Simple and Highly Discriminatory VNTR-Based Multiplex PCR for Tracing Sources of *Aspergillus flavus* Isolates

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Abstract

Aspergillus flavus is second only to *A. fumigatus* in causing invasive aspergillosis and it is the major agent responsible for fungal sinusitis, keratitis and endophthalmitis in many countries in the Middle East, Africa and Southeast Asia. Despite the growing challenge due to *A. flavus*, data on the molecular epidemiology of this fungus remain scarce. The objective of the present study was to develop a new typing method based on the detection of VNTR (Variable number tandem repeat) markers. Eight VNTR markers located on 6 different chromosomes (1, 2, 3, 5, 7 and 8) of *A. flavus* were selected, combined by pairs for multiplex amplifications and tested on 30 unrelated isolates and six reference strains. The Simpson index for individual markers ranged from 0.398 to 0.818. A combined loci index calculated with all the markers yielded an index of 0.998. The MLVA (Multiple Locus VNTR Analysis) technique proved to be specific and reproducible. In a second time, a total of 55 isolates from Chinese avian farms and from a Tunisian hospital have been evaluated. One major cluster of genotypes could be defined by using the graphing algorithm termed Minimum Spanning Tree. This cluster comprised most of the isolates collected in an avian farm in southern China. The MLVA technique should be considered as an excellent and cost-effective typing method that could be used in many laboratories without the need for sophisticated equipment.

Introduction

Members of the genus *Aspergillus* are ubiquitous filamentous fungi widely distributed in the environment. About 250 species have been described but only a few (about 20) have been reported to cause opportunistic infections in humans and animals. The most important pathogens in this genus are *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Emericella nidulans* (anamorph: *Aspergillus nidulans*) [1]. *Aspergillus* fungi release large amounts of airborne asexual spores (conidia). As a result of its ubiquitous presence, people and animals are constantly exposed to *Aspergillus* conidia, which can lead to colonisation, allergic manifestations or invasive infection depending on host immunity. Birds are considered to be much more susceptible than mammals [2]. Respiratory aspergillosis remains a major cause of morbidity and mortality in turkey poults in large confinement houses, quails, marine birds that are brought into rehabilitation, captive raptors, and penguins being maintained in zoological parks [3,4]. In Europe and North America, the majority of human and animal aspergillosis are caused by *Aspergillus fumigatus* [1,5,6]. The second most frequent pathogenic species is *A. flavus* [7,8]. In many countries from the Middle East, Africa and Southeast Asia however, *A. flavus* is the principal *Aspergillus* species causing human

invasive forms of aspergillosis but also sinusitis, keratitis, endophthalmitis and cutaneous infections [9–12]. In a recent epidemiological survey in avian farms in the South of China (Guangxi Province), we demonstrated that *A. flavus* was the major airborne filamentous fungus isolated from air, eggshells and the respiratory tract of birds. The species *A. flavus* is also known to cause environmental aflatoxin contamination, particularly in maize and peanuts, which leads to substantial economic loss worldwide [13].

Tracking the sources of contamination is essential to prevent *A. flavus* infection in humans or animals. For that purpose, molecular tools have been proposed [14] but the number of techniques validated for *A. flavus* is much lower than that currently used for *A. fumigatus* in hospitals or animal facilities.

The Multiple Locus Variable-number tandem-repeat Analysis (MLVA) is based on polymorphism of tandemly repeated genomic sequences called VNTR (Variable-Number Tandem-Repeats). VNTRs are classically separated into microsatellites (up to 8 bp) and minisatellites (9 bp and more) [15]. The MLVA technique has been used for the genotyping of many bacterial pathogens. It allows resolving closely related isolates for the investigation of disease outbreaks and provides information on the phylogenetic patterns among isolates [16–18]. The usefulness of MLVA was recently demonstrated for *A. fumigatus* [19]. The MLVA technique

can be performed with simple electrophoretic equipment and the judicious association of several VNTR primers in a single reaction tube may considerably reduce the number of PCRs required for a single isolate.

The objective of the present study was to develop a new typing method based on the detection of VNTRs in the filamentous fungus, *A. flavus*.

Materials and Methods

Origin of Fungal Isolates

In order to develop a scheme for MLVA and select discriminant VNTR markers, a total number of 36 *A. flavus* geographically or temporally unrelated isolates were selected. Thirty isolates were collected from the environment, from the pharynx or the respiratory tract of birds or from human cases of aspergillosis. Six reference strains (UMIP20.65, UMIP954.67, UMIP597.69, UMIP855.64, UMIP1145.76 and NRRL3357) were also included. To test the MLVA technique, a second group of 55 isolates was examined. These isolates represented distinct epidemiological situations: (i) 41 isolates from avian farms in Southern China and (ii) 14 isolates from human cases of invasive aspergillosis in Tunisia.

