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In vitro Antifungal Susceptibility Testing of Clinical and Environmental *Fusarium* Isolates in Iran

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Abstract

Background: Members of the genus *Fusarium* are common soil saprophytes and important plant pathogens which also cause a wide spectrum of diseases ranging from onychomycosis to the life - threatening systemic infections. The antifungal susceptibility patterns of *Fusarium* isolates varies in different species.
Objectives: This study was undertaken to investigate the antifungal susceptibility pattern of environmental and clinical *Fusarium* isolates to conventionally - used azole antifungal drugs in Iran.
Methods: A total of 36 *Fusarium* isolates (16 clinical and 20 environmental) were included in this study. All environmental isolates were obtained from the culture collection of medical mycology laboratory of the School of Public Health at Tehran University of Medical Sciences, Tehran, Iran. Clinical isolates were obtained from patients with onychomycosis and were identified by PCR - sequencing of a fragment of *translation elongation factor 1 alpha* gene. All clinical and environmental isolates were tested for their *in vitro* susceptibility to itraconazole (ITC) and voriconazole (VRC) according to the CLSI M38 - A2 standard. Statistical analysis of data was performed using SPSS version 21.
Results: The majority of clinical isolates were identified as *F. proliferatum* (N = 6) followed by *F. oxysporum* (N = 4), *F. solani* (N = 3), *F. verticillioides* (N = 1), *F. acutatum* (N = 1), and *F. thapsinum* (N = 1). The lowest minimum inhibitory concentration (MIC) values of ITC was observed for environmental *F. verticillioides* isolates (N = 5, GM = 13.93 μg/mL). For VRC, the lowest MICs were recorded for environmental *F. verticillioides* isolates (N = 5, GM = 2.3 μg/mL), and the highest MICs were observed for clinical *F. solani* isolates (N = 3, GM = 10.08 μg/mL). ITC was inactive against all clinical and the majority of environmental *Fusarium* isolates (MICs $\geq$ 16 μg/mL). Significant differences were observed between MICs of ITC and VRC against environmental *F. proliferatum* and *F. verticillioides* isolates ($P < 0.001$ for both). Similarly, the susceptibility of clinical *F. proliferatum*, *F. oxysporum* and *F. solani* isolates to VRC was significantly different ($P = 0.023$).
Conclusions: The resistance pattern in *Fusarium* isolates is species specific and, therefore, identification at the species level is important for choosing the proper antifungal treatment.

Keywords: *Fusarium*, Onychomycosis, Antifungal Agents, Iran

1. Background

Members of the genus *Fusarium* are ubiquitous common soil saprophytes and important plant pathogens and can cause fusariosis in humans and animals (1, 2). In humans, a range of diseases including superficial and cutaneous infections in healthy individuals, locally invasive or disseminated infections exclusively in immunocompromised patients, and allergic or invasive sinusitis have been reported as caused by *Fusarium* species (3-5). Onychomycosis is a cutaneous infection which is considered to be a major public health problem globally.

According to the literature (6, 7), *Fusarium* species are common causative agents of non - dermatophyte mould (NDM) onychomycosis. In addition, some species can produce mycotoxins such as zearalenone and fumonisin which have been shown to be associated with diseases in humans (8). Moreover, *Fusarium* species as plant pathogens cause diseases in agricultural products which result in significant economic loss (9). Moreover, consumption of these contaminated agricultural products can cause serious side effects in humans and animals (9).

In recent years, significant contamination of wheat and maize fields, particularly in humid and semi - humid areas of Iran, have been reported (10).

Extensive use of azole compounds in agriculture to prevent fungal contamination can develop drug - resistant isolates either in the patient or in the environment. *Fusarium* species currently show remarkable resistance to most currently-available antifungal agents. Studies have reported different susceptibility patterns within *Fusarium* species (11-13). Amphotericin B, Voriconazole (VRC), itraconazole (ITC) and various combinations of these have been reported with varying success rates against these species (14, 15).

Although, the distribution of *Fusarium* species varies globally, *Fusarium solani*, *F. oxysporum* and *F. verticillioides* are the most frequent causes of fusariosis (16, 17). Molecular phylogenetic studies have revealed that this genus comprises species complexes and the majority of *Fusarium* isolates cannot be identified to the species level using traditional morphological methods (4). For this reason, DNA sequence - based molecular tools are increasingly used for accurate and reliable species determination (18).

