Luliconazole, a New Antifungal, vs Amphotericin B, Voriconazole, Posaconazole and Caspofungin against Clinical and Environmental *Aspergillus* Nigri Complex

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Running title: Luliconazole against black Aspergilli

ABSTRACT

Black Aspergilli are, the most causes of aspergillosis and *Aspergillus niger and A. tubingensis are two more frequently isolates. Although, amphotericin B was a gold standard for the treatment of invasive fungal infection for several decades, it replaced by several new antifungals. Furthermore, a novel antifungal, luliconazole, appears to offer the potential for improved therapy for aspergillosis. The aim of the present study was to compare the effect of a novel antifungal agent, luliconazole, with classical antifungal* against clinical and environmental strains of black Aspergilli. Sixty seven strains of black Aspergilli were identified using morphological and molecular tests (β-Tubulin gene). Antifungal susceptibility test was applied according to CLSI M38 A2. The results were reported as MIC range, MIC_{50}, MIC_{90} and MIC_{GM}. In the present study, *A. niger* was the common isolate followed by, *A. tubingensis* and 54.1% (clinical) and 30% (environmental) of isolates were resistant to caspofungin. The highest resistant rate was found in amphotericin B for both clinical (86.5%) and environmental (96.7%) strains. Clinical strains of *Aspergillus* were more sensitive to voriconazole (86.7%) than environmental strains (70.3%). On the other hand, 83.8% of clinical and 70% of environmental isolates were resistant to posaconazole, respectively. It is found that the lowest MIC range, MIC_{50}, MIC_{90}, and MIC_{GM} was attributed to luliconazole in clinical strains. In conclusion, luliconazole vs. routine antifungal is a potent antifungal for *A. niger* complex *in vitro*. The MIC range, MIC_{50}, MIC_{90} and MIC_{GM} of luliconazole against black Aspergilli were the lowest among the representative tested antifungals.

Keywords: Black Aspergilli, Luliconazole, Clinical and environmental isolates, Antifungal profile

Introduction

Aspergillus species are the commonest environmental saprophytic fungi that have worldwide distribution. The Genus *Aspergillus* containing several sections, including, *Aspergillus* section Nigri, section Flavi, section Terrei, section Fumigati, and section Clavati (1, 2). Although, all sections are contributing in the involving aspergillosis, the most cases of disease are usually caused by different species of *Aspergillus* in the sections of Nigri and Flavi. Black Aspergilli (section Nigri) are often grown in different environmental conditions, whereas, they are more prevalent at tropical and subtropical areas.

The section Nigri (*Aspergillus niger*, sensu lato) contains 19 species including, *Aspergillus niger*, *A. tubingensis*, *A. awamory*, *A. welwitschiae*, *A. acidus*, *A. brasiliensis* etc. (3-5). The Aspergilli in this section are comprised of several closely related species, and accurate identification based on sequence analyses of β-tubulin gene (6). *Aspergillus niger* and *A. tubingensis* are two more frequently isolates from clinical materials (4, 7-9). Black Aspergilli cause several types of aspergillosis among predisposed patients. Out of them, otomycosis is the most common cutaneous infection that are usually caused by black Aspergilli.

The increasing of fungal opportunistic infections among receiving intensive therapy immunocompromised, hematological malignancies and transplant patients during last decades is remarkable (10-14). Invasive *Aspergillus* infections are one of the life threatening human disease. On the other hand, some species of *Aspergillus* have inherent resistance to some antifungal agents. Moreover, some species have raised minimum inhibitory concentration (MIC) against specific antifungals. As a results, infection prevention consultant and the best choice antifungal are common clinical challenges.

While, amphotericin B was a Gold standard in the first-line treatment of invasive fungal infections for several decades, it has been replaced by several new antifungals including,

voriconazole, posaconazole and caspofungin (13). The randomized clinical trials were considered voriconazole as the primary therapy for invasive pulmonary aspergillosis (15). Further studies have shown that posaconazole is a useful antifungal for invasive fungal infection including aspergillosis (13). On the other hand, during 2-3 last decades, caspofungin was developed to improve the prognosis of invasive aspergillosis (16).

