PH.D. THESIS

CHARACTERIZATION AND GENETIC VARIABILITY TESTING OF AGRICULTURALLY AND CLINICALLY IMPORTANT AFLATOXIGENIC ASPERGILLI

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Introduction

Members of *Aspergillus* section *Flavi* have both clinical and agricultural importance. The significance of infections caused by *Aspergillus* species has increased in recent years among immunocompromised patients. *A. flavus* is the second most important causative agent of invasive aspergillosis after *A. fumigatus* and frequently causes cutaneous and wound aspergillosis. In tropical and subtropical developing countries Aspergilli are the second most common cause of mycotic keratitis. The main risk factor for infection is trauma by vegetable matter during agricultural activities. Among *Aspergillus* species, mainly *A. flavus*, *A. terreus*, *A. fumigatus* and *A. niger* have been isolated from fungal keratitis cases. As *Aspergillus* species could present very diverse antifungal susceptibility, to choose the appropriate clinical therapy, it is essential to identify Aspergilli by molecular methods.

In addition, *A. flavus* is a widespread pathogen of various economically important crops, including maize, cotton and peanut, and causes serious yield losses throughout the world. Furthermore, it is one of the major species of the *Aspergillus* genus being able to produce mycotoxins such as aflatoxins, which are produced by at least 20 species of the genus *Aspergillus*. Aflatoxins are classified as Group 1 carcinogens (carcinogenic to human) by the International Agency for Research on Cancer (IARC). Among the known aflatoxin forms (aflatoxins B_1, B_2, G_1, G_2, M_1), aflatoxin B_1 is the most toxic and potent hepatocarcinogenic natural compound ever characterized. Moisture and high temperature support aflatoxin production, therefore *A. flavus* produces aflatoxins in warmer climates, typically in tropical and subtropical regions. Recently, an alteration has been observed in the presence of aflatoxin producing fungi in several European countries. Climate changes and global warming may lead to the occurrence of aflatoxin producing fungi in countries with temperate climate. Moreover, in recent years, a series of aflatoxin contaminations has been observed in agricultural commodities, including maize and milk in several European countries.

Objectives

Since *Aspergillus* species have great influence in health and agriculture, we identified and examined the genetic variability and mating-type locus genes of potential aflatoxin producers (mainly *A. flavus* isolates) from different habitats using various techniques.

1. Collection of *Aspergillus* species with potential aflatoxin producing ability and genotypic analysis of the isolates.

Collection of clinical and environmental *Aspergillus* isolates from various geographic origin and sequence-based species identification of these isolates based on part of the calmodulin gene.

2. Examination the antifungal susceptibility of the clinical isolates.

Antifungal susceptibility testing of clinical isolates frem South India using Etest method.

3. Testing the aflatoxin producing abilities of the isolates.

Examination of aflatoxin production using thin layer chromatography and HPLC methods.

4. Investigation of the genetic variability of *A. flavus* isolates from different habitats using UP-PCR and microsatellite typing methods.

There are a lot of molecular typing methods which can help to identify the genotypic variations in Aspergilli. We have choosen the UP-PCR (Universally Primed PCR) and microsatellite length polymorphism analyses to investigate the genetic variability in *A. flavus* isolates deriving from different habitats.

5. Examination of the mating-type locus genes.

A. flavus is a heterothallic fungus, and its strains typically contain one of the two mating-type genes: *MAT1-1* or *MAT1-2*. We have examined the carried *MAT* idiomorphs with specific primers.

