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Genotyping and Antifungal Susceptibility Testing of *Aspergillus flavus* Isolated from Clinical Specimens

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Abstract

Background: After *Aspergillus fumigatus*, *A. flavus* is the second leading cause of invasive and non-invasive aspergillosis. These fungi are of significant epidemiological importance in provinces with dry and hot climates.

Objectives: In the present study, antifungal susceptibility testing (AFST) and genotyping of sixty-five *A. flavus* clinical isolates originating from patients in Mazandaran and Tehran were performed.

Methods: Antifungal susceptibility testing of 65 clinical isolates of *A. flavus* was conducted against amphotericin B (AMB), itraconazole (ITR), voriconazole (VOR), posaconazole (POS), isavuconazole (ISA), luliconazole (LUL), lanoconazole (LAN), and 5-fluorocytosine according to the Clinical Laboratory Standards Institute (CLSI) method (M38-A2). The minimum inhibitory concentrations (MICs) were determined for each antifungal drug against all strains. Additionally, microsatellite typing using six variable number tandem repeat (VNTR) markers was performed to assess the genetic diversity and potential relationships among the clinical strains.

Results: Luliconazole had the lowest geometric mean MIC (0.020 µg/mL), followed by LAN (0.021 µg/mL), POS (0.089 µg/mL), ISA (0.115 µg/mL), ITR (0.220 µg/mL), VOR (0.244 µg/mL), AMB (0.870 µg/mL), and 5-fluorocytosine (58.76 µg/mL). Microsatellite typing revealed sixty-five distinct sequence genotypes. Statistically, there was no significant relationship between genotypes and AFST profiles ($P \geq 0.05$).

Conclusions: Luliconazole and lanoconazole demonstrated the greatest *in vitro* activity among all tested antifungals. However, most *A. flavus* strains exhibited reduced sensitivity to AMB. Microsatellite genotyping indicated no genetic similarity among the clinical strains, revealing high genetic diversity among *A. flavus* isolates obtained from clinical samples.

Keywords: *Aspergillus flavus*, Antifungal Susceptibility Testing, MLVA Genotyping

1. Background

The genus *Aspergillus* comprises more than 339 species of filamentous fungi, but only a few, including *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans*, are recognized as human pathogens (1, 2). Six species of the *Aspergillus* section *Flavi*, which are closely related both morphologically and phylogenetically, include *A. flavus*, *A. parasiticus*, *A. nomius*, *A. oryzae*, *A. sojae*, and *A. tamarii* (3, 4). The most common causative agent of aspergillosis is *A. fumigatus*; however, *A. flavus* is the second leading cause of invasive and non-invasive aspergillosis (5-9).

Aspergillus flavus can cause various clinical forms of infections, including otomycosis, keratitis, onychomycosis, sinusitis, bronchiectasis, and invasive aspergillosis (10). Whether specific isolates are preferentially associated with certain clinical manifestations of aspergillosis remains a critical unresolved question (11, 12). Molecular species typing methods, such as random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism

(AFLP), and multiple-locus variable number tandem repeat analysis (MLVA), are effective tools in microbiology laboratories and for monitoring hospital infections to determine genetic diversity (12, 13). The MLVA method identifies closely related strains for analyzing disease outbreaks and provides data on genetic diversity profiles (11, 14).

In recent years, antifungal agents such as itraconazole (ITR), amphotericin B (AMB), voriconazole (VOR), posaconazole (POS), isavuconazole (ISA), and caspofungin (CAS) have been approved for the treatment of invasive and non-invasive aspergillosis (8, 15). However, triazole resistance is increasingly reported in clinical and environmental isolates of Aspergillus species worldwide (16). Luliconazole (LUL) and lanoconazole (LAN) are two new imidazole antifungals with broad-spectrum activity against common human fungal pathogens, including *Malassezia* spp., *Trichophyton* spp., *Candida* spp., and *A. fumigatus* (15). These antifungals have been approved by the US Food and Drug Administration (FDA) for the topical treatment of dermatophytosis (17). Antifungal susceptibility testing (AFST) procedures, following the guidelines of the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI), are essential for detecting drug resistance and determining optimal therapies for invasive and non-invasive aspergillosis (8, 15).

