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Genotyping of clinical and environmental *Aspergillus flavus* isolates from Iran using microsatellites

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Summary

Aspergillus flavus is the second most important *Aspergillus* species causing human infections in tropical countries. Despite an increasing number of infections of *A. flavus* in Iran, the molecular epidemiology of clinical and environmental strains has not been well studied. We used a panel of nine microsatellite markers to analyse the genetic relatedness of *A. flavus*. Microsatellite typing of 143 (n = 119 clinical and n = 24 environmental) isolates demonstrated 118 different genotypes. A possible outbreak at a pulmonary ward was discovered. The discriminatory power for the individual markers ranged from 0.4812 to 0.9457 and the panel of all nine markers combined yielded a diversity index of 0.9948. This high-resolution typing method assists in better understanding of the molecular epidemiology of *A. flavus*.

Key words: *Aspergillus flavus*, microsatellite typing, Iran.

Introduction

Aspergillus flavus is a saprophytic soil fungus but also an opportunistic animal and human pathogen causing aspergillosis.[1] This species is the second most important *Aspergillus* species associated with colonisation, allergic symptoms and invasive disease. The incidence seems to be increasing in the immunocompromised population.[2] Like other *Aspergillus* species, *A. flavus* has a worldwide distribution although geographical factors and climatic conditions are important determinants of the local incidence of *A. flavus* infections.[3] This species is of major epidemiological importance in regions with a dry and hot climate.[4] For instance, in certain countries with semi-arid and arid dry weather conditions such as India, Iran, Saudi Arabia, Qatar and Sudan, *A. flavus* is the main aetiological agent in patients with invasive aspergillosis, fungal rhinosinusitis and keratitis.[4–8] Nonetheless, little information is available about the genetic typing of this fungus isolated from different sources in Iran.[9,10] Recently, different genotyping methods have been employed to understand colonisation patterns in patients and epidemiological relationships between environmental and clinical isolates.[11,12] Genotyping methods with high discriminatory power, an objective interpretation of the typing results and inter-laboratory reproducibility is required. Microsatellite analysis based on short tandem repeats (STR's) has these characteristics.[11] Molecular typing using microsatellites offers high discriminatory power and high reproducibility including easy exchange of results.[13,14] This study aimed at applying microsatellite typing to investigate the epidemiology of clinical and environmental *A. flavus* isolates from Iran.

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Materials and methods

Isolates

A total of 143 *A. flavus* isolates (clinical and environmental) were evaluated for their genetic relatedness. The isolates were maintained at the Invasive Fungi Research Centre (IFRC) collection, Sari, Iran. Stock cultures were inoculated on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) at 25 °C for 7 days to induce adequate sporulation. All strains were preliminarily identified as *A. flavus* by colony morphology and microscopic characteristics and confirmed by partial sequencing of the β-tubulin gene fragment.[15]

DNA isolation

Isolates were grown on Sabouraud dextrose agar plates at 30 °C until sporulation. For the isolation of DNA a sterile pre-wetted cotton swab was saturated with conidia and resuspended in a vial containing 350 µl lysis buffer and ceramic beads (Roche Diagnostics, Almere, The Netherlands) and subjected to mechanical lysis by using a MagNA Lyser instrument (Roche Diagnostics) for 30 s at 4500 g. After an inactivation-step for 15 min at 100 °C, 200 µl of the DNA was extracted and purified by using the MagNA Pure 96 platform (Roche Diagnostics). The yield and purity of the DNA were determined by UV absorbance measurements.

Microsatellite typing

The *A. flavus* isolates were genotyped by using a recently described microsatellite typing scheme that consists of three multiplex PCRs that target each three loci.[16] To discern the three loci within one multiplex PCR from each other the forward primers were labelled with FAM-, JOE- and HEX-fluorophores at the 5′-side. The PCR setup and programme were followed as described before.[16] After amplification, the PCR products were prepared for fragment analysis by diluting them 100-fold with ddH_2O, subsequently 1 µl of this diluted PCR product was added to 8.9 µl ddH_2O and 0.1 µl of CC-500-ROX marker (Promega, Leiden, The Netherlands). The samples were boiled for 1 min at 100 °C and after cooling down to room temperature the fragment sizes were determined by using an ABI3500xL Genetic Analyzer platform (Applied Biosystems, Foster City, CA, USA) as per manufacturer recommendations. The genetic relatedness between the *A. flavus* isolates was investigated by comparing the profiles with BioNumerics v6.6 software (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was performed by treating the data as categorical values followed by applying the UPGMA algorithm to generate the dendrogram, or the option to generate a minimum spanning tree directly from the categorical data. The discriminatory power of the microsatellite markers was calculated using Simpson's index of diversity (*D*) as described previously.[11] A '*D*' value of 1 indicates that all isolates are different, whereas a '*D*' value of 0 indicates that all isolates are identical.

