden zijn ontwikkeld is de volgende stap het vinden van een geschikte therapie. Daartoe werd *in vitro* gevoeligheid voor antimycotica bepaald, om verschillen in behandelbaarheid tussen *Fusarium* soorten te onderzoeken. Op basis van de in dit proefschrift gegenereerde data waren amfotericine B en voriconazol de beste antimycotica met het breedste spectrum van *in vitro* activiteit, gevolgd door posaconazol – maar de gevoeligheid van *Fusarium* voor bijna alle middelen is lager dan de in meeste andere fungi. Daarom blijft fusariose moeilijk te behandelen, en nieuwe geneesmiddelen met verbeterde veiligheid en werkzaamheid blijven nodig.

Gezien het matige succes van monotherapie hebben we geprobeerd of een combinatie van geneesmiddelen tot betere effectiviteit zou kunnen leiden. Daartoe werd de *in vitro* activiteit van de antimycotica alleen en in combinatie onderzocht. Een succesvol model was de combinatie van voriconazol en natamycine voor *Fusarium* keratitis. Resultaten werden geanalyseerd op basis van de FIC index. Het bleek dat MICs van deze verbindingen afzonderlijk respectievelijk >4 en 4–8 mg / L waren, maar de geteste combinaties waren in 70% van de stammen synergistisch. Echter, er is klinisch onderzoek nodig om te bewijzen dat deze *in vitro* resultaten ook in de praktijk werkzaam blijken om fusariose door intrinsiek multiresistente *Fusarium* soorten te genezen.

Acknowledgments

Despite that this section is usually mentioned at the end, it is leading the completion of the PhD thesis, since the latter has been realized through the support of many people who contributed in different aspects for the initiation, performance and completion of these studies. Those people I would like to thank wholeheartedly here.

Prof. dr. Sybren de Hoog, promotor and supervisor of this thesis, without you this dissertation would not have been possible, which is obvious. Thank you for giving me the opportunity to do scientific research and to explore the field of mycology. Also thanks for accepting me as a PhD student in the Netherlands. Especially thank you for the way you have let me do this, with your advice and decisiveness, but also for the freedom and the trust you have given me to accomplish this. Thank you also for introducing a new style in my work, more organized and cooler, especially when problems occurred and deadlines were approaching and you reply to me 'Don't worry, everything can be solved'. For all this and for your support to obtain my PhD, beste Sybren, dank je wel.

Dr. Jacques F. G. M. Meis, co-promotor of this thesis, I would also like to thank you, without you the innovative part of this work was not possible. Thanks for the enthusiasm and the passion with which you let me meet with antifungal susceptibility testing and its applications at the Canisius Wilhelmina Hospital (CWZ) in Nijmegen. Also I would like to thank you for sharing with me your scientific experience and motivating me for greater efforts as well as for the critical comments in manuscripts even after they were submitted. I would like to thank you also for introducing me to CWZ and the opportunities to attend many scientific meetings and explore the scientific world with your valuable advice, and insightful discussions during the whole period of the study. Beste Jacques, dank je wel.

Many thanks to **Prof. dr. Steph Menken** for promoting this thesis at the University of Amsterdam and the nice environment that he made in all our meetings. Thanks for guidance through this project, which meant that I could make this successfully at the UvA. Beste Steph, dank je wel.

Dr. Ferry Hagen, co-author of papers of this thesis, I would like to thank you for your exciting ideas, teaching me AFLP and your critical comments in my manuscripts. Beste Ferry, dank je wel.

Dr. Benjamin Stielow, I would like to thank you for introducing the molecular biology world to me and offering a thoughtful guidance during my PhD project. Danke schoen, lieber Benjamin.

Dr. Anne-Cécile Normand, I would like to thank you for introducing me to the mass spectrometry (MALI TOF MS) and its applications. I also would like to express my sincere gratitude to **Professor Renaud Piarroux**, Head of Parasitology and Mycology at Assistance Publique-Hôpitaux de Marseille in Marseilles, while I was working on MALDI-TOF MS

project in France. I also would like to thank **Dr. Stephane Ranque** for interesting discussions that we had about MALDI-TOF MS. Merci Anne-Cécile, Stephane et Renaud.

Dr. Joseph Meletiadis, co-author of papers of this thesis, I would like to thank you for teaching me different drug combinations models and for your new ideas during our discussion and for your critical comments in my manuscripts. Dear Joseph,. ευχαριστω

I am very grateful to **Bert Gerrits van den Ende**, **Kasper Luijsterburg, Dr. Anne van Diepeningen** for their support especially during the first year of my PhD and their helpful technical assistance during my experiments. I really enjoyed working in the BYP group, thank you so much for everything. **Anne** is a co-author of papers of this thesis.

