PH.D. THESIS

INVESTIGATION AND CHARACTERIZATION OF *FUSARIUM* STRAINS ISOLATED FROM HUMAN MYCOTIC KERATITIS

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INTRODUCTION

The genus *Fusarium* is a large group of hyaline filamentous fungi. They are widely distributed in soil as harmless, saprophytic organisms. However, some members of this genus are capable of causing infection in plants, animals and humans. According to the latest surveys, *Fusarium* species are the most frequently isolated causative agents of fungal keratitis in South India. The members of the genus and especially the *Fusarium solani* species complex usually show poor susceptibility to clinically applied antifungal drugs, and the antifungal susceptibilities of different *Fusarium* species complexes vary. Thus, the management of *Fusarium* keratitis is still challenging. The misidentification of the causative agent and the subsequent application of an inappropriate antifungal therapy may even result in the loss of vision. Using molecular techniques in laboratory practice instead of conventional morphological methods can make the identification process faster and more accurate. New antifungals and alternative treatments would also be necessary to treat *Fusarium* infections.

An alternative way to cure these types of infections could be the use of alternative adjunctive therapeutic agents, such as essential oils. Essential oils or their constituents may be used as monotherapy, but in combination with conventional drugs a more improved antifungal efficacy could be reached. The antifungal effect of essential oils against filamentous fungi was previously reported in the literature, however the focus was mainly set on dermatophytes, phytopathogenic and postharvest pathogenic fungi and susceptibility data of human pathogenic fungal isolates and especially of *Fusarium* isolates are limited. Keratitis-associated *Fusarium* species have not been investigated yet in this respect.

We also have limited knowledge about the virulence factors of *Fusarium* species that contribute to their pathogenicity. Extracellular enzymes may play an important role in the infection facilitating tissue invasion.

OBJECTIVES

The main objectives of our work were:

- to identify *Fusarium* isolates derived from human keratitis using molecular methods and to investigate their phylogenetic relationships,
- to find a new, rapid and reliable identification method for the *F. solani* species complex, the taxon most commonly isolated from *Fusarium* keratitis cases,
- to determine the *in vitro* susceptibilities of clinical *Fusarium* isolates to commonly used antifungal agents,

- to study the *in vitro* inhibitory effects of essential oils on *Fusarium* species complexes isolated from human keratitis,
- to investigate the *in vitro* interactions between antifungal drugs as well as between antifungals and essential oil components,
- to examine and compare the extracellular enzyme activities of clinical and environmental isolates of the *F. solani* species complex.

METHODS

DNA based techniques:

- Purification of genomic DNA
- Agarose gel electrophoresis
- Polymerase Chain Reaction (PCR)
- DNA sequencing, sequence analysis
    - Nucleotide sequence analysis (BLAST)
    - Nucleotide and amino acid sequence alignment (ClustalX, BioEdit)
    - Phylogenetic analysis (PhyML, MrBayes)

***In vitro* antifungal susceptibility testing:**

- CLSI M38-A2 microdilution method
- Checkerboard titration method

Light- and fluorescent microscopy:

- FUN1 staining (FUN1 viability staining kit)
- Detection of apoptotic/necrotic events (Annexin V-FITC Apoptosis Detection Kit)

Extracellular enzyme activity testing:

- Keratinase activity testing in liquid media
- Casease, cellulase, elastase, lipase, phospholipase and pectinase activity testing in solid media

Analytical methods:

- Gas Chromatography-Mass Spectroscopy

RESULTS

1. Molecular identification and phylogenetic analysis of *Fusarium* isolates derived from human keratitis.

A total of 70 *Fusarium* isolates derived from human keratomycosis were identified based on the BLAST searches of the partial sequences of translation elongation factor 1α (*TEF1*) and β-tubulin (*TUB*) genes. The results of BLAST searches were confirmed by phylogenetic analysis using the same two sequences. These analyses generated a tree which can be separated into five well supported clades: Clade 1 included all the members of the FSSC, Clade 2 the *Fusarium dimerum* species complex, Clade 3 the *Fusarium fujikuroi* species complex, Clade 4 the *Fusarium oxysporum* species complex, and Clade 5 all the *Fusarium incarnatum-equiseti* species complex isolates. Our phylogenetic analysis revealed that one isolate identified previously as the member of the *F. oxysporum* species complex using BLAST search belongs to the *F. fujikuroi* species complex in fact. Thus the final results of the identification process were the following: 53 isolates confirmed as members of the *F. solani* species complex, 6-6 isolates belonged to the *F. dimerum* species complex and *F. fujikuroi* species complex, 3 isolates to the *F. oxysporum* species complex and 2 to the *F. incarnatum-equiseti* species complex. At species level 6 isolates were identified as *Fusarium delphinoides* (*F. dimerum* species complex) and 3 as *Fusarium napiforme* (*F. fujikuroi* species complex). To our knowledge, this is the first report in the world of *F. napiforme* as the causative agent of human keratomycosis.