2. Objectives

There is limited data on the genetic diversity and antifungal susceptibility profiles of clinical isolates of *A. flavus* from Iran. As a result the aim of the current study was to perform *in vitro* AFST and genotyping of clinical *A. flavus* strains using the MLVA method.

3. Methods

3.1. Sample Collection

This descriptive cross-sectional study was conducted over a period of 24 months (2021 - 2023). A total of 65 isolates of *A. flavus* were collected from clinical samples, including otomycosis (24 isolates, 36.92%), onychomycosis (17 isolates, 26.15%), bronchoalveolar lavage (BAL) (13 isolates, 20%), and sinus samples (11 isolates, 16.93%), from hospitals in Mazandaran and Tehran.

3.2. DNA Extraction

DNA extraction of *A. flavus* strains was performed using a glass bead and phenol:Chloroform:Isoamyl alcohol method (25:24:1, v/v) (Sigma-Aldrich, Germany). A fresh colony of *A. flavus* (2 - 5 days old, incubated at 35°C) was used. In each sterile microtube (1.5 mL), 200 microliters of lysis buffer (containing Triton X-100, 1% sodium dodecyl sulfate, 100 mM sodium chloride, 10 mM Tris-hydrochloric acid with pH = 8, 1 mM ethylenediaminetetraacetic acid (EDTA) with pH = 8) and 300 mg of glass beads (diameter 0.4 - 0.6 mm) were added.

A swab exposed to sterile distilled water was used to collect spores of *A. flavus*. The contents of the microtube were vortexed for 30 seconds and then incubated at 70°C for 30 minutes. Subsequently, 200 µL of the phenol:chloroform:isoamyl alcohol mixture was added. The microtube was incubated at 25°C for 5 minutes and centrifuged at 14,000 rpm for 10 minutes at 4°C. The optical density (OD) of the DNA samples (to measure the amount of DNA) was then determined using a spectrophotometric method. The extracted DNA was stored in a freezer at -20°C (18).

3.3. Strain Identification and PCR Sequencing

The strains were identified based on macroscopic and microscopic morphological features and confirmed through β-tubulin region sequence analysis, as described previously (19).

3.4. Multiple-Locus Variable Number Tandem Repeat Analysis Typing of Aspergillus flavus by Multiplex PCR Method

Multiple-locus variable number tandem repeat analysis typing was performed on all *A. flavus* strains using six VNTR markers (AFLA1, AFLA3, AFLA7, AFPM3, AFPM4, and AFPM7), as previously described by Hadrich et al. (11). The PCR amplification reaction, with a final volume of 25 µL, was prepared by combining 1 µL of extracted DNA, 1 µL of each primer at a concentration of 10 pmol/µL (Table 1), 12.5 µL of Taq DNA Polymerase 2x master mix red (Amplicon, Denmark), and 9.5 µL of distilled water.

The multiplex PCR reaction was carried out in a thermal cycler (Applied Biosystems Simpliamp, USA) following a specified thermal program: An initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 30

Table 1. Characteristics of the Six Variable Number of Tandem Repeats Loci in the *Aspergillus flavus* Isolates

VNTR Markers	Primer Sequences (5' to 3')	Repeat Unit	Range of Repeat Number	Fragment Size
AFLA1	F:CGTTGGCATGTTATCGTCAC	AC	14	180 - 290
	R:CTACTGAATGGCGGGACCTA			
AFLA3	F:CTGAAAGGGTAAGGGGAAGG	TAGG	11	164 - 272
	R:CACGCGAACTTATGGGACTT			
AFLA7	F:GCGGACACTGGATGAATAGC	TAG	13	121 - 293
	R:AACAAATCGGTGGTTGCTTC			
AFPM3	F:CCTTTCGCACTCCGAGAC	(AT)6AAGGGCG(GA)	10	188 - 274
	R:CACCACCAGTGATGAGGG			
AFPM4	F:AGCGATACAGTTTTAACACC	CA	7	184 - 210
	R:TCTTGCTATACATATCTTCACC			
AFPM7	F:TTGAGGCTGCTGTGGAACGC	AC	13	188 - 256
	R:CAAATACCAATTACGTCCAACAAGGG			

Abbreviations: VNTR, variable number of tandem repeats; F, forward; R, reverse.

seconds, with a final extension step at 72°C for 15 minutes. The PCR products were electrophoresed on a 1.5% agarose gel, and the resulting gel electrophoresis was photographed and recorded under UV light.

