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ORIGINAL ARTICLE

Use of cell surface protein typing for genotyping of azole--resistant and --susceptible *Aspergillus fumigatus* isolates in Iran

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Summary

Aspergillus fumigatus is the leading cause of mortality in severely immunocompromised individuals. Understanding pathogen dispersion and relatedness is essential for determining the epidemiology of nosocomial infections. Therefore, the aim of this study was to investigate the diversity and putative origins of clinical and environmental azole-susceptible and -resistant *A. fumigatus* isolates from Iran. In all, 79 isolates, including 64 azole--susceptible and 15 azole--resistant isolates, were genotyped using the cell surface protein (CSP) gene. Seven distinct repeat types (r01, r02, r03, r04, r05, r06 and r07) and 11 different CSP variants (t01, t02, t03, t04A, t06A, t06B, t08, t10, t18A, t18B and t22) were observed. Interestingly, t06B, t18A and t18B were exclusively present in azole--resistant isolates. The Simpson's index of diversity (D) was calculated at 0.78. Resistant isolates were genetically less diverse than azole--susceptible isolates. However, azole-resistant *A. fumigatus* without TR_{34}/L98H were more diverse than with TR_{34}/L98H. The limited CSP type diversity of the TR_{34}/L98H isolates versus azole--susceptible isolates suggests that repeated independent emergence of the TR_{34}/L98H mechanism is unlikely. It has been suggested that CSP types might have a common- ancestor that developed locally and subsequently migrated worldwide.

KEYWORDS
Aspergillus fumigatus, azole-resistant, cell surface protein typing, Iran

1 | INTRODUCTION

Aspergillus fumigatus is a ubiquitously distributed saprophytic mould and the leading cause of invasive aspergillosis.[1] The latter disorder is associated with immunocompromising conditions, that is, neutropenia, hematopoietic stem cell transplantation (HSCT), solid organ transplantation (SOT) and chemotherapy.[2,3] Although triazole antifungal drugs (voriconazole and posaconazole) are currently the first--line therapy in the management and prophylaxis of aspergillosis,[4] surveillance studies and case series suggest a global presence of clinical and environmental azole-resistant *A. fumigatus* isolates in Africa, Australia, Asia, Europe, Latin--America, the Middle East and United States.[5-11] Azole resistance may develop either in patients who are treated with long--term azole therapy or in the environment through the exposure of the fungus to azole fungicides used in agriculture.[12,13] Since 2013, the prevalence of azole-resistant *A. fumigatus* isolates has increased from 3.3 % to 6.6 % in Iran.[6] Molecular typing methods have been used to study nosocomial outbreaks, understanding genetic diversity, relationships between clinical and environmental isolates, and patterns of either colonisation or invasion.[14,15] To date, little information is available on these aspects in the Iranian *A. fumigatus* population. Cell surface protein (CSP) typing is a strategy that overcomes many of the problems encountered

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during data comparison involving DNA sequence typing.[16,17] The CSP locus comprises a series of tandem 12--mer repeat motifs. Distinction between isolates arises from insertion or deletion of repeat types, resulting in differences in the number and organisation of repeats. Additional sequence variation resulting in strain discrimination can be observed in the flanking regions of the tandem repeats.[17] Single-locus sequence typing (CSP typing) is a simple, rapid and discriminatory tool that can be readily shared among laboratories,[18] and is a useful first--line approach to typing *A. fumigatus* isolates and determining relationships at the sub--population level.[19] The DNA sequence variation in the CSP gene has resulted in 27 described CSP types in the entire *A. fumigatus* population so far.[20] Therefore, in the current study, we used CSP typing, to determine the extent of genetic diversity in azole--resistant and -susceptible *A. fumigatus* isolates from Iran in comparison with previously published CSP types from different continents.