The availability of a novel antifungal, luliconazole, appears to offer the potential for improved therapy for a wide range of invasive fungal infections, including aspergillosis. Luliconazole was basically introduced as anti-dermatophytic infections in Japan and India (17, 18). However it is newly under evaluation by several researchers for *A. fumigatus* (6, 19), *A. terreus* (6, 20), *A. flavus* (6, 21), *A. niger* (6) and *A. tubingensis* (6).

Objectives

The aim of the present study was to compare the effect of a novel antifungal agent, luliconazole, with amphotericin B, voriconazole, posaconazole and caspofungin against clinical and environmental strains of black Aspergilli.

Materials and Methods

Ethics statement

This project was approved by the ethical committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1396.1066).

Fungal isolates

Thirty seven clinical strains of black Aspergilli were previously isolated from clinical materials, identified based on morphology characteristics and preserved at Medical Mycology laboratory affiliated to Ahvaz Jundishapur University of Medical Sciences. Environmental strains (30 strains) of black Aspergilli were trapped from airborne spores using Sabouraud dextrose agar, SDA (BioLife, Italia) plates. Primary screening of black Aspergilli strains was applied based on colony morphology (Black colony) and micromorphology. In the

present study all strains (clinical and environmental) were subcultured on SDA and re-identified using molecular tests.

DNA extraction

All strains (clinical and environment isolates) were cultured on SDA plates and incubated at 29ºC for 24-48 hours. Mycelia were collected in cryo-tubes containing 300 µL lysis buffer and 0.46 g glass beads and kept at 4ºC for 72 hours. The tube contents were homogenized using a SpeedMill PLUS Homogenizer (Analytikjena, Germany) for 6 minutes (3 cycles) and boiled at 100ºC for 20 minutes. 300 µL of sodium acetate (3 molar) was added to each tube and stored at -20ºC for 10 minutes. Supernatants were removed after a centrifugation at 12000 rpm for 10 min. DNA was purified using phenol-chloroform-isoamyl alcohol according to Makimura et al., protocol (22). Finally purified DNA was preserved at -20 ºC for further tests.

Molecular identification

In the present study, β-Tubulin gene was used for the molecular detection of strains using primers pair, βt2a (forward), 5' GGTAACCAAATCGGTGCTGCTTTC 3' and βt2b (reverse) 5' ACCCTCAGTGTAGTGACCCTTGGC 3' (23). PCR products subjected for sequence analysis and then sequences were manually verified by MEGA6 software package and aligned using the CLUSTALW algorithm. All sequences were compared to reference sequences in the GenBank (NCBI) and CBS database via the nucleotide BLAST ™ algorithm to obtain a definitive identification (similarity values ≥ 99%). Finally, all nucleotide sequences representative were deposited in the GenBank database.

Antifungal susceptibility assay

Twofold serial dilutions of antifungals including, luliconazole (APIChem Technology, China) (from 0.25 to 0.00012 µg/mL), amphotericin B (Sigma - Aldrich, Germany) (from 16 to 0.125 µg/mL), voriconazole (Sigma - Aldrich, Germany) (from 4 to 0.0078 µg/mL), posaconazole (Sigma - Aldrich, Germany) (from 4 to 0.0312 µg/mL), and caspofungin (Sigma - Aldrich,

Germany) (from 1 to 0.0078 µg/mL) were prepared in RPMI 1640 (Bio Idea, Iran). Antifungal susceptibility test was applied according to CLSI M38 A2 (24). A standard suspension (0.5 McFarland) of 48 - 72 hours cultures on SDA was prepared in sterile saline (0.85%) with 0.2% Tween 20 (Merck, Germany). Then, 100 µL of diluted suspension (1:50) and 100 µL of serial dilutions of each antifungal purred in 96 well microplates. Microplates incubated at 35ºC for 24 - 72 h and results were recorded as MIC. Finally, MIC range, MIC_{50}, MIC_{90} and MIC_{GM} were calculated. CLSI or EUCAST have not been defined any clinical or epidemiologic breakpoints/cutoffs for amphotericin B, voriconazole, posaconazole, caspofungin and *Aspergillus* species. In the present study, strains susceptibility / resistance to each antifungals was evaluated according to proposed breakpoints by several researchers (Table 1) (25-30).