Methods

DNA based techniques:

- DNA extraction
- Purification of DNA fragments
- Agarose gel electrophoresis
- Polimerase chain reaction (PCR)
    - Genotypic analysis based on calmodulin gene
    - UP-PCR analysis
    - Microsatellite analysis
    - Examination of mating-type (MAT) genes
- DNA sequencing and analysis of sequence data

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

- E-test method

Aflatoxin producing ability testing:

- High Performance Liquid Chromatography (HPLC)
- Thin Layer Chromatography (TLC)

Genetic variability testing:

- UP-PCR analysis
- Microsatellite analysis

Phylogenetic analysis:

- Alignments (MAFFT v7.266, MEGA6)
- Phylogenetic trees (MAEGA6, RAxML)
- Cluster analysis was carried out using the PhylTools program for UP-PCR data
- Cluster analysis was carried out using the Microsatellite Toolkit program on microsatellite data

Results

1. Collection of *Aspergillus* species with potential aflatoxin producing ability and genotypic analysis of the isolates.

In this study, we have investigated 194 isolates deriving from South India, Croatia, Serbia, and Hungary which were identified as *A. flavus* based on their morphological characters. The species identification based on their calmodulin sequences proved that 182 isolates belonged to *A. flavus*, 5 to *A. tamarii*, 1 to *A. pseudotamarii* and 1 to *A. terreus,* which is not a member of section *Flavi*. It was the first occasion to detect *A. pseudotamarii* as the causative agent of keratitis (Baranyi *et al*. 2013a). *A. pseudotamarii* can only produce B-type aflatoxins similarly to *A. flavus*. In addition, we have identified one *A. minisclerotigenes* isolate from an infected coconut from South India. This species is able to produce both B- and G-type aflatoxins. Moreover, we have identified two *A. parasiticus* isolates from indoor air from Croatia, one *A. nomius* isolate from a Hungarian cheese product and one *A. pseudonomius* isolate from maize seed from Serbia. To our knowledge, we are the first who detected the Central European presence of the species able to produce G-type aflatoxins as well (Baranyi *et al*. 2015).

2. Examination of antifungal susceptibility in clinical isolates.

There are a lot of opportunistic human pathogenic species among the members of Aspergilli which could present very diverse antifungal susceptibility. We have tested an alternative procedure, E-test method instead of the conventional microdilution method. During the process, we have investigated the antifungal susceptibility of clinical strains, including 24 *A. flavus*, 4 A. *tamarii* and 1 *A. pseudotamarii* isolates, all isolated from keratitis cases in India. The susceptibility of the isolates to echinocandins (anidulafungin, micafungin and caspofungin), azoles (itraconazole, voriconazole, posaconazole and fluconazole) and amphotericin B was investigated. The examined isolates were almost equally susceptible to echinocandins; nevertheless, microcolonies were observed in inhibition zones. In the case of amphotericin B, the *A. flavus* isolates were less susceptible in comparison with the *A. tamarii* and *A. pseudotamarii* isolates. Fluconazole was not able to inhibit Aspergilli, while the other triazoles could efficiently block the growth of the *A. flavus*, A. *tamarii*, and *A. pseudotamarii* isolates. The most efficient ones were posaconazole and voriconazole. In accordance with earlier research in the subject, we have found that this method could be effective in choosing the appropriate clinical therapy.

3. Testing the aflatoxin producing ability of the isolates.

In addition to their clinical importance some members of section *Flavi* are able to produce aflatoxins. According to the examinations of the isolates for their aflatoxin producing ability, we observed differences between the populations: 47% of the clinical isolates and 50% of the environmental isolates from South India were able to synthesize aflatoxins. However, only three (one clinical and two maize isolates from Hungary) of the *A. flavus* isolates derived from Central Europe (Serbia, Croatia, and Hungary) showed aflatoxin producing ability. The *A. nomius*, *A. pseudonomius* and *A. parasiticus* isolates were able to produce aflatoxins in different ratios and amounts depending on the applied media and incubation temperatures. In recent years, although aflatoxins have been observed in some grain and milk products in Central European countries, our results show that most of the isolates examined by us does not produce aflatoxins. Further studies would be necessary to research the aflatoxin producing *A. flavus* strains both in Europe and in Hungary.