Ilse Curfs-Breuker, I would like to thank you for kind technical support and teaching me the principle of antifungal susceptibility testing and the knowledge that I gained from our unlimited discussion during my work at CWZ.

I would like to thank all my friends of the BYP group at the CBS for making my stay at CBS joyful and for the pleasant atmosphere created by you to complete this work. Thanks **Dr. Sarah, Dr. Kittipan, Karolina, Leandro, Balazs, Min Chen, Dr. Joanna, Dr. Azadeh, Mansoureh, Luana, Yanping, Leo, Mariana** and **Dr. Somayeh**. Also I would like to thank all the students and the guests whom performed either their work or internships in the BYP laboratories at CBS, we had nice moments and collaborations especially, **Dr. Peiying**, **Dr. Patricia, Dr. Burcu, Thomas, Kimberley, Domenico, Elenora, Engin and Cinzia**

I would like thank all our collaborators, those who provided us with *Fusarium* strains from all over the world especially **Prof. Bonifaz, Prof. Teun, Bart**, **Husam** and **Dr. Saad**

Many thanks to all the staff members of CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands for your great support during my study. I would like to express my deepest gratitude and appreciation to all staff members of the culture collection, because without you not only my project, but also all research at CBS would not have been possible. Special thanks to **Marlene Setropawiro, Manon Verweij**, **Rico Göbel**, **Dr. Alejandra Giraldo** and **Trix** for your entire predisposition to help me with anything that I asked.

I would like to thank **Ministry of Health** of Oman for the financial support (Formal Agreement no. 28/2014), also I am grateful to the staff members of **IBED** Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, the Netherlands, especially the management team for handling all my paper works at the UvA from the start to the end.

Finally, I would like to express my special appreciation to my dear father **Mohammed,** my dear mother **Moza** and my family for their support during this period. My deepest gratitude goes to my wife, **Fatima** for her understanding, patience and support. I have no words to thank my dear son, **Said**, I am very happy and glad with every progress you make with your mum during my PhD period.

Curriculum vitae

Abdullah M. S. Al-Hatmi was born on the 1st January 1977, in Al-Hajr, Ibri, Oman. From an early age, he had an interest in science and medicine. This led him to complete his BSc in Bio-medicine in 2006 at the University of Wales, Cardiff, the UK. During his BSc studies, he received a grant from Ministry of Health in Oman. In 2008, Abdullah started his MSc in medical and clinical microbiology at the Sultan Qaboos University, College of Medicine with a grant from the same University. In the last year of his MSc studies, he got another grant from Ministry of Health in Oman to do his master's research project at the University of Sydney working at the molecular mycology laboratory with Prof. Wieland Meyer. There Abdullah fell in love with molecular biology and fungi and he wished to know everything related to infections caused by nasty fungi. During his master, Abdullah also attended the medical mycology course done by CBS at the University of Peking in China where he met Prof. Dr. G.S. de Hoog. After the course, Abdullah understood that fungi can cause lots of different types of infections in human. In 2013, Abdullah received a joined grant from the Ministry of Health, Oman and IBED, the Netherlands and got admission for PhD with Prof. Dr. G S de Hoog and joined the Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam and CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands. His research focused on phylogeny, diagnostics and antifungal susceptibility of clinically relevant *Fusarium* species under supervision of Prof. Dr. G.S. de Hoog, Prof. Dr. S.B.J. Menken and Dr. Jacques F. G. M. Meis.