Table 1: Defined breakpoints of amphotericin B, voriconazole, posaconazole and caspofungin for *Aspergillus niger* sensu lato

Antifungals	**MIC (µg/mL)**	
	Sensitive	**Resistance**
Amphotericin B	≤2	>2
Posaconazole	≤0.5	>0.5
Voriconazole	≤1	>1
Caspofungin	≤0.06	>0.06
Luliconazole	Undefined	Undefined

MIC, Minimum inhibitory concentration

Statistical analysis

The Chi-squared test using the Social Science Statistics software (Online) was applied to determine the significant between variables and P value < 0.05 is considered as significance level.

Results

Molecular detection of isolates

In the present study, 37 clinical strains of black Aspergilli were detected using molecular and sequencing method. *Aspergillus niger* (21, 56.8%) was the common strain followed by, *A.*

tubingensis (11, 29.8%), *A. luchuensis* (1, 2.7%), and black *Aspergilli* (4, 10.8%) (Table 2). Furthermore, out of 30 environmental black Aspergilli isolates, 15 (50%) was identified as *A. niger* followed by, *A. tubingensis* (13, 43.3%), *A. piperis* (1, 3.3%) and black Aspergilli (1, 3.3%). However, we could not identified four clinical and one environmental black Aspergilli, using molecular technique due to lack enough DNA.

Table 2: Clinical and environmental black Aspergilli with accession numbers

Sources	**Morphological identification**	**Molecular identification**	**Accessions numbers**
Clinical isolates (37 isolates)	*Aspergillus niger* sensu lato	*A. niger*, sensu stricto (21)	LC441155, LC441156, LC441157, LC441158, LC441159, LC441160, LC441161, LC441162, LC441163, LC441164, LC441165, LC441166, LC441167, LC456320, LC456323, LC456326, LC456335, LC456336, LC456337, LC456339, LC456341
		A. tubingensis (11)	LC441168 , LC441169, LC441170, LC441171, LC456297, LC456298, LC456301, LC456302, LC456303, LC456338, LC456340
		A. luchuensis (1)	LC456304
	Unidentified black *Aspergilli* (4)	***	***
Environmental isolates (30 isolates)	*Aspergillus niger* sensu lato	*A. niger*, sensu stricto (15)	LC456317, LC456318, LC456319, LC456321, LC456322, LC456324, LC456325, LC456327, LC456328, LC456329, LC456330, LC456331, LC456332, LC456333, LC456334
		A. tubingensis (13)	LC456299, LC456300, LC456306, LC456307, LC456308, LC456309, LC456310, LC456311, LC456312, LC456313, LC456314, LC456315, LC456316
		A. piperis (1)	LC456305
	Unidentified black *Aspergilli* (1)	***	***

Clinical isolates

The minimum effective concentration (MEC) range for all clinical *Aspergillus* species was 1 - 0.0078 µg/ml for caspofungin (Table 3). In addition, the 50% and 90% MEC (MEC_{50}, MEC_{90})

values were 0.125 and 0.5 µg/ml for caspofungin. In the present study, 54.1% of isolates were resistant to caspofungin. The results have shown that the MIC range of amphotericin B for tested isolates was 16 - 0.25 µg/mL. However, MIC_{50}, MIC_{90} was similar, 8 µg/mL. The highest resistant rate was found in amphotericin B (86.5%). All clinical isolates were inhibited at the ranges 4 - 0.0078 and 4 - 0.0625 µg/mL of voriconazole and posaconazole, respectively. However, the MIC_{GM} for voriconazole (0.77 µg/mL) was lower than posaconazole (1.45 µg/mL). In our study, 29.7% and 83.8% of isolates were resistant to voriconazole and posaconazole, respectively. It is found that the lowest MIC range (0.125 - 0.00024 µg/mL), MIC_{50} (0.00195 µg/mL), MIC_{90}, (0.125 µg/mL) and MIC_{GM} (0.00295 µg/mL) was attributed to luliconazole.