Results

The collection of 143 isolates was analysed by using the complete panel of nine STR markers. An overview of the origin of isolates is depicted in Table 1. The Simpson's diversity index for the individual markers ranged from 0.4812 to 0.9457. The panel of all nine markers combined yielded a diversity index of 0.9948. The STR typing of 143 Iranian *A. flavus* isolates demonstrated 118 different genotypes. Among all genotypes, 102 genotypes were only found once and 21 clusters of related genotypes could be identified differing only at a single locus (Fig. 1). Analysis of the data showed that eleven genotypes were shared between two isolates, two genotypes were shared between three isolates and one genotype was shared by eight isolates. Thirteen genotype clusters contained only clinical isolates and one cluster only environmental isolates (Fig. 2). One hundred and seven patients were sampled once. Of these, 83 had a unique genotype. Six patients were sampled twice (on two different days) and in four patients with aspergillosis, the same

Table 1 Sources of *Aspergillus flavus* isolates recovered from clinical and environmental specimens.

Source (*n*)		No. of isolates (%)
Clinical samples (119)	Nose discharge	19 (15.9)
	Sputum	7 (5.8)
	Cutaneous (Nail, skin, liquid)	27 (22.6)
	BAL	18 (15.1)
	Sinus	39 (32.77)
	Biopsy	2 (1.6)
	Ear swabs	4 (3.36)
	Other	3 (2.5)
Environment samples (24)	Outdoor air	2 (8.3)
	Hospital air	12 (50)
	Hospital soil	1 (4.1)
	Hospital surface	9 (37.5)

BAL, broncho alveolar lavage.