2. Objectives

The goal of the present study was to assess the susceptibility pattern of clinical and environmental *Fusarium* isolates to azole antifungal drugs conventionally used in Iran. In addition, clinical *Fusarium* isolates collected from patients with onychomycosis were identified using a sequence - based method.

3. Methods

3.1. Fungal Isolates

A total of 36 *Fusarium* isolates were included in this study. Among them, 20 environmental isolates (15 *F. proliferatum* and 5 *F. verticillioides* isolates) had been previously identified (19) and obtained from the culture collection of medical mycology laboratory of the School of Public Health at Tehran University of Medical Sciences in Tehran, Iran. The remaining 16 clinical isolates were collected from patients with onychomycosis who referred to the medical mycology laboratory of the School of Public Health at Tehran University of Medical Sciences in Tehran, Iran. All the clinical isolates were identified using the molecular method.

3.2. Molecular Identification

All 16 clinical isolates were cultured on sabouraud dextrose agar (SDA) medium plates (Merck, Germany) and incubated at 30°C for 2 - 4 days. The total genomic DNA was extracted using the glass - bead phenol chloroform method as described elsewhere (20). PCR was performed to amplify a fragment of *translation elongation factor 1 alpha (TEF1-α)* gene using the primer pair EF1 (5'-ATGGGTAAGGARGACAAGAC-3') and EF2 (5'-GGARGTACCAGTSATCATGTT-3'). The thermal cycling conditions were as follows: 5 min of initial pre - incubation at 94°C, followed by 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 58°C for 35 sec, and extension at 72°C for 1 min with a final extension at 72°C for 7 min.

The PCR products were electrophoresed on 1% (w/v) agarose gel and then were visualized under an UV illuminator. Afterwards, all PCR products were subjected to sequencing (Macrogen Inc., Korea). For species identification of the isolates, the *TEF1* - α sequences were compared to GenBank data using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Molecular Evolutionary Genetics Analysis (MEGA) software (version 6.0) was used for phylogenetic analysis of the sequences (21).

3.3. Antifungal Susceptibility Testing

All clinical and environmental isolates were tested for *in vitro* antifungal susceptibility according to the Clinical and Laboratory Standards Institute (CLSI) broth micro - dilution method (M38 - A2) (22). Stock solutions of the ITC (Sigma - Aldrich, USA) and VRC (Lyka, India) were prepared in DMSO (Sigma - Aldrich, Germany) and diluted using RPMI 1640 (Sigma - Aldrich, USA) medium buffered at pH 7.0 with morpholinepropanesulfonic acid (Sigma - Aldrich, Germany) according to the CLSI M38 - A2. The final concentrations tested ranged from 0.03125 to 16 μg/mL. The density of the conidia suspensions were spectrophotometrically adjusted to a final concentration of 0.4×10^4 to 5×10^4 CFU/mL according to CLSI M38 - A2 (22) and the minimum inhibitory concentration (MIC) values were determined after 48 h of incubation at 35°C. *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as quality control strains. All tests were performed in duplicate.

3.4. Ethical Approval

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Tehran University of Medical Sciences's human research committee (Ethics approval code: IR.TUMS.REC. 1394 - 763).

3.5. Statistical Analysis

Statistical analysis of data were performed using ANOVA and t-test in SPSS version 21 and P less than 0.05 was considered significant.

4. Results

4.1. Molecular Identification

The *TEF1* - α sequences of 16 clinical isolates (F1 - F16) were deposited into GenBank and received accession numbers. According to the results of the sequence analysis, the majority of clinical isolates were identified as *F. proliferatum* (N = 6) followed by *F. oxysporum* (N = 4). The detailed results of the molecular identification and the GenBank accession numbers are shown in Table 1. The phylogenetic tree of the 16 clinical *Fusarium* isolates (F1 - F16) and 15 GenBank sequences based on the partial *TEF1* - α gene is shown in Figure 1.

4.2. Antifungal Susceptibility Testing

The MICs of ITC and VRC for the quality control strains (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258) were within expected ranges (22); therefore, the results of antifungal susceptibility testing were reliable. According to the results of the present study, ITC was inactive against all clinical as well as the majority of environmental *Fusarium* isolates (MICs $\geq$ 16 μg/mL). The lowest MICs of ITC was observed for environmental *F. verticillioides* isolates (N = 5, GM = 13.93 μg/mL).