After identifying the VNTR loci of the *A. flavus* isolates, a dendrogram was created using PHYLOViZ version 2.0 software. The Simpson's Index of Diversity (SID) for each locus across all tested isolates was calculated using the Comparing Partitions online software (20).

3.5. Antifungal Susceptibility Testing

The broth microdilution method, as described by CLSI-M38A2, was used to determine the minimum inhibitory concentrations (MICs) of AMB, ITR, VOR, POS, ISA, luliconazole (LUL), and lanoconazole (LAN) (Sigma-Aldrich, USA) (21). The final concentrations of polyene and triazole antifungals were prepared in the range of 0.032 - 16 µg/mL. For flucytosine (5-FC), the concentration range was 0.125 - 64 µg/mL, and for LAN and LUL, it was 0.016 - 8 µg/mL.

Conidial suspensions were obtained from sporulated *A. flavus* grown on Sabouraud dextrose agar (SDA) culture (HiMedia, India). The turbidity of the conidia was adjusted spectrophotometrically to optical densities between 0.09 and 0.11 at 530 nm and diluted 1:50 in RPMI 1640 broth (Sigma-Aldrich, USA). In each well of a microplate, 100 µL of the final conidial suspension was added to 100 µL of each antifungal concentration.

The MIC was defined as the lowest drug concentration that inhibited growth by 100% after 48 hours, compared to the growth of the controls. *Candida parapsilosis* ATCC22019 and *C. krusei* ATCC6258 isolates were used as quality control strains (22).

4. Results

The mean age in the present study was 53.4 years (range: 27 - 79 years). Of the participants, 38 (58.46%) were male, and 27 (41.54%) were female. The β-tubulin gene sequences for all *A. flavus* strains were deposited in GenBank and registered under the National Center for Biotechnology Information (NCBI) accession numbers PQ415656, PQ415659–PQ415711 and PQ422102–PQ422113.

The results of AFST for eight antifungal drugs across all *A. flavus* strains are shown in Table 2. The geometric mean minimum inhibitory concentration (GM-MIC), from lowest to highest, was obtained as follows: LUL (0.020 µg/mL), LAN (0.021 µg/mL), POS (0.089 µg/mL), ISA (0.115 µg/mL), ITR (0.220 µg/mL), VOR (0.244 µg/mL), AMB (0.870 µg/mL), and 5-fluorocytosine (58.76 µg/mL) (Table 2).

Multiple-locus variable number tandem repeat analysis analysis performed with PHYLOViZ software identified 65 genotypes (sequence types). None of the *A. flavus* isolates were identical in terms of allelic profiles (Figure 1). Based on a cut-off value of 1.5, 19 clusters and 4 singleton were determined for the 65 clinical isolates of *A. flavus*. According to the goeBURST phylogenetic tree, out of the 65 isolates, only 5 (7.7%) differed in 2 loci, while the remaining isolates differed in at least 3 or 4 loci.