We have also examined the effects of media and temperatures on the aflatoxin production of three *A. flavus*, two *A. parasiticus*, one *A. nomius*, one *A. pseudonomius*, one *A. minisclerotigenes* and one *A. pseudotamarii* isolate. These strains were cultivated in YES media on 25, 30 and 35 °C temperatures, and most of the strains preferred 30 °C for aflatoxin production, except for the two *A. parasiticus* isolates. After cultivation in BHI and RPMI media, the isolates could produce only low amounts of aflatoxins in most cases, except for the *A. minisclerotigenes* and the *A. parasiticus* isolates.

4. Investigation of the genetic variability of *A. flavus* isolates from different habitats using UP-PCR and microsatellite typing methods.

Having investigated the genetic variability by microsatellite methods, we could not observe the complete separation of the isolates from different habitats. However, some isolates deriving from maize and indoor air formed separate clades, and the mixed occurrence of the clinical and environmental isolates from India were noticed. In the case of UP-PCR, 8 primers were used altogether and a binomial matrix consisting of 74 sites were analyzed using neighbor joining analysis. On the phylogenetic tree, the four populations formed different clades according to their geographical origin.

Data from the partial calmodulin gene sequences and UP-PCR and microsatellite analyses were combined to create a phylogenetic tree. The four populations separated to well-defined clades (although with low bootstrap values in most cases). Phylogenetic analysis showed that the isolates deriving from indoor air and from maize formed a well-defined cluster with a high support value, while those originating from environmental and clinical samples from India segregated from the European isolates.

5. Examination of the mating-type locus genes.

A. flavus was presumed to be strictly asexual; however, recently there have been a lot of evidence confirming the ability of sexual reproduction. *A. flavus* is a heterothallic fungus, and its strains typically contain one of the two mating-type genes: *MAT1-1* or *MAT1-2*. We amplified a 396 bp fragment of the *MAT1-1* gene and a 270 bp of the *MAT1-2* gene to identify the mating-type genes in the isolates. The dominant presence of the *MAT1-1* idiomorph was observed in the examined isolates, except for the clinical isolates deriving from Hungary, where the *MAT1-2* idiomorph dominated. Nine of the isolates carried both *MAT* allels, which may prove the presence of heterokaryosis as it was suggested in former studies.

Summary

1. In this study, we have identified 194 isolates of *Aspergillus* section *Flavi* from different habitats based on calmodulin gene sequences: 182 isolates belonged to *A. flavus*, 5 to *A. tamarii*, 1 to *A. pseudotamarii,* 1 to *A. minisclerotigenes*, 1 to *A. pseudonomius*, 1 to *A. nomius*, 2 to *A. parasiticus* species.
2. We are the first to detect the occurrence of *A. pseudotamarii* as the causative agent of keratitis, and the presence of *A. nomius* and *A. pseudonomius* in Central Europe.
3. Almost 50 % of the clinical and environmental strains from South India produced aflatoxins; however, only 3 strains from Europe have aflatoxin producing ability.
4. The *A. nomius*, *A. pseudonomius* and *A. parasiticus* isolates were able to produce aflatoxins in different ratios and amounts depending on the applied media and incubation temperatures.
5. In BHI and RPMI media, the isolates could only produce low amounts of aflatoxins in most cases, except for the *A. minisclerotigenes* and the *A. parasiticus* isolates.
6. The *A. flavus*, *A. tamarii* and *A. pseudotamarii* isolates have shown diverse susceptibility to the used antifungal agents; nevertheless, microcolonies were observed in inhibition zones.
7. In genetic variability testing, data from the partial calmodulin gene sequences and UP-PCR and microsatellite analyses were combined, and all the four examined populations separated to well-defined clades on the phylogenetic tree. The European isolates separated from those originated from India with high bootstrap values.

8. During the examination of mating-type genes, the dominant presence of the *MAT1-1* idiomorph was observed in the isolates, except for the clinical isolates deriving from Hungary, where the *MAT1-2* idiomorf dominated.
9. Nine of the isolates carried both *MAT* allels, which may prove the presence of heterokaryosis.

The results summarized in this Ph.D. thesis were published in the following articles

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