In comparison to ITC, better activity was obtained using VRC against all *Fusarium* isolates. The lowest MICs were recorded for the environmental *F. verticillioides* isolates (N = 5, GM = 2.3 μg/mL), and the highest MICs were observed for the clinical *F. solani* isolates (N = 3, GM = 10.08 μg/mL). Additional data on the antifungal susceptibility results are shown in Table 2.

4.3. Statistical Analysis

According to the results of statistical analysis, the MICs of the environmental *F. proliferatum* (N = 15) and *F. verticillioides* (N = 5) isolates for ITC and VRC were significantly different (P < 0.001 for both). Similarly, there were statistically significant differences between the MICs of the clinical *F. proliferatum* (N = 6), *F. oxysporum* (N = 4), and *F. solani* (N = 3) isolates for VRC (P = 0.023). However, the MICs of these species for ITC were not statistically different (P = 0.072). The source of isolates was a factor which affected the results of antifungal susceptibility testing of VRC. In this regard, the MICs of VRC between the environmental and clinical *F. proliferatum* isolates were significantly different (P = 0.02) while the same condition was not observed for ITC (P = 0.334).

5. Discussion

Fusarium species are plant pathogens distributed worldwide and cause diseases in many agriculturally important crops. The cause of the damaging effect of their presence as food contaminants is their production of highly toxic secondary metabolites (8, 10). In addition, *Fusarium* species cause a very wide spectrum of diseases in humans, ranging from superficial infections to disseminated infections with high morbidity and mortality rates (4). Onychomycosis is a common type of cutaneous infection affecting both the fingernails and toenails. *Fusarium* spp. have been reported frequently as common causative agents of NDM onychomycosis in Iran and other countries (6, 24-26).

Although conventional methods such as microscopic examination and culture are available in most laboratories for the diagnosis of onychomycosis, it is difficult to differentiate the *Fusarium* species on the basis of morphological features. Therefore, molecular methods have been developed for accurate and reliable identification of fungal pathogens to the species level (18, 27).

In this study, 16 clinical *Fusarium* isolates (F1 - F16) were identified using PCR - sequencing of the *TEF1* - α gene. The majority of isolates were found to be *F. proliferatum* and were similar to the environmental strains obtained from the culture collection. These results are indicative of the notable pathogenic potential as well as the increased prevalence of this species in the environment. Among the 16 clinical isolates, six distinct species were identified. Considering the different susceptibility patterns of the various species (11-13), the high diversity of clinical isolates in our study highlights the need for accurate identification of etiologic *Fusarium* species and antifungal susceptibility testing in order to prescribe efficient treatment.

Overall, *Fusarium* spp. show high MICs to available antifungal agents; hence choosing the optimal treatment strategy is a challenge (13, 27-29). According to the CLSI, filamentous fungi with MIC values of $\leq$ 8 μg/mL are categorized as "susceptible" (22). Based on this standard, ITC was inactive against all clinical and the majority of environmental *Fusarium* isolates. The results obtained in this study are in agreement with results of other studies that reported low activity of ITC against *Fusarium* species (13, 15, 30, 31). On the other hand, in the present study, almost all *Fusarium* species had high MICs above the published epidemiological cut - off values and were insensitive to ITC with MICs $\geq$ 16 μg/mL which can lead to treatment failure in patients infected with these strains.

Among the new triazoles, VRC is a recommended drug (32) with moderate antifungal activity (MIC values of 1 - 8 μg/mL) depending on the species complex (27, 33). In

Table 1. Demographic Data of Patients and Characteristics of 16 Clinical *Fusarium* Isolates Used for Antifungal Susceptibility Testing

Isolate number	Gender/Age	Isolation Source	Molecular Identification	GenBank Accession No.
F 1	Male/43	Nail scraping	*Fusarium solani*	KY801929
F 2	Female/62	Nail scraping	*Fusarium oxysporum*	KY801930
F 3	Male/30	Nail scraping	*Fusarium oxysporum*	KY801931
F 4	Female/54	Nail scraping	*Fusarium oxysporum*	KY801932
F 5	Female/45	Nail scraping	*Fusarium acutatum*	KY801933
F 6	Female/32	Nail scraping	*Fusarium proliferatum*	KY801934
F 7	Female/63	Nail scraping	*Fusarium proliferatum*	KY801935
F 8	Female/57	Nail scraping	*Fusarium solani*	KY801936
F 9	Female/33	Nail scraping	*Fusarium proliferatum*	KY801937
F 10	Female/62	Nail scraping	*Fusarium thapsinum*	KY801938
F 11	Male/65	Nail scraping	*Fusarium oxysporum*	KY801939
F12	Female/78	Nail scraping	*Fusarium proliferatum*	KY927140
F13	Female/64	Nail scraping	*Fusarium proliferatum*	KY927141
F14	Male/38	Nail scraping	*Fusarium proliferatum*	KY927142
F15	Male/53	Nail scraping	*Fusarium verticillioides*	KY962812
F16	Female/34	Nail scraping	*Fusarium solani*	KY962813

