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TR_{34}/L98H mutation in *Aspergillus fumigatus* isolate: First report in Azerbaijan

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Abstract

Purpose. *Aspergillus fumigatus* is the most common etiological agent of aspergillosis. Intensive use of azoles in treatment and agriculture resulted in the emergence of isolates resistant to azoles. In regions with a prevalence of environmental azole resistance exceeding the 10% threshold empirical treatment regimen with azole-echinocandin/liposomal amphotericin is recommended. The aim of the current investigation was to evaluate the prevalence of azole resistant *A.fumigatus* isolates in the environment and clinical samples of patients applied to hospitals in the Azerbaijan Republic and to detect mutations responsible for resistance.

Methods. Clinical and environmental samples were collected during 2017–2019 period. Identification of *Aspergillus spp*. colonies was performed by phenotypic (lactophenol cotton blue mount – LCBM) and genotypic methods (ITS – internal transcribed spacer regions) methods. The susceptibility testing of isolates to itraconazole (ITR), voriconazole (VOR), and posaconazole (POS) was performed in accordance with the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Resistant isolates were investigated for the presence of mutation in the *cyp51A* gene, the target gene of the azoles.

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Results. Resistance was detected in one environmental isolate and in four clinical isolates. Among 50 isolates, three were resistant to all azoles, two were resistant to POS only. Genetic analysis revealed in one isolate *cyp51A* mutation – TR_{34}/L98H.

Conclusion. The findings indicate a lower environmental resistance rate (0.4%) contrasted with a higher rate in clinical isolates (21%) in Azerbaijan. Further, comprehensive studies involving more isolates from patients and environment are necessary.

Keywords – *Aspergillus fumigatus* – azole resistance – chronic pulmonary aspergillosis – EUCAST – invasive aspergillosis – MIC

Acronyms

IA – invasive aspergillosis, CPA – chronic pulmonary aspergillosis, ITR – itraconazole, VOR – voriconazole, POS – posaconazole, ISA – isavuconazole, MIC – minimum inhibitory concentration, EUCAST – European Committee on Antimicrobial Susceptibility Testing, HSCT – hematopoietic stem cell transplant, ESCMID – European Society for Clinical Microbiology and Infectious Diseases, ECMM – European Confederation of Medical Mycology, ESCMID – European Society for Clinical Microbiology and Infectious Diseases, ERS – European Respiratory Society, IDSA – Infectious diseases society of America, ITS – internal transcribed spacer regions, BLAST – Basic Local Alignment search Tool

Introduction

Aspergillus fumigatus is an ubiquitous mould producing a large amounts of spores. Inhaled spores are eliminated by the immune system of a healthy individual (Lestrade et al. 2019). In immunocompromised patients and those with chronic pulmonary diseases, this mould can cause a wide spectrum of conditions including colonisation, allergic bronchopulmonary aspergillosis (ABPA), severe asthma with fungal sensitisation (SAFS), chronic pulmonary aspergillosis (CPA), and invasive aspergillosis (IA) (Kosmidis & Denning 2015). The small diameter of the conidia facilitates their passage to the low respiratory tract airways, making *A.fumigatus* the most common etiological agent of aspergillosis. Prolonged neutropenia and immune suppression induced by corticosteroid treatment are the main risk factors for IA. Neutropenia results in angioinvasion and dissemination of the pathogen via blood; while corticosteroids cause impairment of phagocytosis, excessive inflammation, and tissue necrosis in recipients of haematopoietic stem cell (HSCT) and solid organ transplant (Dagenais & Keller 2009). CPA occurs in patients with preexisting pulmonary diseases (chronic obstructive pulmonary disease - COPD, pulmonary tuberculosis, sarcoidosis) without severe immunosuppression (Kosmidis & Denning 2015).

Triazoles: itraconazole (ITR), voriconazole (VOR), posaconazole (POS), and isavuconazole (ISA) – are used in the treatment of diseases caused by *Aspergillus* species. They target the enzyme 14α–demethylase encoded by the *cyp51A* gene, hindering the synthesis of ergosterol, a vital component of the cell membrane that regulates its fluidity and permeability. This results in altered membrane structure and permeability, leading to the accumulation of toxic methylated sterols within fungal cell (Berger et al. 2017, Scorzoni et al. 2017).