2. A new, rapid and reliable identification method for the *F. solani* species complex.

A new, rapid method for the identification of the was developed based on our *in silico* observations that the recognition site of *Eco*RI restriction endonucelase is found only in the partial *TEF1* sequences of the strains belonging to the *F. solani* species complex. To prove this experimentally, the partial *TEF1* of 80 *Fusarium* isolates was digested by *Eco*RI, which resulted in the two expected fragments in case of all the *F. solani* species complex isolates. False negative or false positive results were not observed. We also compared the specificity of our method with a previously described *F. solani* species complex-specific PCR method: our method identified 100%, while the *F. solani* species complex-specific PCR identified only the 93.75% of the examined isolates correctly.

3. *In vitro* susceptibilities of clinical *Fusarium* isolates to commonly used antifungal agents.

In vitro antifungal susceptibilities of clinical isolates to seven commonly used antifungals (amphotericin B, clotrimazole, econazole, itraconazole, natamycin, terbinafine, and voriconazole) were determined by the broth microdilution method. Terbinafine, natamycin and amphotericin B were the most effective antifungal drugs against the majority of the investigated isolates. Seventy percent of the isolates were inhibited by natamycin in a concentration range which can be achieved in the eye during therapeutic conditions. The largest part of each species complex showed high minimal inhibitory concentration (MIC) values to clotrimazole, econazole and voriconazole in the examined concentration range. With one exception (a *F. solani* species complex strain) itraconazole proved to be ineffective against the investigated isolates. Similar results were found in the tests with itraconazole. No specific clades with differing antifungal susceptibility could be observed.

4. *In vitro* inhibitory effects of essential oils on *Fusarium* species complexes isolated from human keratitis.

To reveal the potential of essential oils as monotherapeutic agents against clinical *Fusarium* isolates, the MIC values of nine essential oils (cinnamon, juniper, lemon eucalyptus, lime, marjoram, sage, teatree, thyme, wintergreen) were determined using broth microdilution tests. The tested essential oils exhibited different anti-*Fusarium* activities. Cinnamon oil proved to be the most effective essential oil against all investigated *Fusarium* isolates. The main component of cinnamon oil, trans-cinnamaldehyde was also involved in these tests: it showed a similarly strong antifungal activity. The other essential oils exerted relatively lower antifungal activities. Lime oil proved to be the least effective against the investigated *Fusarium* isolates. The antifungal effect of the tested essential oils varied in a species complex-independent manner.

We also investigated the germination ability, metabolic activity and viability of *Fusarium* conidia in the presence of cinnamon oil and its main component trans-cinnamaldehyde using light and fluorescent microscopic techniques. Based on our observations, cinnamon oil and trans-cinnamaldehyde significantly decreased the metabolic activity, viability and inhibited the germination of *Fusarium* conidia.

5. *In vitro* interactions between antifungal drugs as well as between antifungals and essential oil components.

Because their high *in vitro* efficacy and their differing modes of action, drug interactions were investigated between natamycin and terbinafine in the case of 42 isolates using the checkerboard microdilution method. The combined application of these two drugs displayed either synergistic interactions (in 71.8% of the strains) or the two compounds did not interact with each other (28.2%). In the case of the examined members of the *F. oxysporum* and *F. incarnatum-equiseti* species complexes, only synergistic interactions were observed between natamycin and terbinafine. Antagonistic interactions were not revealed between the two compounds.

The *in vitro* combinations of natamycin and trans-cinnamaldehyde were also tested. In most of the cases, interactions were not observed between natamycin and trans-cinnamaldehyde. Synergistic interaction was revealed between the two compounds in one case only. Antagonism was not detected at all. Although the low level of synergism, we observed an enhanced fungal growth inhibition when these agents were applied in combination.

6. Extracellular enzyme activities of clinical and environmental isolated of the *F. solani* species complex.

The extracellular casease, cellulase, elastase, keratinase, lipase, phospholipase and pectinase activities of 67 *F. solani* species complex isolates (23 clinical isolates from India, 19 soil isolates from India, 11 plant isolates from India, and 14 plant isolates from different geographical regions of the world) were investigated. We observed a significantly higher keratinase and casease activity in the case of the clinical isolates compared to the plant isolates, while plant isolates showed significantly higher lipase activity than clinical and soil isolates.