Table 2. The Results of Antifungal Susceptibility Testing of Iranian Clinical and Environmental *Fusarium* Isolates

Sample Type (N)	*Fusarium* Species	Antifungal Drugs (μg/mL)									
		Itraconazole					Voriconazole				
		MIC	Range	GM	MIC50	MIC90	MIC	Range	GM	MIC50	MIC90
Clinical (16)	*F. proliferatum* (6)	-	> 16	> 16	> 16	> 16	-	2 - 8	4	4	8
	F. oxysporum (4)	-	16 - > 16	> 16	16	> 16	-	2 - 8	4	4	8
	F. solani (3)	-	> 16	> 16	> 16	> 16	-	8 - 16	10.08	8	16
	F. verticillioides (1)	16	-	-	-	-	4	-	-	-	-
	F. acutatum (1)	> 16	-	-	-	-	4	-	-	-	-
	F. thapsinum (1)	> 16	-	-	-	-	2	-	-	-	-
Environmental (20)	*F. proliferatum* (15)	-	16 - > 16	>16	> 16	> 16	-	2 - 16	6.65	8	8
	F. verticillioides (5)	-	8 - 16	13.93	16	16	-	2 - 4	2.3	2	4

Abbreviations: GM, geometric mean; MIC, minimum inhibitory concentration.

the present study, VRC showed variable antifungal activity against all tested isolates. Clinical *F. proliferatum* isolates (N = 6) showed lower MIC values (range: 2 - 8 μg/mL, GM: 4 μg/mL) for VRC when compared to the environmentally obtained isolates (N = 15; range: 2 - 16 μg/mL, GM: 6.65 μg/mL). This is probably due to previous exposure of the environmental isolates to azole compounds in agriculture; however, for this aspect, further studies are required.

In this study, two rare species belonging to the *F. fujikuroi* complex, *F. acutatum* and *F. thapsinum*, were identified among the clinical isolates. In general, these two rare species have the potential to act as causative agents of onychomycosis. For instance, *F. acutatum* has been reported from nail infections as an emerging human opportunist which has thus far been detected in Asia and appears to be restricted to the Middle East (34, 35). In other studies, *F. thapsinum*, has been reported as causing eumycetoma and has been also isolated from environmental sources (23, 35). Their patterns of *in vitro* susceptibility to antifungal agents showed high MIC values for ITC (> 16 μg/mL) for both species, which demonstrated resistance of these two rare species. MIC values of VRC for the *F. acutatum* and *F.*