Aspergillus isolates were microscopically identified after cultivation on Malt Agar plates at 37°C until conidia formation. For all isolates, the species identification was confirmed by amplification and sequencing of partial *ß-tubulin* gene using primer set ßtub1 and ßtub2 [20,21].

DNA Isolation

For each isolate, conidia were collected from the pure culture and transferred into a microtube for extraction. DNA extraction was performed with QIAamp DNA miniKit (Qiagen) according to the manufacturor's instructions.

Selection of VNTR Markers

The availability of sequences of *A. flavus* (strain NRRL3357) (http://www.aspergillusflavus.org/) allowed us to identify exhaustively tandem-repeat sequences using the Tandem Repeat Finder on-line software (http://tandem.bu.edu/trf/trf.html) [22]. Loci with tandem repeats consisting of more than 20 nucleotides and more than 3 repeats were selected. Primers were designed using Primer Express® 2.0 software (Applied Biosystems). PCR were performed in a total volume of 15 µl containing 1–5 ng of DNA, 1x mixed buffer and 0.5 µM of each primer. The initial denaturation step at 95°C for 10 min was followed by 35 cycles consisting of denaturation at 95°C for 40 s, primer annealing at 61°C for 40 s, and elongation at 72°C for 40 s. The final extension step was at 72°C for 10 min. Six microliters of amplification product were loaded onto a 2% standard agarose gel. Gels stained with ethidium bromide were visualized under UV light, and photographed. The size marker used was a Quick-load 100-bp ladder (New England BioLabs, Ipswich UK).

Taking into account the maximum and minimum size of the repeats, combinations of VNTR markers were finally tested to obtain a multiplex technique with a clear and unambiguous separation of amplicons on agarose gels. All the combinations were tested by double, triple and fourfold PCR. The primer annealing temperature was the same (61°C) for all multiplex PCRs.

Specificity

To test the specificity of the MLVA technique, isolates from other *Aspergillus* species were also included: 4 reference strains of the *Flavi* section (*A. parasiticus* UMIP1142.76, *A. tamarii*

UMIP1017.70 and *A. oryzae* UMIP1042.72, UMIP1141.76), 3 strains of other sections of the genus *Aspergillus*: *A. fumigatus* (CBS 14489), *A. niger* (CBS 733.88) and *A. nidulans* (CBS 589.65).

Stability and Reproducibility

The stability of the VNTR markers was estimated by analysis of 5 distinct isolates of *A. flavus* subcultured 12 times in 2 months.

The reproducibility of the method was assessed by the analysis of 25 isolates in 2 different units situated in two different buildings of the Animal Health Laboratory of ANSES (Agence Nationale de Sécurité Sanitaire) at Maisons-Alfort, France, and by 2 different technicians.

Discriminatory Power

The discriminatory power was calculated by using the Simpson index of diversity [23].

Clustering Analysis

Amplicon size was determined with Quantity One software package version 4.6.9 (Bio-Rad Laboratories, USA). The number of repeats in each allele was derived from the amplicon size. The size of flanking sequences was subtracted from the band size and the number was divided by the repeats size. The result of this calculation corresponded to the number of repeats. Data were analyzed with Bionumerics software package version 6.5 (Applied-Maths, Saint-Martens-Latem, Belgium) as a character dataset. Two different techniques were used to represent the relationships between isolates [18]: a phenogram using phenetic UPGMA method and a graphing algorithm termed Minimum Spanning Tree (MST). The priority rule for constructing MST was set in order that the type that had the highest number of single-locus variants (SLVs) would be linked first. A cutoff value of maximum differences of one VNTR out of 8 was applied to define cluster in the MST method.

Results and Discussion

The use of the Tandem Repeat Finder software allowed the detection of 24 tandem-repeat loci (with a repeat unit larger than 20 bp) within the genome of *A. flavus*. Out of 24 tandem-repeat loci, 14 had a homology of more than 90% between the different repeats and a number of repeats higher than 3. Only 8 loci were finally deemed suitable for genotyping because they displayed variation (more than 3 alleles among the 36 tested isolates) and were present in all the isolates. Final markers were located on 6 different chromosomes (1, 2, 3, 5 7 and 8). Characteristics of final VNTRs and respective primer sets are listed in table 1. For multiple MLVA analysis, 4 pairs of markers were finally chosen: the combinations of AFL2-66 and AFL3-236, AFL1-75 and AFL5-66, AFL5-81 and AFL8-135, AFL7-57 and AFL7-78. These combinations allowed a clear and unambiguous separation of amplicons on agarose gels. Multiple (more than 2)-bands patterns were never detected.