Simpson's VNTR diversity index indicated that the AFPM7 marker, with SID = 0.901, was the most effective

Table 2. MIC50, MIC90, Geometric Mean Minimum Inhibitory Concentration, and MIC Ranges Values of 65 Clinical *Aspergillus flavus* Isolates to Eight Antifungal Agents

Criteria	Antifungal Drugs							
	AMB	ITC	VRC	PSC	ISA	LUL	LAN	5 - FC
MIC50 (µg/mL)	1	0.25	0.25	0.063	0.125	0.016	0.016	64
MIC90 (µg/mL)	2	0.5	0.5	0.25	0.25	0.032	0.032	64
GM	0.870	0.220	0.244	0.089	0.115	0.020	0.021	58.766
MIC Range (µg/mL)	0.125 - 4	0.032 - 0.5	0.063 - 0.5	0.032 - 0.25	0.032 - 0.5	0.016 - 0.25	0.016 - 0.25	64.0 - 64.0

Abbreviations: AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; PSC, posaconazole; ISA, isavuconazole; LUL, luliconazole; Lan, lanoconazole; 5-FC, 5-flucytosine; MIC, minimum inhibitory concentration; GM, geometric mean.

marker for differentiating among all strains.

5. Discussion

Epidemiologically, the prevalence of *A. flavus* is more frequently reported in countries with dry and semi-arid climates, such as India, Iran, Saudi Arabia, Qatar, and Sudan (10, 15, 23, 24). A retrospective study in Iran showed that the prevalence of *A. flavus* exceeds that of other *Aspergillus* species (10). Our results indicated that the MIC50 of 65 isolates of *A. flavus* was as follows: Amphotericin B (1 µg/mL), ITR (0.25 µg/mL), VOR (0.25 µg/mL), POS (0.063 µg/mL), ISA (0.125 µg/mL), luliconazole (0.016 µg/mL), lanoconazole (0.016 µg/mL), and 5-fluocytosine (64 µg/mL). The *in vitro* AFST data indicated that all the tested antifungals demonstrated good activity, except for AMB and 5-fluocytosine.

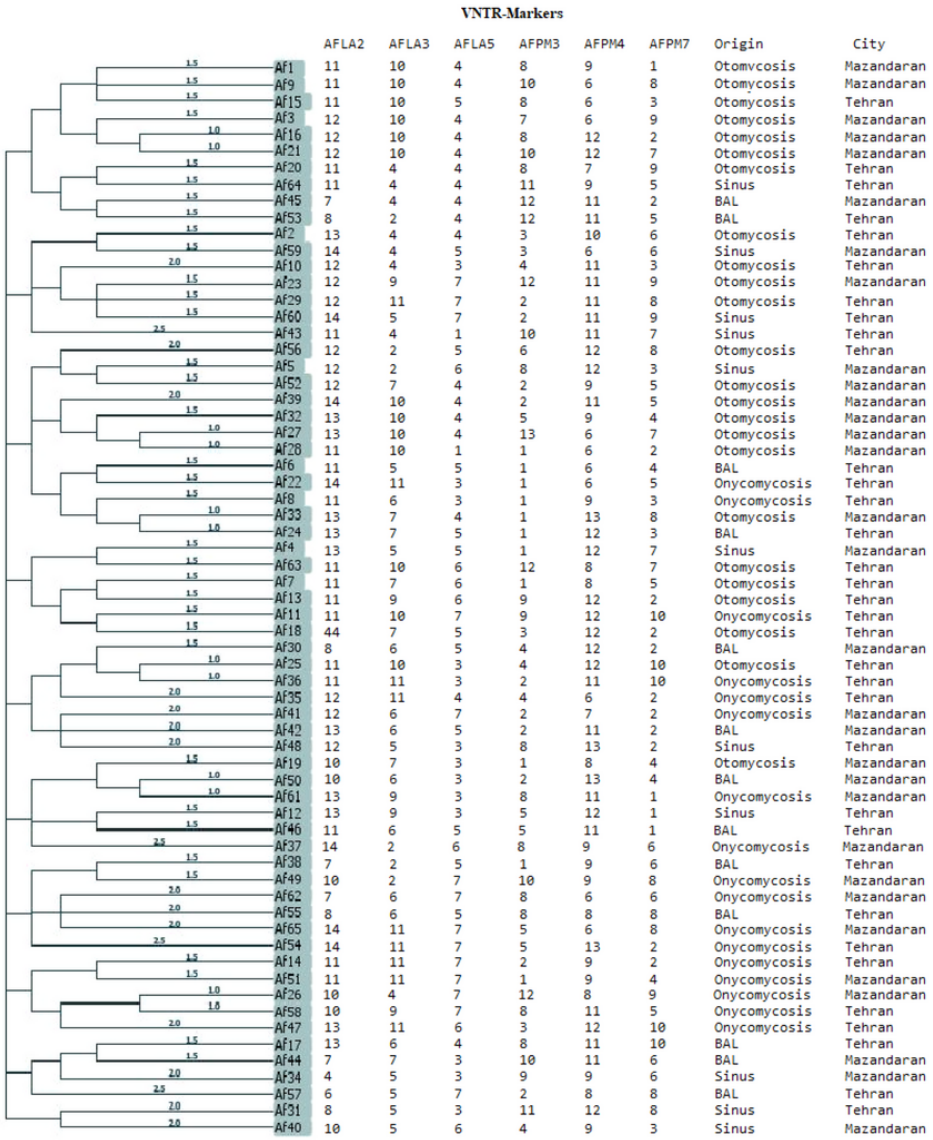
Gheith et al. reported MIC50 values for clinical isolates of *A. flavus* isolated from patients with hematologic malignancies in Tunisia as follows: Amphotericin B (6 µg/mL), ITR (0.5 µg/mL), VOR (0.19 µg/mL), POS (0.19 µg/mL), and CAS (0.64 µg/mL) (25). Pfaller et al. reported MIC50 values of ITR (0.5 µg/mL), POS (0.25 µg/mL), ravuconazole (0.5 µg/mL), and VOR (0.5 µg/mL) against 76 *A. flavus* isolates (26).