The Joint Clinical Guidelines of the European Society for Clinical Microbiology and Infectious Diseases, the European Confederation of Medical Mycology and the European Respiratory Society (ESCMID–ECMM–ERS) recommend the identification of *Aspergillus* species to a complex level. VOR and ISA are considered first-line drugs in the treatment of IA (Ullmann et al. 2018). Infectious Diseases Society of America (IDSA) recommends voriconazole for primary treatment (strong recommendation) and isavuconazole/liposomal amphotericin B (AMB) as an alternative therapy (moderate recommendation) (Patterson et al.

2016). Oral ITR and VOR are considered for the treatment of CPA (Denning et al. 2016). Both guidelines recommend an epidemiological survey of azole resistance (Patterson et al. 2016, Ullmann et al. 2018).

Intensive use of azoles in treatment and agriculture resulted in the emergence of isolates resistant to azoles. The first resistant isolate was reported in 1997 and was associated with a point mutation in the *cyp51A* gene (M220R) (Denning et al. 1997). Other mutations leading to substitutions at G54, P216, F219, M220, G138, Y431, and G448 have been reported. Point mutations are frequently observed in patients undergoing extended azole treatment. (Camps et al. 2012, Verweij et al. 2016). Another common type of mutation is associated with the formation of tandem repeats (TR) in promoter regions in combination with *cyp51A* point mutations. This type of mutation is believed to be selected for by the massive use of triazole fungicides in agriculture (TR_{34}/L98H, TR_{53}, and TR_{46}/Y121F/T289A) (Verweij et al. 2009, Snelders et al. 2012, Berger et al. 2017, Hollomon 2017, Chowdhary & Meis 2018).

The mortality rate due to azole resistant isolates in patients with IA varies between 50–100%. Investigations have revealed that mortality from azole-resistant isolates is 23%–31% higher compared to azole-susceptible individuals (Lestrade et al. 2019). As there is no specific risk factor that allows the prediction of azole resistance, monitoring of patients is recommended. In case of aspergillosis treatment failure, antifungal susceptibility test with detection of minimum inhibitory concentration (MIC) should be performed and if resistance to azole is detected, therapy should be adjusted. However, laboratories in low–resource settings and non-academic hospitals (Lestrade et al. 2016) do not perform routine antifungal susceptibility testing of moulds. Thus, empirical treatment regimens were recommended in regions with a prevalence of environmental azole resistance exceeding the 10% threshold (Verweij et al. 2015, Ullmann et al. 2018).

This is the first investigation related to isolates resistant to azoles of *A.fumigatus* in the Republic of Azerbaijan. There is no laboratory in Azerbaijan that routinely performs MIC tests of isolated moulds and there is no published data on resistant clinical or environmental isolates in the republic. Therefore, the objective of the current investigation was to evaluate the prevalence of azole resistant isolates of *A.fumigatus* in the environment and clinical samples of patients applied to hospitals of the Azerbaijan Republic and to detect mutations responsible for resistance.

Materials & Methods

Environmental samples were collected from eight regions of the Azerbaijan Republic (Baku-Absheron, Daghlig Shirvan, Ganja-Qazakh, Shaki–Zaqatala, Lankaran, Quba-Khachmaz, Aran and Nakhchivan) during 2017–2019 period.

A total of 229 samples were collected from the environment, namely public gardens (79), hospital gardens (39), vegetable fields (38), private gardens (25), cereal fields (13), hospital air (11), orchards (7), fruit gardens (3), sunflower fields (2), vineyards (2) olive grove (1), saffron field (1) peanut field (1), and other sites (7).

2 grams from each soil sample were suspended in 8 ml of distilled water containing 1% Tween 20 and chloramphenicol (0.5 g/l). After 1 hour of sedimentation, 100 µl of supernatant was inoculated on Sabouraud Dextrose agar (SDA) plates supplemented with chloramphenicol (0.5 g/l) (Pronadisa, Spain). Plates were incubated for 72 hours at 37°C and evaluated for the presence of *Aspergillus spp*. Colonies (Snelders et al. 2009, Riat et al. 2018).

Clinical samples from patients applied to Clinical Microbiological Laboratory of the department of Medical Microbiology and Immunology, Educational-Therapeutic and Educational-Surgical Clinics of Azerbaijan Medical University and Scientific Research Institute of Lung Diseases of Azerbaijan Republic were collected during 2017–2019 period.

Specimens were inoculated on 2 SDA (Pronadisa, Spain) plates containing chloramphenicol (0.5 g/l) and incubated at 37°C.