When VNTR primer sets were tested with DNA from *A. fumigatus*, *A. niger* and *A. nidulans* no amplification was observed. On the contrary with DNA from *Aspergillus* species from the Flavi section, amplification was obtained with 4 to 5 out of 8 markers (AFL1-75, AFL2-66, AFL5-66 and AFL8-135 for *A. parasiticus* and *A. oryzae*; AFL1-75, AFL2-66, AFL3-236, AFL7-78 and AFL8-135 for *A. tamarii*). As a consequence, the observation of 8 amplicons following the combination of 8 VNTRs should be considered as specific of *A. flavus* and 3 markers (AFL1-75, AFL2-66 and AFL8-135) may be specific of the section *Flavi*.

Table 1. Characteristics of VNTR markers for fingerprinting of Aspergillus flavus.

VNTR markers	Primer sequences (5′ to 3′)	Unit repeat size (bp)	Range of repeat number	Simpson diversity index*	Marker location (non coding region or name of the gene if coding)
AFL1-75	GTCAGAGTGCTGTTGGGCG CGTCTCCCAGGCCGTTAGT	75	0–8	0.494	Chromosome 1, non coding
AFL2-66	CGCGAATGTCGATGATCACT AACAGGTAGGGCTGGGTTCC	66	3–6	0.618	Chromosome 2, GPI anchored protein, putative
AFL3-236	CAGAATTTTCAGTTAGCAAAGTGCTC TAAGACTTGGAGATATGTGACAAGGCTATA	236	1–3	0.541	Chromosome 3, non coding
AFL5-66	TCCACAGGCTGTATCGTTATCCT CAGTGACCCTTTCGGTGAAGAC	66	0–5	0.740	Chromosome 5, conserved hypothetical protein
AFL5-81	GGTTGCATCACAGTTATAGCGCT CCGGCACGACTGTGGAC	81	2–9	0.802	Chromosome 5, conserved hypothetical protein
AFL7-57	CACCGCAATGGAGCACAAG TGGTCGAGCTGTTCCTGGA	57	6–17	0.818	Chromosome 7, hypothetical protein
AFL7-78	GCTTCGTCATTGGCCCAT CTCGATCAATGTGTACTCATAAATGCT	78	2–6	0.655	Chromosome 7, conserved hypothetical protein
AFL8-135	GGTTTGCAGTGAGGATCTGCT GATGTGAGCCAGGCCATTG	135	0–3	0.398	Chromosome 8, conserved hypothetical protein

*Each index was calculated with the results from the 36 unrelated Aspergillus flavus isolates including 6 reference strains.
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The 35 samples (5 isolates subcultured 7 times in 2 months) used for the evaluation of stability were typed by MLVA and yielded exactly the same MLVA pattern for each isolate. The 50 samples used for the evaluation of reproducibility (25 isolates tested by 2 different technicians in 2 different laboratories) yielded exactly the same MLVA pattern.

Simpson diversity index was first calculated for each VNTR and for the panel of 8 markers tested on the 30 unrelated isolates and 6 reference strains. The index for individual markers ranged from 0.398 to 0.818. A combined loci index calculated with all of 8 markers yielded an index of 0.998.

A total number of 91 A. flavus isolates, including 6 reference strains were typed with the panel of 8 VNTRs. This analysis yielded 78 different genotypes, which corresponds to a combined loci index of 0.993. Among all genotypes, 71 were only found once. Five genotypes were shared by two isolates, one genotype was shared by three isolates and one genotype was shared by 7 isolates. Analysis of the details of those isolates in a single genotype revealed that 7 of them were isolated from eggshells in May and June 2010 in the same avian farm in China (farm A). Three isolates collected from eggshells and pharyngeal swabs (in laying hens) from the same farm shared a single but distinct genotype. Two isolates from another avian farm in China (farm B) shared a single genotype. The same situation was detected for two pairs of isolates collected in a hospital in Tunisia: one genotype corresponded to two isolates collected in bronchoalveolar lavages (from two different patients) and another one corresponded to two isolates collected from nasal swabs (from two distinct patients). More surprisingly, two unrelated isolates (one isolate from an avian farm in Nanning, China and the reference strain UMIP597.69) shared the same genotype. Although an identical genotype can occur by chance in unrelated isolates, a probable explanation for this result is that a contamination occurred during subculture.

UPGMA analysis did not allow a clear clustering of the isolates (data not shown) whereas the graphing algorithm termed Minimum Spanning Tree (MST) demonstrated one major cluster of isolates (figure 1). This cluster comprised 19 out of 32 isolates collected in the avian farm A in Guilin, China. Additional but smaller clusters could be defined for isolates collected in the hospital in Sfax or from avian farm B in China.