Table 3: The antifungal susceptibility pattern of 37 clinical strains of black Aspergilli

Caspofungin	**No**		**(µg/mL)**			
		MEC range	**MEC_{50}**	**MEC_{90}**	**MEC_{GM}**	**R (%)**
A. niger	21	1-0.0078	0.125	0.5	0.099	11(52.4%)
A. tubingensis	11	0.5-0.032	0.125	0.5	0.133	7(63.6%)
A. luchuensis	1	0.032	-	-	-	-
Black *Aspergilli*	4	0.25-0.0625	-	-	-	2(50%)
Total	37	1-0.0078	0.125	0.5	0.107	20(54.1%)
Amphotericin B	**No**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**MIC_{GM}**	**R (%)**
A. niger	21	8-0.25	8	8	4.56	17(81%)
A. tubingensis	11	16-4	8	8	8	11(100%)
A. luchuensis	1	1	-	-	-	-
Black *Aspergilli*	4	8-4	-	-	-	4(100%)
Total	37	16-0.25	8	8	5	32(86.5%)
Voriconazole	**No**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**MIC_{GM}**	**R (%)**
A. niger	21	2-0.0625	1	2	0.99	5(23.8%)
A. tubingensis	11	4-0.5	1	2	1.20	4(36.4%)
A. luchuensis	1	0.0078	-	-	-	-
Black *Aspergilli*	4	2-0.5	-	-	-	2(50%)
Total	37	4-0.0078	1	2	0.77	11(29.7%)
Posaconazole	**No**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**MIC_{GM}**	**R (%)**
A. niger	21	4-0.0625	2	2	1.26	17(81%)
A. tubingensis	11	4-0.125	2	4	2.13	10(90.9%)
A. luchuensis	1	0.5	-	-	-	-
Black *Aspergilli*	4	4-0.25	-	-	-	4(100%)
Total	37	4-0.0625	2	4	1.45	31(83.8%)
Luliconazole	**No**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**MIC_{GM}**	**R (%)**

A. niger	21	0.125-0.00024	0.00195	0.125	0.00378	-
A. tubingensis	11	0.125-0.00024	0.00195	0.00391	0.00251	-
A. luchuensis	1	0.00098	-	-	-	-
Black *Aspergilli*	4	0.00391-0.00049	-	-	-	-
Total	37	0.125-0.00024	0.00195	0.125	0.00295	-

MEC, Minimum effective concentration; MIC, Minimum inhibitory concentration; R, Resistant

Environmental isolates

Table 4 summarizes the *in vitro* susceptibilities of 30 environmental *Aspergillus* Nigri against caspofungin. The MEC range, MEC_{50}, MEC_{90} and MEC_{GM} were 0.5 - 0.0078, 0.0625, 0.25, and 0.0507 µg/ml, respectively. Furthermore, 30% of environmental strains were resistant to caspofungin. As shown, the MIC range for amphotericin B was 16 - 2 µg/ml followed by, MIC_{50}, MIC_{90} and MIC_{GM} were 8, 8 and 6.063 µg/ml, respectively. Moreover, 96.7% of strains were resistant to antifungal. Totally, the MIC range voriconazole for environmental isolates of *Aspergillus* was 2 - 0.0625 µg/ml, whereas MIC_{90} 2 µg/ml, MIC_{50} 0.5 and MIC_{GM} 0.4665 µg/ml). Our results indicated that only 4(13.3%) of strains were resistant to voriconazole. In the present study, tested isolates were inhibited at MIC range 4 - 0.0625 µg/ml by posaconazole. Furthermore, the MIC_{50}, MIC_{90} and MIC_{GM} were 2, 4 and 1.2599 µg/ml, respectively. In addition, 70% of strains were resistant to posaconazole. The same as clinical isolates, the lowest MIC range was 0.00781 - 0.00049 µg/ml for luliconazole. Moreover, the MIC_{50}, MIC_{90} and MIC_{GM} were 0.00195, 0.00391 and 0.00195 µg/ml, respectively.