Shivaparkash et al. analyzed the AFST profiles of triazoles against 188 isolates of *A. flavus* collected from India using the CLSI method. Posaconazole exhibited the highest activity (GM MIC, 0.123 mg/L), followed by ITR (GM MIC, 0.177 mg/L), ISA (GM MIC, 0.697 mg/L), and VOR (GM MIC, 1.167 mg/L) (27). In the study by Vanathi et al., MICs against *A. flavus* were reported as follows: Amphotericin B (0.5 - 16 µg/mL), VOR (0.025 - 4 µg/mL), ITR (0.125 - 8 µg/mL), and POS (0.047 - 0.25 µg/mL) (28).

Although no drug susceptibility breakpoints exist for *A. flavus*, there is a consensus on the epidemiological cutoff values (ECVs) for *A. flavus* strains: Posaconazole 0.5 mg/L, ITR 1 mg/L, VOR 1 mg/L, ISA 1 mg/L, and AMB 4 mg/L (19). In the present study, all azoles tested showed good activity against all *A. flavus* strains, consistent with previous reports (29-32). Our AFST results indicated that luliconazole and lanoconazole demonstrated low MICs (GM = 0.020 µg/mL, with a range of MIC = 0.016 - 0.25 µg/mL) against all *A. flavus* strains. Similarly, in a study by Abastabar et al. (33), luliconazole and lanoconazole exhibited the lowest MICs against sensitive and resistant *A. fumigatus* isolates compared to those of other antifungal drugs. The analysis of our AFST data revealed that the GM MIC value of luliconazole was lower than that of lanoconazole against all tested strains.

Although no preparation for systemic administration of these antifungals is currently available, *in vivo* studies in animal models have demonstrated that these antifungals are highly effective for managing invasive aspergillosis compared to other drugs (34). Our results indicated that the MICs for AMB were higher than those for other antifungals, consistent with the study by Moslem and Zarei Mahmoudabadi, which reported MICs of AMB ≥ 8 µg/mL (35). These findings align with previous studies conducted in Europe (36, 37) and the Middle East (8, 38, 39). These differences may be attributed to variations in strains isolated from different specimens, the sample sizes of investigated strains, antifungal treatments, different AFST guidelines, and varying breakpoints applied for MIC determination.