SUMMARY

1. Our results confirmed that the members of the *F. solani* species complex are the most frequently isolated causative agents of fungal keratitis in South India.
2. We also report the first isolation of *F. napiforme* (*F. fujikuroi* species complex) from human keratomycosis.
3. A new method based on a specific *Eco*RI site in the *TEF1* gene was developed for the rapid identification of *F. solani* species complex.
4. The isolates showed relatively high MIC values to the examined agents.

5. Terbinafine, natamycin and amphotericin B proved to be the most effective drugs, followed by voriconazole.
6. In most of the cases, natamycin MICs were in the range of its effective concentrations, that can be reached in the eye during therapy.
7. Cinnamon oil showed outstanding activity against *Fusarium* strains.
8. The main component of cinnamon oil, trans-cinnamaldehyde showed similar effect as the oil.
9. The antifungal effect of the tested antifungal agents and essential oils varied in a species complex-independent manner.
10. In the interaction tests, mainly synergistic interactions were detected between natamycin and terbinafine, suggesting that this combination could be a promising base of an effective therapy in the treatment of *Fusarium* keratitis.
11. We observed an enhanced fungal growth inhibition when trans-cinnamaldehyde and natamicin were applied in combination.
12. The presence of cinnamon oil and trans-cinnamaldehyde significantly decreased the metabolic activity, viability and inhibited the germination of *Fusarium* conidia.
13. Enzyme activity tests revealed that isolates from plants had significantly higher lipase activity than clinical and soil isolates.
14. We observed a significantly higher keratinase and casease activity in the case of clinical isolates compared to those that were isolated from plants.

PUBLICATIONS

Publications in referred journals summarizing the results of this Ph.D. Thesis:

Homa M, Fekete IP, Böszörményi A, *et al*. 2015. Antifungal effect of essential oils against *Fusarium* keratitis isolates. *Planta Med* 81 (14): 1277-1284. (IF=1.99; Citation WOS/Scopus: 1/3)

Kredics L, Narendran V, Shobana CS, ..., **Homa M**, *et al*. 2015. Filamentous fungal infections of the cornea: A global overview of epidemiology and drug sensitivity. *Mycoses* 58 (4): 243-260. (IF=2.332; Citation WOS/Scopus: 8/10)

Homa M, Shobana CS, Singh YRB, *et al*. 2013. *Fusarium* keratitis in South India: causative agents, their antifungal susceptibilities and a rapid identification method for the *Fusarium solani* species complex. *Mycoses* 56 (5): 501-511. (IF=1.805; Citation WOS/Scopus: 13/14)

Congress abstracts summarizing the results of this Ph.D. Thesis:

Kredics L, **Homa M**, Baranyi N, *et al*. 2016. Environmental Ascomycetes in human corneal infections. In: Škrbić B (ed.) 18th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Book of Abstracts. Novi Sad: University of Novi Sad (ISBN:978-86-6253-059-2). *p. 23-24.*

Kredics L, Varga J, Anita R, ..., **Homa M**, *et al*. 2015. Newly identified causal agents of fungal keratitis. In: 6th Congress of European Microbiologists (FEMS 2015). *Paper FEMS-1317*.

Homa M, Fekete IP, Böszörményi A, *et al*. 2015. Antifungal effect of essential oils against clinically relevant fusaria. *Nat Volatiles & Essent Oils* 2 (3): 85.

Kredics L, **Homa M**, Baranyi N, *et al*. 2015. Widening spectrum of filamentous fungi causing mycotic keratitis. *Acta Microbiol Immunol Hung* 62 (Supplement 1): 172-173. (IF=0.568)

Homa M, Kredics L, Narendran V, *et al*. 2014. A newly identified causal agent of mycotic keratitis: *Fusarium napiforme.* http://www.tnoaabstracts.com/ViewFreePaperByNo.aspx?id=384.

Homa M, Singh YRB, Shobana CS, *et al*. *In vitro* antifungal effect of essential oils on *Fusarium* isolates derived from keratomycosis. In: 24th European Congress of Clinical Microbiology and Infectious Diseases (24thECCMID). *Paper R514.*

Homa M, Fekete IP, Singh YRB, *et al*. 2014. *In vitro* antifungal activity of essential oils against *Fusarium* spp. isolated from human keratitis. *Russian Journal of Infection and Immunity* Special Issue (Sept.): 38.

Homa M. 2014. Investigation of South-Indian *Fusarium* isolates from human keratitis. *Acta Biol Szeged* 58: 81.