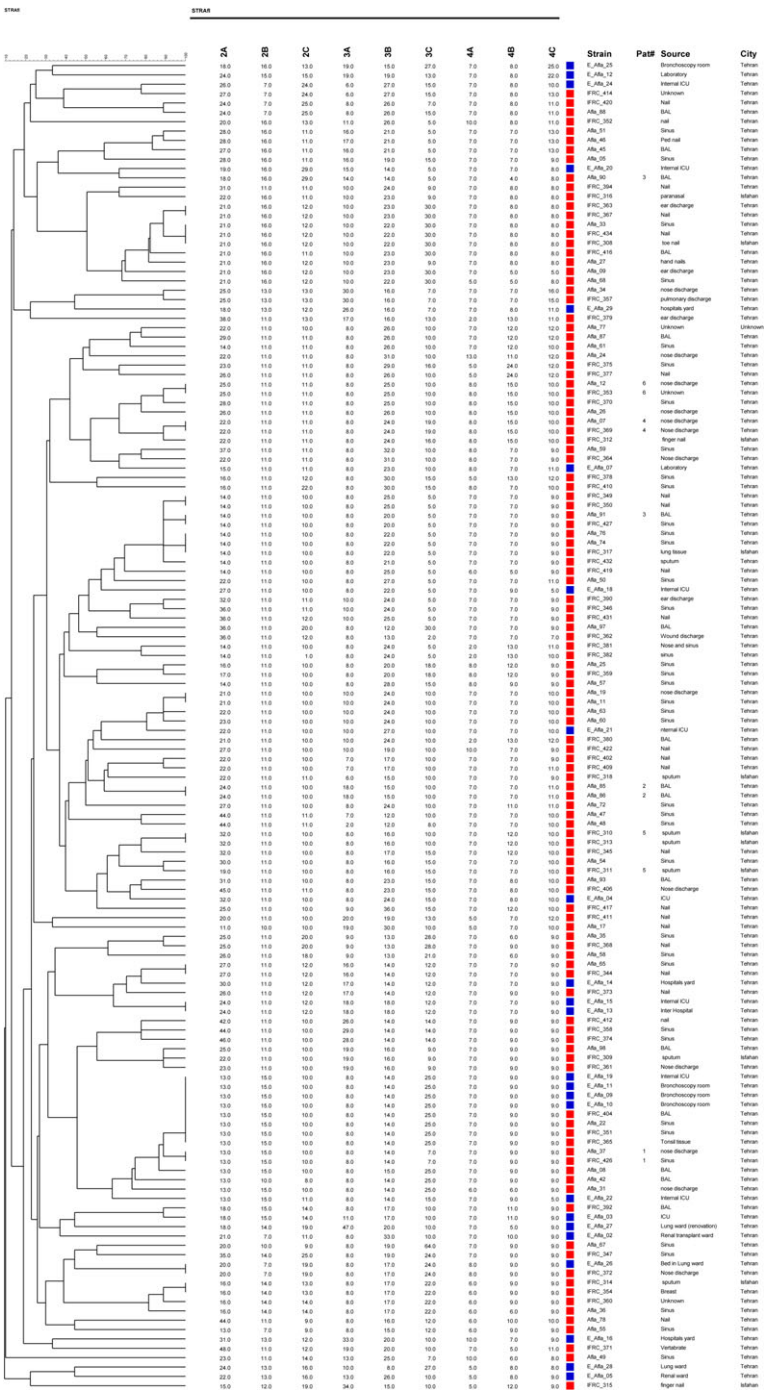

Figure 1 Dendrogram based on profiles of nine STR markers from 143 *Aspergillus flavus* isolates. The dendrogram is based on a categorical analysis of nine microsatellite markers in combination with UPGMA clustering. The scale bar above the dendrogram indicates the percentage identity between the genotypes. Red colour representing clinical isolates and blue colour representing environmental isolates.

genotype was found in clinical samples and two patients were infected/colonised by different genotypes. Four isolates recovered from clinical samples from four patients and another four environmental isolates were of the same genotype. Also in one case, an identical clinical isolate and an environmental isolate was found. The high genetic diversity of Iranian *A. flavus* isolates with isolates from other countries was also

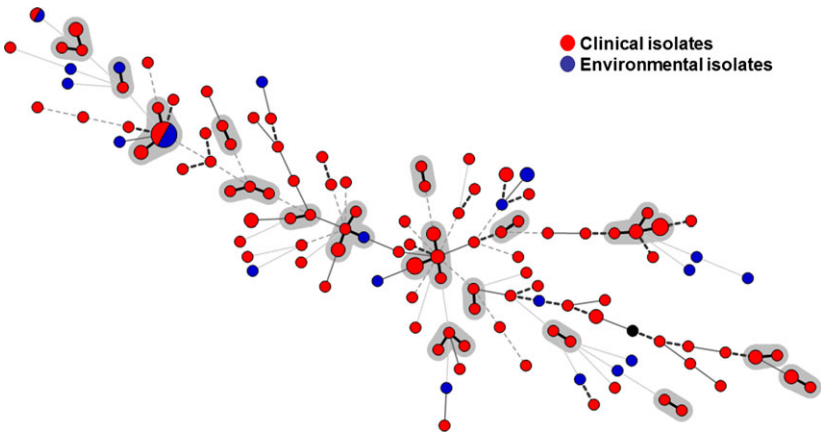

Figure 2 Minimum spanning tree (MST) representing the genotypic diversity of *Aspergillus flavus* isolates from Iran. MST representing the genotypic diversity of 143 clinical and environmental *A. flavus* isolates and a reference isolate using microsatellite typing. The number of allelic mismatches among STR profiles was used as distance. Each circle represents a unique genotype (Gt). The size of the circle is correlated with the number of isolates possessing the corresponding Gt. Dark, dashed and thin connecting bars corresponds to one, two or >2 different markers observed between linked Gt. Gts with a shaded background contain a minimum of two isolates that differ maximum in one microsatellite marker as the possible result of microevolutionary events and are likely to be clonally related. The black genotype is from the reference strain *A. flavus.*

observed in STR typing. Two clinical isolates from Iran were identical to genotypes found in clinical isolates from the Netherlands and Indonesia. Moreover, an isolate from India and a clinical isolate from Iran were in the same clonal cluster (see the dual-coloured circles in Fig. 3).

Discussion

Aspergillus flavus is the second common cause of invasive and non-invasive aspergillosis on a global scale.[3,4,17] Although not completely understood, the prevalence of *A. flavus* in the environment depends greatly on climatic conditions and this species is particularly prevalent in (sub)tropical countries.[4–7] Sino-orbital or cerebral aspergillosis, and endophthalmitis are emerging diseases and *A. flavus* has been described as the predominant aetiological agent of these infections.[18] In previous studies in Iran, multiple molecular methods have been developed to trace the spread of particular *A. flavus* strains.[9,10] To interpret nosocomial infections of *A. flavus*, discrimination between environmental and clinical strains is necessary for determining the epidemiology and the design of prevention strategies.[12] Several studies have shown that microsatellite typing of *Aspergillus* species has emerged as a reproducible, high discriminatory method with easy exchange of results between different laboratories.[11,12,19,20] Indeed, the employed panel of nine microsatellite markers showed 118 unique genotypes in the present collection of 143 Iranian *A. flavus*. Similar results have been obtained previously from India showing 124 distinct genotypes among 149 *A. flavus* isolates.[16] Likewise Hadrich *et al.* [2] using a combination of six microsatellite markers yielded 48 different genotypes with a 0.994 D value. In our study the combination of nine markers yielded a D value of 0.9948 indicative of the high discriminatory power. Since the genome of *Aspergillus* is haploid, the ability to identify multiple genotypes in a sample gives microsatellite analysis an incomparable advantage over other molecular typing methods like RAPD[10,21] or AFLP.[12] Although the number of environmental isolates was small, an interesting aspect of our study was that four clinical *A. flavus* isolates with four environmental hospital isolates were of the same genotype. Three isolates were recovered from air samples and surface swabs of a hospital bronchoscopy ward, suggesting a common environmental source in these patients. In this study clinical isolates had unique genotypes in the majority of patients. In two patients with aspergillosis, isolates with different genotypes were recovered on two different days. As mentioned above, most isolates were assumed to be unrelated. Therefore, the genetic heterogeneity of *A. flavus* isolates probably reflects the diversity of conidia because they were all collected from different patients either at different wards of hospital, or at different timepoints.[22] The high genetic diversity of *A. flavus* isolates from Iran is comparable to results described from India.[16]