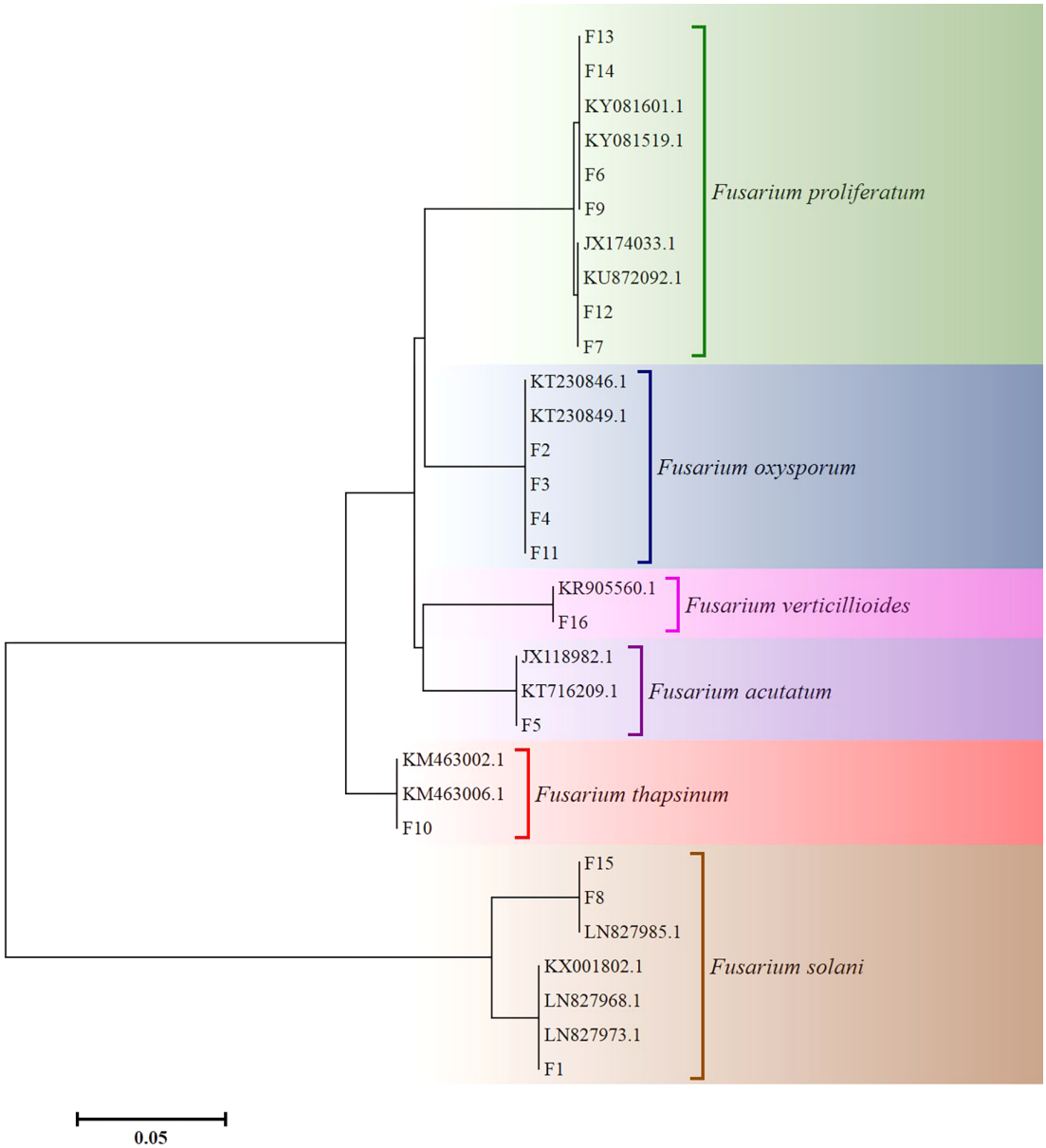

Figure 1. The Phylogenetic Tree of 16 Clinical *Fusarium* Isolates and 15 Genbank Sequences Constructed Using the Neighbor - Joining Method in Molecular Evolutionary Genetics Analysis Version 6.0 (20, 23)

thapsinum strains were 4 and 2 μg/mL, respectively.

Interestingly in this study most clinical isolates of *F. solani* were resistant to the VRC as well as the ITC. The data was similar to that of previous reports of poor activity of antifungal drugs against the *F. solani* species complex. This result is indicative of higher resistance of this species in comparison with other species of the genus *Fusarium* (13, 27, 28). Members of the *F. solani* species complex are among the most common species present in both clinical and environmental sources. Fusarial infections have emerged in recent decades that are related to high mortality, especially in disseminated infections. In addition, VRC is recommended as the first - line therapy for both superficial and disseminated fusariosis in immunocompromised patients (32). This high drug resistance among *Fusarium* species means that *in vitro* analysis of antifungal susceptibility prior to drug administration might be important for choosing the optimal treatment strategy.

In conclusion, poor susceptibility of *Fusarium* isolates to ITC and VRC was observed in this study. The MIC values obtained showed that VRC exhibited significantly better *in vitro* activity against all clinical and environmental *Fusarium* isolates. The present antifungal susceptibility profiles, however, shows that differences in antifungal susceptibility exist within the *Fusarium* species; therefore, accurate identification of *Fusarium* pathogens and determination of their susceptibility profiles is required.

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Footnotes

Authors' Contribution: Farideh Zaini designed and supervised the study. Parivash Kordbacheh, Mahin Safara, and Reza Kachuei provided administrative and technical support. Hamed Aghaei performed the experiments. Shahram Mahmoudi assisted in molecular identification and interpretation of the results. Setareh Agha Kuchak Afshari drafted the manuscript and the final version was prepared by Farideh Zaini and Shahram Mahmoudi.

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