Table 4: The antifungal susceptibility pattern of 30 environmental strains of black Aspergilli

Caspofungin	**No**	**(µg/mL)**				
		MEC range	**MEC_{50}**	**MEC_{90}**	**MEC_{GM}**	**R (%)**
A. niger	15	0.25-0.0078	0.032	0.25	0.0412	3(20%)
A. tubingensis	13	0.5-0.0078	0.0625	0.5	0.0733	6(46.2%)
A. piperis	1	0.0625	-	-	-	-
Black *Aspergilli*	1	0.0078	-	-	-	-
Total	30	0.5-0.0078	0.0625	0.25	0.0507	9(30%)
Amphotericin B	**No**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**MIC_{GM}**	**R (%)**
A. niger	15	16-2	8	16	6.964	14(93%)
A. tubingensis	13	8-4	4	8	5.508	13(100%)
A. piperis	1	4	-	-	-	-

Black *Aspergilli*	1	4	-	-	-	-
Total	30	16-2	8	8	6.063	29(96.7%)
Voriconazole	**No**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**MIC_{GM}**	**R (%)**
A. niger	15	2-0.125	1	2	0.6300	2(13.3%)
A. tubingensis	13	2-0.0625	0.5	2	0.4261	2(15.4%)
A. piperis	1	0.125	-	-	-	-
Black *Aspergilli*	1	0.0625	-	-	-	-
Total	30	2-0.0625	0.5	2	0.4665	4(13.3%)
Posaconazole	**No**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**MIC_{GM}**	**R (%)**
A. niger	15	4-0.5	2	4	1.8234	14(93%)
A. tubingensis	13	4-0.125	2	4	1.1125	7(53.8%)
A. piperis	1	0.5	-	-	-	-
Black *Aspergilli*	1	0.0625	-	-	-	-
Total	30	4-0.0625	2	4	1.2599	21(70%)
Luliconazole	**No**	**MIC range**	**MIC_{50}**	**MIC_{90}**	**MIC_{GM}**	**R (%)**
A. niger	15	0.00781-0.00098	0.00195	0.00391	0.00214	-
A. tubingensis	13	0.00781-0.00049	0.00195	0.00391	0.00195	-
A. piperis	1	0.00195	-	-	-	-
Black *Aspergilli*	1	0.00049	-	-	-	-
Total	30	0.00781-0.00049	0.00195	0.00391	0.00195	-

MEC, Minimum effective concentration; MIC, Minimum inhibitory concentration; R, Resistant

In the present study, caspofungin was significantly more effective against environmental than clinical strains (P = 0.048). However, the inhibitory effect of other antifungals (amphotericin B, posaconazole and voriconazole) against both strains (clinical and environmental) was similar (amphotericin B, P=0.147; voriconazole, P=0.109; posaconazole, P=0.178). When we compared the effect antifungals against *A. niger* and *A. tubingensis* among clinical and environmental strains, it is found that caspofungin was more effective on *A. niger* with environmental sources than clinical strains (P=0.0482). Whereas, the effect of other antifungals against both species was not significant.

Our results showed that 32 (86.5%) of clinical strains have multi drug resistance, 2(5.4%) resistance to one antifungal and 3(8.1%) were fully sensitive to antifungals (Table 5). Two strains of *A. tubingensis*, one *A. niger* and one black Aspergilli were resistant to all antifungals (except luliconazole). On the other hand, in environmental strains, 70% and 30% of strains were multi drug resistance (2-4 antifungals) and drug resistance (one antifungals),

respectively (Table 6). Two strains of *A. niger* and one *A. tubingensis* were resistant to all antifungals (except luliconazole).