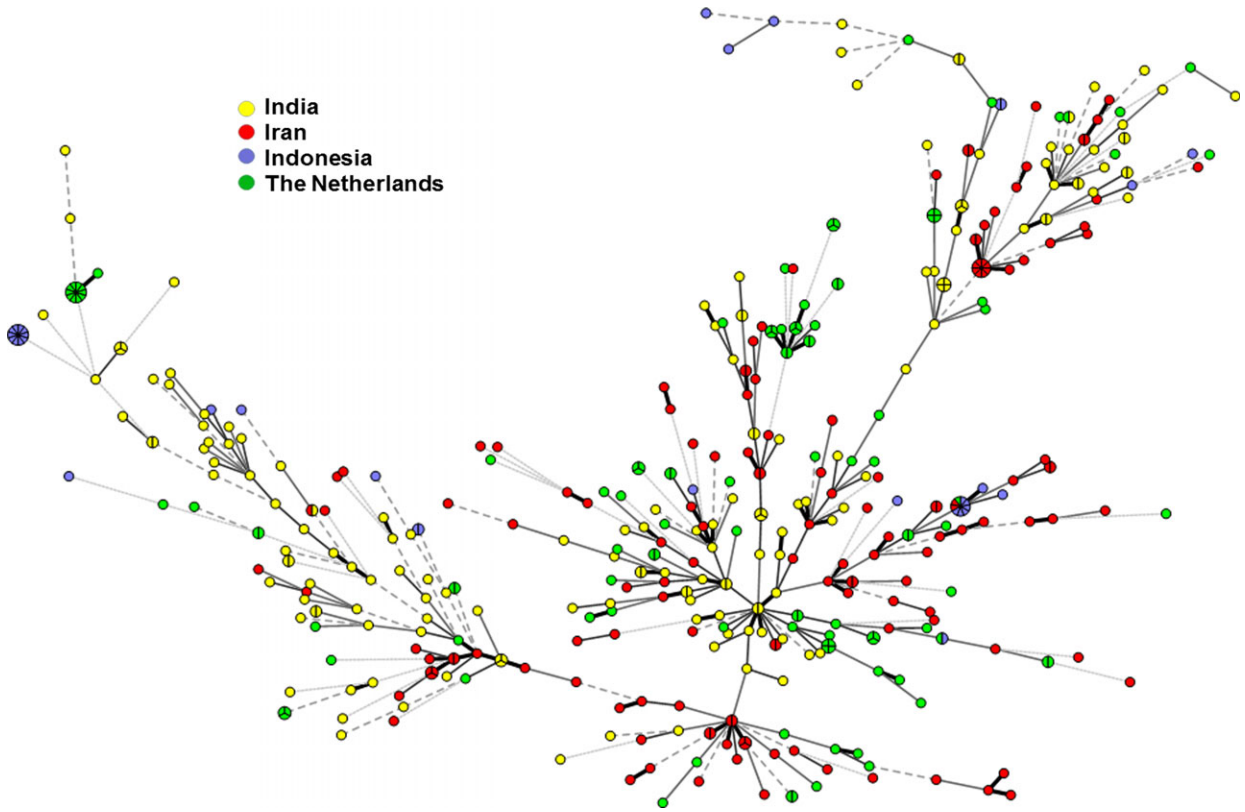

Figure 3 Minimum spanning tree representing the genotypic diversity of *Aspergillus flavus* isolates from Iran and those outside Iran. Circles in yellow indicate Indian isolates (n = 162), green indicating Dutch (n = 114), blue indicate isolates from Indonesia (n = 34) and red indicates Iranian (n = 143) isolates.

In conclusion, microsatellite typing provided excellent discriminatory power in studying the molecular epidemiology of clinical and environmental *A. flavus* isolates in Iran.

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Conflict 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, United Medical and Gilead. All other authors: no potential conflicts of interest.

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