2 | MATERIALS AND METHODS

2.1 | Fungal isolates

A total of 79 clinical (n = 50) and environmental (n = 29) *A. fumigatus* isolates were obtained from the reference culture collection of Invasive Fungi Research Center (IFRC) during 2013--2016. The collection originated from biopsies (n = 9, 18%), lower respiratory tract (n = 31, 62%), sinus (n = 8, 16%) and nail (n = 2, 4%) specimens. Data collection included demographic, clinical and mycological findings. The patients were categorised according to published diagnostic criteria.[21-23] Patients without proven or probable disease were classified as being colonised. In addition, 29 environmental isolates were collected from soil and leaves of gardens surrounding hospitals and from indoor air of hospital wards. The isolates were identified by partial sequencing of the β-tubulin gene.[6,24] Antifungal susceptibility testing was performed according to Clinical and Laboratory Standards Institute guidelines (M38--A2, 2008).[25] The whole *cyp51A* gene of azole--resistant *A. fumigatus* isolates was amplified and sequenced as previously described.[6] Isolates comprising of azole--susceptible (n = 64), resistant with the TR_{34}/L98H mutation (n = 9) and triazole--resistant isolates without mutations in the *cyp51A* gene (n = 6) were included. Stock cultures for the transient working collections were maintained on malt extract agar (MEA, Difco) supplemented with chloramphenicol at 35°C for a period of 3--5 days prior to use.

2.2 | CSP typing

Genomic DNA was extracted using an Ultra Clean Microbial DNA Isolation Kit (Mobio, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was stored at −20°C prior to use. Partial CSP (putative cell surface protein) gene was amplified and sequenced using a forward primer (5′--TTGGGTGGCATTGTGCCAA--3′) and reverse primer (5′--GGAGGAACAGTGCTGTTGGTG A--3′) as previously described.[16-18] Briefly, the amplification was performed on a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with cycles of 5 minutes at 95°C for primary denaturation, followed by 35 cycles at 95°C (30 s), 60°C (30 s) and 72°C (60 s), with a final 7 minutes extension step at 72°C. Reactions were purified using GFX PCR DNA and gel band purification kit (GE Healthcare, Ltd., Buckinghamshire, UK) followed by sequencing using an ABI3730xL Genetic Analyzer (Applied Biosystems). Sequence data were aligned manually using MEGA (5.05)[26] and BioEdit version 7.0.9 (Alignment, BioEdit Sequence 2011) software packages. CSP types were assigned according to the CSP typing nomenclature described previously.[17] The genetic diversity between *A. fumigatus* isolates was analysed -according to Simpson's index of diversity.[15,20]

3 | RESULTS

Among the 50 patients with at least one *A. fumigatus* isolate, the major risk factors were solid organ transplantation, corticosteroid therapy, haematological malignancies, diabetes and chronic obstructive pulmonary disease (COPD). Colonisation was diagnosed in 32 cases (64%) and of 18 patients (36%) with aspergillosis, 4 patients had proven

TABLE 1 Distribution of CSP types in the Iranian *Aspergillus fumigatus* isolates

	Wild-type (n = 64)		Resistant TR_{34}/L98H (n = 9)		Resistant non-TR_{34}/L98H (n = 6)	
Type	Clinical	Environmental	Clinical	Environmental	Clinical	Environmental
t01	17	2	--	--	1	--
t02	1	--	2	3	--	--
t03	6	7	--	2	--	1
t04A	18	6	--	1	--	2
t06A	1	1	--		--	--
t06B	--	--	--	1	--	--
t08	1	--	--	--	--	--
t10	1	2	--	--	--	--
t 18A	--	--	--	--	--	1
t 18B	--	--	--	--	1	--
t22	1	--	--	--	--	--

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TABLE 2 Overview of the identified CSP types in the Iranian isolates *Aspergillus fumigatus*