In a recent review, Hadrich et al. [14] listed the different molecular techniques, which have been tested so far for the typing of A. flavus or for the distinction between the clades or species within the Flavi section. Various DNA fingerprinting systems have been described such as restriction fragment length polymorphism (RFLP) [24–26], random amplified polymorphic DNA (RAPD) [27,28], amplified fragment length polymorphism (AFLP) [29] and, very recently, microsatellite length polymorphism (MLP) [30–
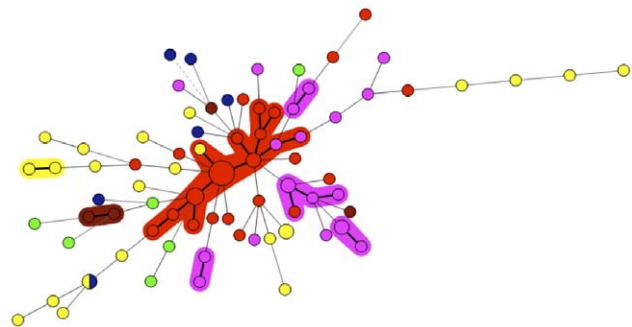

Figure 1. Minimum spanning tree of 91 A. flavus isolates based on categorical analysis of 8 VNTRs. Each circle represents a unique genotype. The diameter of each circle corresponds to the number of isolates with the same genotype. Genotypes connected by a shaded background differ by a maximum of one VNTR marker and could be considered a "clonal complex". Thick connecting lines represent one marker difference; regular connecting lines represent two or three marker differences; thin interrupted lines represent four or more differences. The length of each branch is also proportional to the number of difference. Each epidemiological situation is represented by a specific colour: red for 32 isolates collected in an avian farm (A) in Guilin, China; yellow for 23 isolates collected other avian farms (B, C, D) in Nanning, China; purple for 20 isolates collected in patients and in the hospital of Sfax, Tunisia; green for 6 isolates collected from a stock of mouldy peanuts in Benin; brown for 4 isolates collected from the fur of domestic carnivores in France and blue for 6 reference strains.
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32]. Most of these typing techniques were developed in order to resolve closely related isolates for the purposes of outbreak investigation in hospitals and disease surveillance in humans. In only one study [31], the genetic markers (24 microsatellite loci and the mating type locus) were used to assess population structure and potential gene flow among *A. flavus* vegetative compatibility groups in sympatric populations in Arizona and Texas. The RAPD method was used for *A. flavus* probably because it requires simple equipment and no genomic sequence information, but it suffered from limited discriminatory power and reproducibility. MLP typing methods were proved to be highly discriminant and reproducible. Hadrich *et al.* selected 12 microsatellite markers for the typing of 63 *A. flavus* isolates (15 from Marseille, France and 48 from Sfax, Tunisia, including some of the isolates we examined in the present study) [30]. The use of all the markers yielded 35 different genotypes with a diversity index of 0.970. A 5 markers combination yielded 27 different genotypes with a diversity index of 0.952. In 2011, Rudramurthy *et al.* developed a multicolor microsatellite panel for genotyping of *A. flavus* [32]. Nine microsatellite markers were finally used for the typing of 162 clinical isolates from India. The diversity index for the individual markers ranged from 0.657 to 0.954. The diversity index of the panel of nine markers combined was 0.997. In order to compare MLVA and microsatellite typing techniques, we examined 20 *A. flavus* isolates from patients from Sfax hospital. This preliminary analysis indicated that the MLVA method yielded a larger number of genotypes than the microsatellite technique proposed by Hadrich *et al.* [30] (17 MLVA patterns versus 11 microsatellite

profiles). Both techniques confirmed that iterative samplings on the same patients generally yielded to the isolation of different *A. flavus* genotypes. In further investigations a larger number of *A. flavus* isolates should be examined to obtain a precise comparison between MLVA and microsatellite typing techniques.

In conclusion, we developed a new molecular typing method for *A. flavus* based on the study of 8 VNTR markers. Size differences between alleles of the 8 selected VNTRs were large enough to allow a multiplex amplification and further efficient differentiating on agarose gel. This makes the present MLVA scheme easy to implement in laboratories with basic molecular biology equipment. The method showed a good reproducibility, which could be increased by the production of an internal ladder (including an example of each allele amplicon size). The MLVA was shown to be rapid and very discriminant. The VNTR patterns were incorporated in a specific database (http://minisatellites.u-psud.fr/MLVAnet/). On this website, it is now possible to compare *A. flavus* VNTR patterns using complete panel of 8 markers or just a selection of them. This database also allows to build dendrograms with the query [33].

Author Contributions

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