Table 5: Drug resistance against tested antifungals among 37 clinical strains

Species (Clinical)	**Antifungal drugs**				
	LUL	**POS**	**VOR**	**AMP**	**CAS**
Aspergillus niger	0.125	R	S	R	R
A. niger	0.125	S	S	R	R
A. niger	0.125	S	S	R	R
A. niger	0.125	R	S	R	R
A. tubingensis	0.125	R	S	R	R
A. niger	0.01561	R	S	R	R
A. niger	0.00781	R	S	R	R
A. niger	0.00781	R	S	S	S
A. tubingensis	0.00391	R	R	R	R
Black Aspergilli	0.00391	R	S	R	S
A. tubingensis	0.00391	R	R	R	R
A. niger	0.00391	R	S	R	R
A. niger	0.00195	R	R	R	S
A. tubingensis	0.00195	R	R	R	S
A. tubingensis	0.00195	R	R	R	S
Black Aspergilli	0.00195	R	R	R	S
A. tubingensis	0.00195	R	S	R	R
A. niger	0.00195	R	S	R	R
A. tubingensis	0.00195	R	S	R	R
A. niger	0.00195	R	R	R	S
A. tubingensis	0.00195	R	S	R	S
A. niger	0.00195	R	R	R	S
Black Aspergilli	0.00195	R	R	R	R
A. niger	0.00195	R	S	R	S
A. tubingensis	0.00195	S	S	R	R
A. niger	0.00098	R	S	R	S
A. niger	0.00098	R	S	R	R
A. niger	0.00098	R	S	S	S
A. niger	0.00098	R	R	R	R
A. tubingensis	0.00098	R	S	R	S
A. niger	0.00098	R	R	R	S
A. niger	0.00098	R	S	R	R
A. luchuensis	0.00098	S	S	S	S
Black Aspergilli	0.00049	S	S	R	R

A. tubingensis	0.00024	R	S	R	R
A. niger	0.00024	S	S	S	S
A. niger	0.00024	S	S	S	S

LUL, Luliconazole; POS, Posaconazole; VOR, Voriconazole; AMP, Amphotericin B; CAS, Caspofungin; R, Resistance: S, Sensitive

Table 6: Drug resistance against tested antifungals among 30 environmental strains

Species (Environmental)	**Antifungal drugs**				
	LUL	**POS**	**VOR**	**AMP**	**CAS**
Aspergillus niger	0.00781	R	S	R	S
A. tubingensis	0.00781	R	R	R	S
A. niger	0.00391	R	S	R	S
A. niger	0.00391	R	S	R	S
A. niger	0.00391	R	R	R	R
A. tubingensis	0.00391	R	R	R	R
A. niger	0.00391	R	S	R	S
A. niger	0.00195	R	S	R	S
A. tubingensis	0.00195	R	S	R	R
A. niger	0.00195	R	S	R	S
A. tubingensis	0.00195	S	S	R	S
A. niger	0.00195	R	S	R	S
A. niger	0.00195	R	S	S	S
A. tubingensis	0.00195	S	S	R	S
A. niger	0.00195	R	S	R	R
A. tubingensis	0.00195	S	S	R	S
A. tubingensis	0.00195	R	S	R	R
A. tubingensis	0.00195	R	S	R	R
A. tubingensis	0.00195	R	S	R	S
A. tubingensis	0.00195	R	S	R	R
A. niger	0.00195	R	S	R	S
A. tubingensis	0.00195	S	S	R	S
A. piperis	0.00195	S	S	R	S
A. niger	0.00098	S	S	R	S
A. niger	0.00098	R	S	R	S
A. tubingensis	0.00098	S	S	R	S
A. niger	0.00098	R	R	R	R
A. niger	0.00098	R	S	R	S
Black Aspergilli	0.00049	S	S	R	S
A. tubingensis	0.00049	S	S	R	R

LUL, Luliconazole; POS, Posaconazole; VOR, Voriconazole; AMP, Amphotericin B; CAS, Caspofungin; R, Resistance: S, Sensitive

Discussion

In the present study, the *Aspergillus* strains isolated from clinical and air borne samples were species typed using classical morphological features and molecular methods. Moreover, their susceptibilities to amphotericin B, caspofungin and three azole antifungals including luliconazole, voriconazole, and posaconazole were assayed. *Aspergillus tubingensis*, *A. luchuensis* and *A. piperis* were identified as the cryptic species of *A. niger* sensu lato by the sequence analysis of β-tubulin gene. Several reports have shown that *A. niger* is generally as common causative agent of otomycosis and one of the most important agent for invasive aspergillosis (8, 10, 31-33). However, new molecular techniques are indicating that this species comprises 19 cryptic species (4, 6, 9).