CSP type	Codon			Tandem repeat succession	Codon			Prevalence		
	−15	−14	−1		+1	+2	+3	Clinical (%)	Environmental (%)	Overall (%)
t01	GTG	GTC	CCG	01-01-01-01-05-03-01-06-03-07	CCA	CCT	CCA	36	6.9	25.3
t02	GTG	GTC	CCG	01-01-02-03-04-05-03-01-06-03-07	CCA	CCT	CCA	6	10.3	7.6
t03	GTG	GTC	CCG	01-02-03-04-06-03-07	CCA	CCT	CCA	12	34.5	20.2
t04A	GTG	GTC	CCG	01-02-03-04-05-03-01-06-03-07	CCA	CCT	CCA	36	31	34.1
t06A	GTG	GTC	CCG	01-01-01-02-03-04-05-03-01-06-03-07	CCA	CCT	CCA	2	3.4	2.5
t06B	GTG	CTC	CCG	01-01-01-02-03-04-05-03-01-06-03-07	CCG	CCT	CCT	--	3.4	1.3
t08	GTG	CTC	CCG	01-01-01-02-03-04-05-03-04-05-03-01-06-03-07	CCG	CCT	CCT	2	--	1.3
t10	GTG	GTC	CCG	01-01-01-05-03-01-06-03-07	CCA	CCT	CCA	2	6.9	3.8
t18A	GTG	GTC	CCG	01-01-05-03-01-06-03-07	CCA	CCT	CCA	--	3.4	1.3
t18B	GCG	CTC	CCG	01-01-05-03-01-06-03-07	CCA	CCT	CCA	2	--	1.3
t22	GTG	GTC	CCG	01-01-02-03-01-06-03-07	CCA	CCT	CCA	2	--	1.3

invasive aspergillosis. Based on the epidemiological cut--off values (ECV) for triazoles,[27] 15 (19%) isolates were resistant (itraconazole MIC ≥ 4 μg/mL). Of these, nine isolates had the TR_{34}/L98H mutation, whereas the remaining six isolates had no mutation in the *cyp51A* gene.

CSP genotyping of 79 azole--resistant and --susceptible *A. fumigatus* isolates revealed 100% typeability (Table 1). We observed 11 different CSP variants (t01, t02, t03, t04A, t06A, t06B, t08, t10, t18A, t18B and t22) (Table 2). The most commonly observed CSP variant among the clinical isolates were t04A, t01 and t03 among environmental isolates. The CSP type t04A, with 27 isolates, was the most prevalent in clinical and environmental isolates. It comprised of 18 clinical and nine environmental isolates and eight out of 18 clinical isolates were obtained from the respiratory tract. Of 18 clinical isolates with t01 as the sec-ond common CSP type in clinical specimens, 10 were isolated from the respiratory tract, five from sinuses and three from biopsies. There was no obvious correlation between any particular genotype and the underlying disease of the patient. Two patients with proven invasive aspergillosis had CSP type t02 and the other two cases t04A and t06A. CSP genotyping revealed that the azole--susceptible wild--type control isolates were spread across eight CSP types. These four types (t06A, t08, t10 and t22) were found specifically in the wild--types (Table 1). In addition, nine mutant isolates with TR_{34}/L98H were distributed over four CSP types (t02, t03, t04A and t06B); remarkably t02 was the pre-dominant type in azole--resistant mutant isolates. In contrast, six azole-- resistant non-TR_{34}/L98H isolates grouped in five CSP types (t01, t03, t04A, t18A and t18B). Moreover, t06B, t18A and t18B were only found in azole--resistant either TR_{34}/L98H or non--TR_{34}/L98H isolates. All in all, four types (t01, t02, t03 and t04 A) were observed among wild--type and resistant isolates. There was no significant difference in the prevalence of CSP variants between clinical and environmental isolates ($P > .05$). The Simpson's index of diversity (D) was calculated at 0.78. The partial β--tubulin gene sequences of all isolates have been deposited in GenBank under the accession numbers KU885393 to KU885422.

4 | DISCUSSION

Molecular typing is an indispensable technique for outbreak analysis, environmental monitoring and the study of local and global epidemiology.[14] Several genotype--based methods such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) have been used to[28-31] distinguish closely related organisms at the species to strain level. Methods based on the detection of single nucleotide polymorphisms (SNPs), such as multi--locus sequencing typing (MLST), are used extensively because the high levels of resolution, the ability to characterise a large number of isolates rapidly and absence of any subjective interpretation of banding patterns.[28] For these reasons, CSP typing is a powerful, highly reproducible and stable approach to type *A. fumigatus* iso-