Homa M, Randhir Babu Singh Y, Shobana CS, *et al*. 2014. Fluctuation in species diversity and antifungal susceptibility of *Fusarium* keratitis isolates over time. In: Cotoraci C, Ardelean A (ed.) 16th Danube-Kris-Mures-Tisa (DKMT) Euroregion Conference on Environment and Health: Book of Abstracts. Arad: "Vasile Goldis" University Press, 2014. (ISBN:978-973-664-707-9) *p. 25.*

Homa M, Singh YRB, Shobana CS, *et al*. 2014. Isolation and identification of *Fusarium* keratitis isolates. In: van Diepeningen A (ed.) Pre-Conference Workshop on *Fusarium*: Taxonomy, New Tools for Identification, Infection & Treatment. *p. 9.*

Homa M, Singh YRB, Shobana CS, *et al*. 2014. Fluctuation in species diversity and antifungal susceptibility. In: van Diepeningen A (ed.). Pre-Conference Workshop on *Fusarium*: Taxonomy, New Tools for Identification, Infection & Treatment. *p. 14.*

Homa M, Singh YRB, Selvam KP, *et al*. 2013. *Fusarium napiforme*, a new emerging pathogen from human keratomycosis. *Acta Microbiol Immunol Hung* 60 (Supplement): 149-150. (IF=0.780)

Galgóczy L, **Homa M**, Nagy GL, *et al*. 2013. *Fusarium* keratitis in South India: Identification, phylogeny, antifungal susceptibility and evaluation of a rapid identification method for the *Fusarium solani* species complex. *Acta Microbiol Immunol Hung* 60 (Supplement): 22. (IF=0.780)

Homa M, Shobana CS, Galgóczy L, *et al*. 2013. Investigation of enzymatic activity of environmental and clinical *Fusarium solani* isolates. Power of Microbes in Industry and Environment, Book of Abstracts. *p. 58.*

Homa M, Shobana CS, Kredics L, *et al*. 2012. Investigation of the enzyme activities of *Fusarium solani* isolates from plant and human infections. 14th DKMT, Szeged, Hungary, CD-ROM.

Homa M, Shobana CS, Galgóczy L, *et al*. 2012. Investigation of the lipase, elastase and keratinase activities of plant and human pathogen *Fusarium solani* isolates. 3rd Central

European Summer Course on Mycology: Biology of Pathogenic Fungi, Book of Abstracts. *p. 67.*

Galgóczy L, **Homa M**, Nagy GL, *et al*. 2012. *Fusarium* keratitis in South India: Identification, phylogeny, antifungal susceptibility and evaluation of a rapid identification method for the *Fusarium solani* species complex. In: A Magyar Mikrobiológiai Társaság 2012. évi Nagygyűlése, Book of Abstracts. *p. 13.*

Homa M, Shobana CS, Galgóczy L, *et al*. 2012. Molecular investigation of South-Indian *Fusarium* isolates from keratomycosis and their susceptibilities to conventional antifungal agents. *Mycoses* 55 (s4): 95-338. (IF=1.278)

Shobana CS, Galgóczy L, **Homa M**, *et al*. 2011. Molecular investigation of *Fusarium* isolates from keratomycosis. *Clin Microbiol Infec* 17 (Supplement s4): 463. (IF=4.540)

OTHER PUBLICATIONS:

Other publications in referred journals:

Galgóczy L, **Homa M**, Papp T, Manikandan P, Vágvölgyi C. 2016. *In vitro* antifungal activity of cysteine derivatives and their combinations with antifungal agents against clinically relevant *Scedosporium* species. *Int J Clin Med Microbiol* DOI: 10.15344/ijcmm/2016/111. (IF= 0; Citation WOS/Scopus: 0/0)

Homa M, Galgóczy L, Tóth E, *et al*. 2016. *In vitro* susceptibility of *Scedosporium* isolates to N-acetyl-L-cysteine alone and in combination with conventional antifungal agents. *Med Mycol* 54 (7): 776-779. (IF= 2.644; Citation WOS/Scopus: 0/0)

Hassan AS, Al-Hatmi AM, Shobana CS, ..., **Homa M**, *et al*. 2016. Antifungal susceptibility and phylogeny of opportunistic members of the genus *Fusarium* causing human keratomycosis in South India. *Med Mycol* 54 (3): 287-294. (IF= 2.644; Citation WOS/Scopus: 0/1)