In the present study, all clinical strains were found to be dissimilar, with distinctive genotype profiles. The strains were collected from different patients in two separate regions of Iran. Consistent with our findings, high genetic diversity in *A. flavus* has been observed in clinical isolates obtained from humans (9) and animal infections (40). Moreover, a prior study by Mohammadi et al. indicated that clinical and environmental *A. fumigatus* isolates clustered separately from each other (41). In line with the present study, Hadrich et al. used a



	AFLA2	AFLA3	AFLA5	AFPM3	AFPM4	AFPM7	Origin	City
Af1	11	10	4	8	9	1	Otomycosis	Mazandaran
Af9	11	10	4	10	6	8	Otomycosis	Mazandaran
Af15	11	10	5	8	6	3	Otomycosis	Tehran
Af3	12	10	4	7	6	9	Otomycosis	Mazandaran
Af16	12	10	4	8	12	2	Otomycosis	Mazandaran
Af21	12	10	4	10	12	7	Otomycosis	Mazandaran
Af20	11	4	4	8	7	9	Otomycosis	Tehran
Af64	11	4	4	11	9	5	Sinus	Tehran
Af45	7	4	4	12	11	2	BAL	Mazandaran
Af53	8	2	4	12	11	5	BAL	Tehran
Af2	13	4	4	3	10	6	Otomycosis	Tehran
Af59	14	4	5	3	6	6	Sinus	Mazandaran
Af10	12	4	3	4	11	3	Otomycosis	Tehran
Af23	12	9	7	12	11	9	Otomycosis	Mazandaran
Af29	12	11	7	2	11	8	Otomycosis	Tehran
Af60	14	5	7	2	11	9	Sinus	Tehran
Af43	11	4	1	10	11	7	Sinus	Tehran
Af56	12	2	5	6	12	8	Otomycosis	Tehran
Af5	12	2	6	8	12	3	Sinus	Mazandaran
Af52	12	7	4	2	9	5	Otomycosis	Mazandaran
Af39	14	10	4	2	11	5	Otomycosis	Mazandaran
Af32	13	10	4	5	9	4	Otomycosis	Mazandaran
Af27	13	10	4	13	6	7	Otomycosis	Mazandaran
Af28	11	10	1	1	6	2	Otomycosis	Mazandaran
Af6	11	5	5	1	6	4	BAL	Tehran
Af22	14	11	3	1	6	5	Onycomycosis	Tehran
Af8	11	6	3	1	9	3	Onycomycosis	Tehran
Af33	13	7	4	1	13	8	Otomycosis	Mazandaran
Af24	13	7	5	1	12	3	BAL	Tehran
Af4	13	5	5	1	12	7	Sinus	Mazandaran
Af63	11	10	6	12	8	7	Otomycosis	Tehran
Af7	11	7	6	1	8	5	Otomycosis	Tehran
Af13	11	9	6	9	12	2	Otomycosis	Tehran
Af11	11	10	7	9	12	10	Onycomycosis	Tehran
Af18	44	7	5	3	12	2	Otomycosis	Tehran
Af30	8	6	5	4	12	2	BAL	Mazandaran
Af25	11	10	3	4	12	10	Otomycosis	Tehran
Af36	11	11	3	2	11	10	Onycomycosis	Tehran
Af35	12	11	4	4	6	2	Onycomycosis	Tehran
Af41	12	6	7	2	7	2	Onycomycosis	Mazandaran
Af42	13	6	5	2	11	2	BAL	Mazandaran
Af48	12	5	3	8	13	2	Sinus	Tehran
Af19	10	7	3	1	8	4	Otomycosis	Mazandaran
Af50	10	6	3	2	13	4	BAL	Mazandaran
Af61	13	9	3	8	11	1	Onycomycosis	Mazandaran
Af12	13	9	3	5	12	1	Sinus	Tehran
Af46	11	6	5	5	11	1	BAL	Tehran
Af37	14	2	6	8	9	6	Onycomycosis	Mazandaran
Af38	7	2	5	1	9	6	BAL	Tehran
Af49	10	2	7	10	9	8	Onycomycosis	Mazandaran
Af62	7	6	7	8	6	6	Onycomycosis	Mazandaran
Af55	8	6	5	8	8	8	BAL	Tehran
Af65	14	11	7	5	6	8	Onycomycosis	Mazandaran
Af54	14	11	7	5	13	2	Onycomycosis	Tehran
Af14	11	11	7	2	9	2	Onycomycosis	Tehran
Af51	11	11	7	1	9	4	Onycomycosis	Mazandaran
Af26	10	4	7	12	8	9	Onycomycosis	Mazandaran
Af58	10	9	7	8	11	5	Onycomycosis	Tehran
Af47	13	11	6	3	12	10	Onycomycosis	Tehran
Af17	13	6	4	8	11	10	BAL	Tehran
Af44	7	7	3	10	11	6	BAL	Mazandaran
Af34	4	5	3	9	9	6	Sinus	Mazandaran
Af57	6	5	7	2	8	8	BAL	Tehran
Af31	8	5	3	11	12	8	Sinus	Tehran
Af40	10	5	6	4	9	3	Sinus	Mazandaran