Courses and workshops

1. 28th Meeting of the *Fusarium* working group of the Koninklijke Nederlandse Planteziektenkundige Vereniging at CBS (October 2013).
2. *Fusarium* workshop, Kansas State University – USA, (June 2013).
3. Emerging Zygomycetes workshop, a new problem in the clinical lab, CBS-Utrecht, (April 2013).
4. ISHAM working group medical barcoding workshop, CBS-Utrecht (April 2013).
5. One Fungus: Which gene? Amsterdam, The Netherlands (April 2013).
6. Photoshop course, CBS-Utrecht (April 2013).
7. Introduction to phylogenetic analysis course in Wageningen University/ Current trends in phylogenetics, Wageningen (October 2013).
8. 28th Meeting of the *Fusarium* working group, India (January 2014).
9. 10th National conference of the society for Indian human and animal mycologists, SIHAM, India (January 2014).
10. Medical mycology course at the Institute Pasteur, Paris, (March 2014).
11. Diversity and barcoding of medical fungi: Novel achievement and masterclass (April 2014).
12. CBS Spring symposium: Genera and genomes (April 2014).
13. 28th Meeting of the *Fusarium* working group (October 2014).
14. Resistance and persistence in *Aspergillus fumigatus* masterclass (March 2015).
15. Diagnosis and management of fungal infections both in the West and the East, ESCMID postgraduate education course, Delhi, India (March 2015).
16. CBS-KNAW congress, The second international workshop on Ascomycete systematics, (April 2015).
17. Workshop neglected pathogens, CBS-KNAW (April 2015).
18. 19th Congress of ISHAM in Melbourne, Australia, (May 2015).
19. 7th Congress on Trends in Medical Mycology, Lisboa, Portugal (October 2015).
20. Transdisciplinary ECMM antifungal stewardship workshop, Wesel, Germany (January 2016).
21. The 7th Advances Against Aspergillosis international conference. Manchester, UK., (March 2016).
22. Fungi and Global Challenges, CBS-KNAW (April 2016).
23. The 1st ISHAM-Gilead forum on fungal infections in the Middle East (May 2016).
24. ESCMID ECDC Observerships, Stockholm, Sweden (September 2016).
25. Medical mycology course, CBS-Utrecht (October 2016)
26. Workshop on clinical aspects of dermatophytes (October 2016).
27. In Charge workshop (October 2016).

Membership

1. Young member of International Society of Human and Animal Mycology (ISHAM) since 2012.
2. Member of European Society of Clinical Microbiology and Infectious Diseases (ECCMID) since 2013.
3. Member of Royal Netherlands Society for Microbiology (KNVM) since 2013.
4. Member of research committee at the Ministry of Health (Oman) since 2011.

Awards and grants

1. Recipient of a PhD scholarship from IBED; Institute of Biodiversity and Ecosystem Dynamics, University of Amsterdam and Ministry of Health, Oman, January 2013.
2. Recipient of the Johanna Westerdijk travel grant, University of Utrecht (2013, 2014).
3. Recipient of the best poster prize for young ISHAM members, Melbourne, Australia, May 2015.
4. Recipient of a travel grant from the ISHAM for ISHAM meeting in Melbourne, Australia, May 2015.
5. Recipient of a travel grant from the ECCMID for participation in the molecular diagnostic pathogenic fungi, India, March 2015.
6. Recipient of a travel grant from the ECCMID for TIMM meeting in Lisbon, Portugal. October 2015.
7. Recipient of ESCMID ECDC observership visit to the European Centre for Disease Prevention and Control (ECDC), Sweden, September 2016.

List of publications

1. Kebabcı N, van Diepeningen AD, Ener B, Ersal T, Meijer M, **Al-Hatmi AM**, Ozkocaman V, Ursavaş A, Cetinoğlu ED, Akalın H (2014) Fatal breakthrough infection with *Fusarium andiyazi*: New multi-resistant aetiological agent cross-reacting with *Aspergillus* galactomannan enzyme immunoassay. Mycoses, 57: 249-255.
2. van Diepeningen AD, **Al-Hatmi AM**, Brankovics B, de Hoog GS (2014) Taxonomy and Clinical Spectra of *Fusarium* Species: Where Do We Stand in 2014? Curr Clin Micro Rpt, 1: 10–18.
3. **Al-Hatmi AM**, Bonifaz A, de Hoog GS, Vazquez-Maya L, Garcia-Carmona K, Meis JF, van Diepeningen AD (2014) Keratitis by *Fusarium temperatum*, a novel opportunist. BMC Infect Dis, 14: 588.
4. Ersal T, **Al-Hatmi AM**, Cilo BD, Curfs-Breuker I, Meis JF, Özkalemkaş F, Ener B, van Diepeningen AD (2015) Fatal Disseminated Infection with *Fusarium petroliphilum*. Mycopathologia, 179: 119-124.
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7. Yang Y, Chen M, Li Z, **Al-Hatmi AM**, Ye Q, Chen H, Wang S, Liao W, Wang J (2015) Genome sequence of *Penicillium capsulatum* strain ATCC 48735, a rare *Penicillium* species used in paper manufactories but that recently caused invasive infection. Genome Announc, 3(3): e00307-15. DOI: 10.1128/genomeA.00307-15.
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*Shared first authorship