Homa M, Galgóczy L, Tóth E, *et al*. 2015. *In vitro* antifungal activity of antipsychotic drugs and their combinations with conventional antifungals against *Scedosporium* and *Pseudallescheria* isolates. *Med Mycol* 53 (8): 890-895. (IF=2.644; Citation WOS/Scopus: 0/0)

Shobana CS, Mythili A, **Homa M**, *et al*. 2015. *In vitro* susceptibility of filamentous fungi from mycotic keratitis to azole drugs. J Mycol Med 25 (1): 44-49. (IF=0,756; Citation WOS/Scopus: 3/4)

Galgóczy L, Bácsi A, **Homa M**, Virágh M, Papp T, Vágvölgyi C. 2011. *In vitro* antifungal activity of phenothiazines and their combination with amphotericin B against different *Candida* species. Mycoses 54 (6):737-743. (IF=2,247; Citation WOS/Scopus: 1/5)

Other congress abstracts:

Homa M, Hegedűs K, Fülöp Á, *et al*. 2016. *In vitro* activity of diltiazem and verapamil hydrochloride with conventional antifungal agents against clinically important filamentous fungi. In: Škrbić B (ed.) 18th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Book of Abstracts. Novi Sad: University of Novi Sad (ISBN: 978-86-6253-059-2). *p. 65.*

Homa M, Galgóczy L, Vágvölgyi C, Papp T. 2015. Antifungal activity of non-antifungal drugs against *Scedosporium* and *Pseudallescheria* isolates. *Acta Microbiol Immunol Hung* 62 (Supplement 1): 31. (IF=0.568)

Homa M, Galgóczy L, Vágvölgyi C, Papp T. 2014. Antifungal activity of non-antifungal drugs against *Scedosporium* and *Pseudallescheria* isolates. In: A Magyar Mikrobiológiai Társaság 2014. évi Nagygyűlése és EU FP7 PROMISE Regional Meeting. *p. 22.*

Galgóczy L, **Homa M**, Vágvölgyi C, Papp T. 2014. Beyond the conventional antifungal agents: Antifungal activity of non-antifungal drugs against *Scedosporium* and *Pseudallescheria* isolates. In: de Hoog S, Meyer W, Lackner M, Tintelnot K (eds.) Diversity and Barcoding of Medical Fungi: Novel Achievements and Masterclass. A meeting of the ISHAM Working Groups on Barcoding and *Scedosporium*: Abstract and Programme book. *p. 16.*

Rónavári A, Balázs M, **Homa M**, *et al*. 2014. Investigation of the reactivity and the effect of different nanoscale zero valent iron on microbial populations in cis-1,2 dichloroethylene (cDCE) contaminated groundwater. In: Cotoraci C, Ardelean A (eds.) 16th Danube-Kris-Mures-Tisa (DKMT) Euroregion Conference on Environment and Health: Book of Abstracts. Arad: "Vasile Goldis" University Press, 2014. (ISBN: 978-973-664-707-9) *p. 63.*

Homa M, Szekeres A, Budai A, *et al*. 2014. A novel method for measurement of mycotoxins secreted by *Fusarium* species. In: Manikandan P, Shobana CS, Randhir Babu Singh Y,

Shafeeq Hassan A (eds.) Abstracts of the tenth annual meeting of the Society for Indian Human and Animal Mycologists. *p. 46.*

Randhir Babu Singh Y, Shobana CS, **Homa M**, *et al*. 2014. Epidemiology and antifungal susceptibility of *Fusarium* and *Aspergillus* causing keratitis infection in South Indian population. In: Manikandan P, Shobana CS, Randhir Babu Singh Y, Shafeeq Hassan A (eds.) Abstracts of the tenth annual meeting of the Society for Indian Human and Animal Mycologists; *p. 51.*

Virágh M, Kovács L, Pusztai Zs, **Homa M**, Galgóczy L, Vágvölgyi C. 2011. Isolation of thaumatin-like antimcrobial protein encoding genes in *Rhizopus* species. In: 2nd CEFSER workshop, Book of Abstracts. *p. 82.*

Galgóczy L, Bácsi A, **Homa M**, Virágh M, Papp T, Vágvölgyi C. 2010. *In vitro* interaction between phenothiazines and amphotericin B against different *Candida* species. In: Proceedings of the 11th International Symposium "Interdisciplinary Regional Research", CD-ROM.

Kovács L, Pusztai Zs, **Homa M**, Virágh M, Galgóczy L, Vágvölgyi C. 2010. Identification of thaumatin-like protein encoding genes in *Rhizomucor* isolates. In: Proceedings of the 11th International Symposium "Interdisciplinary Regional Research", CD-ROM.

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