Figure 1. Evolutionary phylogenetic tree creating from the analysis of six variable number tandem repeat (VNTR) marker gene loci of *Aspergillus flavus* isolates. In the tree, none of isolates has completely similar in Multiple locus variable-number tandem-repeat analysis (MVLA) patterns, as a result, these isolates are completely dissimilar.

suitable microsatellite marker for typing 63 isolates of *A. flavus*, employing a combination of 12 markers with a discriminatory power of 0.97, while a combination of 5 markers (AFM7, AFM3, AFLA7, AFLA3, AFLA1) showed a discriminatory power of 0.952 (42). Rudramurthy et al. genotyped 162 clinical isolates of *A. flavus* using 9 microsatellite markers, reporting a polymorphic rate of 33 alleles for these markers. The discriminatory power of each marker ranged from 0.954 to 0.657. Similar to the present study, their genotyping results did not show a significant relationship between the existing genotypes and different clinical forms (43).

Guarro et al., using the microsatellite technique for genotyping *Aspergillus* spp. from a hospital infection, reported 28 genotypes of *A. fumigatus* and 23 genotypes of *A. flavus* (44). Khodavaisy et al. reported the genotyping of 143 clinical and environmental isolates of *A. flavus* using nine microsatellite markers, identifying

118 different genotypes. The discriminatory power of these nine markers for all isolates ranged from 0.9457 to 0.4812 (9).

The differences between our results and those of other studies may be attributed to factors such as the type of strain, geographical region, source of samples, and the number and type of microsatellite markers used. A limitation of the present study is that AFST for echinocandin groups against *A. flavus* strains was not performed. Understanding the associations between the genotypes of strains and clinical disease (12)—which may vary across regions—and therapeutic modalities, including AFST patterns of causative agents against a panel of systemic drug compounds (45), is an important advantage for clinicians, mycology laboratories, and healthcare specialists. Such insights may help guide personalized treatment.

5.1. Conclusions

In conclusion, our results demonstrated that *A. flavus* isolates were highly sensitive to luliconazole, lanoconazole, and POS, whereas AMB did not exhibit strong activity against *A. flavus*. Typing of isolates collected from clinical samples revealed that *A. flavus* possesses a wide genetic diversity. The microsatellite typing method (MLVA assay) showed very high discriminatory power for studying the molecular epidemiology of clinical isolates of *A. flavus*. Additionally, no significant relationship was observed between the different genotypes of *A. flavus* and their AFST profiles.

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Footnotes

Authors' Contribution: All authors contributed to the study conception and design. Material preparation and data collection and analysis were performed by L. A. D., A. N. O., M. T. A., S. M. O. and S. M. H. K. The first draft of the manuscript was written by L. A. D. and M. T. A. All authors read and approved the final manuscript.

Conflict of Interests Statement: All authors approve of this article, and there are no conflicts of interest.

Data Availability: The dataset presented in the study is available upon request from the corresponding author during submission or after its publication. The data are not publicly available because they contain information that could compromise the privacy of the research participants.

Ethical Approval: The present study has been registered in the ethics committee of Islamic Azad University of Tonkabon (IR.IAU.TON.REC.1401.003).

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