The most common cryptic species is *A. niger* (30.2 - 57.0%) sensu stricto, followed by *A. tubingensis* (31.6 - 53.5%) and other cryptic species of *A. niger* sensu lato (4.0 - 8.8%) (4, 6, 16). It is believed that the partial β-tubulin sequencing was superior to ITS sequencing for the species assignment of black Aspergilli (34, 35). Overall, our results were comparable to those of previous studies about the frequency of cryptic species. The most common clinical (86.5%) and environmental (93.3%) species in our data set are *A. niger* sensu stricto and *A. tubingensis*, respectively. Furthermore, with using β-tubulin sequencing, two rare species, *A. luchuensis* and *A. piperis* were respectively identified from clinical and environmental samples.

There are the limited data in *in vitro* efficacy of antifungals against the black Aspergilli both from clinical and environmental sources. While, the clinical and environmental strains had the same MIC ranges for caspofungin, the resistant to antifungal showed the clear differences between clinical and environmental strains (P = 0.048), where the clinical isolates

showed higher resistant rate than the environmental strains. In a report by Badali *et al*., only 6.1% of environmental strains of *A. niger* were resistant to caspofungin and all clinical isolates ranged at 0.008–0.063 μg/ml (9).

The *in vitro* activities of posaconazole, voriconazole, and amphotericin B against clinical *Aspergillus* strains have been reported by Arikan *et al*. (13). They reported that voriconazole was the most active antifungal against *A. niger*. Comparable to our results, voriconazole was more potent than the other tested antifungal (with exception luliconazole) against both clinical and environmental strains. *Aspergillus tubingensis* resistant strains to amphotericin B was very common both in environment and clinical settings, followed by posaconazole, caspofungin, and voriconazole. However, the resistant rate was lower among environmental than clinical strains. Hashimoto et al., finding suggests that *A. tubingensis* is intrinsically resistant to azole antifungals (3). Antifungal susceptibility testing of our *A. tubingensis* strains revealed 90.9% and 53.8% of clinical and environmental isolates were resistant to posaconazole.

During the last decades, some studies have shown a high efficacy of luliconazole against dermatophytes and onychomycosis agents both *in vivo* and *in vitro* (17, 18, 36-38). Furthermore, recently a few studies examined the potency of luliconazole against different species of *Candida*, *A. fumigatus*, *A. terreus* and *Fusarium* species (20, 21, 39). However, the efficacy profile of luliconazole against *A. niger*, complex is unknown. Our results showed that, although the MIC range for strains was externally low, this range for environmental strains (0.00781-0.00049 μg/ml) was lower than clinical strains (0.125 - 0.00024 μg/ml). As shown in table 5, only five clinical strains (*A. niger* sensu stricto, 4 isolates and *A. tubingensis*, 1 isolate) have a MIC = 0.125 μg/ml. In the present study, 100% of environmental and 83.8% of clinical strains had the lowest MICs (MICs < 0.00781 μg/ml) against luliconazole. Moreover, the MIC_{GM} for environmental and clinical strains were 0.00195 and 0.00295 μg/ml, respectively.

Abastabar et al. (19) and Omran et al. (21) were respectively tested luliconazole against *A. fumigatus* and *A. flavus*, and found that the antifungal has the lowest MICs against *A. fumigatus* (0.016 μg/ml) and *A. flavus* (0.062 μg/ml).

In conclusion, luliconazole vs. amphotericin B, voriconazole, posaconazole and caspofungin is a potent antifungal for *A. niger* sensu lato *in vitro*. The MIC range, MIC_{50}, MIC_{90} and MIC_{GM} of luliconazole against black Aspergilli were the lowest among the representative tested antifungals. However, these results must be confirmed by the clinical trials for the efficacy of luliconazole as anti-aspergillosis infections.

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Authors' Contribution

Study concept and design, Ali Zarei Mahmoudabadi; isolation and preparing clinical and environmental isolates, Marzieh Halvaeezadeh and Sahar Hivary; conducting the experiments, Sahar Hivary; data analysis and interpretation of the results, Ali Zarei Mahmoudabadi, Mahnaz Fatahinia, and Sahar Hivary; drafting of the manuscript, Ali Zarei Mahmoudabadi; Critical editing Mahnaz Fatahinia.

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Conflict of interest

No conflict of interest declared.

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