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the CSP gene is much larger than MLST where at least five different alleles were found per gene. In contrast, DNA sequence variation in the CSP gene has resulted in 27 described CSP types in the entire *A. fumigatus* population from Australia, North America, The Netherlands and China so far rendering this gene an interesting target for genotyping.[16,18-20] CSP typing is located in between the MLST markers (low discriminatory) and microsatellite markers (highly discriminatory), providing the ability for typing at the sub--population level.[20] We used CSP typing successfully on all Iranian *A. fumigatus* isolates and a total of 11 CSP variants were identified in 79 isolates. Seven distinct repeat types, that is, r01, r02, r03, r04, r05, r06 and r07 were found among the Iranian isolates. The Simpsons' index of diversity (D) was calculated at 0.78 approximately similar to results from China (16 CSP variants with D--value of 0.83)[20] and Australia (15 CSP variants with D--value of 0.81).[19] Importantly, two newly described types (t18B and t22) among the Chinese isolates[20] and t08 only present among Dutch isolates[17] were also identified in our samples. However, other previously described CSP types (t04B, t05, t07, t09, t11, t12, t13, t14, t15, t16, t17, t19, t20, t21, t23 and t24) were not observed in this study.[16-20] The high prevalence CSP types observed in our study were t04A, t01, t03 and t02, respectively. Although resistant isolates with and without TR_{34}/L98H were genetically less diverse than azole--susceptible wild--type isolates, azole--resistant *A. fumigatus* without TR_{34}/L98H were more diverse than TR_{34}/L98H isolates. This is consistent with a previous report[32] in which TR_{34}/L98H from the Netherlands were also less diverse than susceptible isolates. We show that Iranian azole--resistant isolates with TR_{34}/L98H genotype were found in only four CSP types: t02, t03, t04A and t06B, whereas azole--resistant non--TR_{34}/L98H isolates were distributed in five CSP types, that is, t01, t03, t04A, t18A and t18B. CSP types t02, t03, t04A and t06B in both clinical and environmental TR_{34}/L98H isolates. Camps et al[32] suggested that TR_{34}/L98H isolates are sexually fertile which provides a possible explanation for their genetic diversity. The role of sexual reproduction was recently also found to be responsible for the emergence of more varied mutations.[33] Sexual reproduction played a crucial role in the development of azole resistance with the t02 type being predominantly across the TR_{34}/L98H *A. fumigatus* isolates. A previous microsatellite analysis of environmental Iranian TR_{34}/L98H *A. fumigatus* isolates showed the presence of a single genotype closely related to Dutch and Indian TR_{34}/L98H *A. fumigatus* isolates.[7,21] It is notable that Camps et al[32] showed that *A. fumigatus* isolates with TR_{34}/L98H resistance mechanism had less genetic variation than azole--susceptible isolates or those with a different genetic basis of resistance. Likewise, Chowdhary et al[34,35] reported that all triazole--resistant isolates from India with the same TR_{34}/L98H mutation in the *cyp*51A gene were clonal. In contrast to the genetic uniformity of azole--resistant isolates, the azole--susceptible isolates from patients and the environment in India were genetically very diverse. Resistant *A. fumigatus*, which emerged around the world, can easily spread by producing a large number of airborne asexual conidia. The limited CSP type diversity of the TR_{34}/L98H isolates versus azole--susceptible isolates suggests that it is unlikely that the TR_{34}/L98H mechanism emerged repeatedly and independent. However, the emergence of increasingly azole--resistant isolates of *A. fumigatus* probably demonstrated the ability of this fungus to adapt to antifungal pressures, despite limited genetic diversity. The most commonly observed CSP variants were t01, t02, t03, t04A, t06A and t10 as seen in iso-lates from Australia, The Netherlands, North America, China and Iran, indicating a close genetic relationship despite the wide geo-graphic distances involved. We confirm that CSP typing is a highly discriminatory and powerful tool which is a first--line typing strat-egy for *A. fumigatus* isolates to conduct epidemiological studies. It has been suggested that CSP types might have a common ancestor that developed -locally and subsequently migrated worldwide.

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CONFLICT OF INTEREST

We declare no potential conflicts of interest; the authors alone are responsible for the content and writing of the paper.

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