Colonies resembling *Aspergillus spp.* were investigated using a lactophenol cotton blue mount (LCBM) preparation (Leck 1999). Morphological identification was performed in accordance with taxonomic keys and guides (McClenny 2005, Samson et al. 2014). All isolates phenotypically identified as *A.fumigatus* complex were further incubated at 48°C to separate cryptic species (Riat et al. 2018) and processed for final identification using internal transcribed spacer regions 1 and 4 (ITS1 and ITS4) (Henry et al. 2000, Schoch et al. 2012, Samson et al. 2014).

Identification of *Aspergillus spp.* was performed using ITS 1 and 4. *Aspergillus spp.* isolates were incubated up to one week in order to obtain sufficient growth for DNA extraction. Hyphae obtained from one–week *Aspergillus spp.* culture were suspended in 1 ml of saline and centrifuged at 12000 rpm for 5 minutes. The pellet was suspended in 100 µl of DNA extraction buffer (1M Tris HCl [pH 7.5]), *IGEPAL*® CA-630, Tween 20, Proteinase K[10mg/ml]). Fungal spore suspension in DNA isolation buffer was vortexed, incubated at 56°C for 30 min and heated up to 100°C for 8 min. Oligonucleotide primers ITS 1 and ITS 4 were used for amplification (ITS 1, 5′-TCC GTA GGT GAA CCT GCG G- 3′; ITS 4, 5′-TCC TCC GCT TAT TGA TAT G-3′) (Metabion International, Martinsried, Germany). 2 µl from each test sample was suspended in Master Mix Solution ((2.5 U Taq polymerase (Fermentas), 10X Taq buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2 mM MgCl2, 0.4 pmol primers, 0.2 mM dNTP)) in a final volume of 30 µL. Polymerase chain reaction (PCR) was performed on the Applied Biosystems SimpliAmp thermal cycler. 35 amplification cycles consisting of a denaturation step at 95°C for 30s, annealing step at 50°C for 30s, extension step at 72°C for 1 min, and final extension at 72°C for 8 min were performed (Oryaşın et al. 2013). The amplicons obtained were separated by agarose gel electrophoresis. All amplified ITS fragments were sequenced (Macrogen, http://dna.macrogen.com/eng/ 2019) (Macrogen sequencing facility). Sequences were compared with the those present in the genebank at blast.ncbi.nlm.nih.gov and identified by sequence homology (Oryaşın et al. 2013).

Susceptibility testing. All *A.fumigatus* isolates were tested for susceptibility to ITR, VOR, and POS in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard ("EUCAST definitive document E.DEF9.3.1. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds.," January 2017). Each antifungal was diluted in double strength RPMI 1640 medium supplemented with 2% glucose and dispensed (100µl) in microdilution wells at 2x final concentration. Inoculum suspension was prepared by covering 2–5 days old culture on SDA with sterile water supplemented with 1% Tween 20. Spore suspensions were adjusted to 2 to 5 x 10^6 conidia/ml by counting in a hemocytometer chamber and diluted 10-fold to obtain the final (2–5x10^5 conidia/ml) concentration. Microdilution plates containing antifungals were inoculated with 100 µl of spore suspension and incubated for 2 days at 37°C. The minimum inhibitory concentration (MIC) was detected as the concentration of the drug with no visible growth. Isolates were considered as resistant when MIC was ≥2 mg/l for ITR and VOR, and ≥0.5 mg/l for POS ("European committee on antimicrobial susceptibility testing. Antifungal agents. Breakpoint tables for interpretation of MICs.," 2018).

The genetic analysis of resistant isolates was performed in the Laboratory of Genetics of Wageningen University & Research, the Netherlands. DNA extraction, amplification and sequencing of PCR products were performed in accordance with protocols described earlier (Snelders et al. 2009). Conidia from each isolate were inoculated in GYEP medium (2% glucose, 0.3% yeast extract, 1% peptone) and incubated for 48 hours at 37°C.

The obtained mycelial mats were used for DNA extraction. The mycelium was transferred to a sheet Whatman paper No.1 (to remove excess moisture) and then transferred

to a 50 ml polypropylene tube containing 6 glass beads. After immersion of the tube in liquid nitrogen for 10 seconds, it was thoroughly vortexed for 30 seconds to obtain a powder. Next, 0.8 ml of extraction buffer (200 mM Tris-CI pH 8.0, 0.5 M NaCl, 0.01 M EDTA, 1% sodium dodecyl sulphate) was added and the resulting mixture was gently vortexed. An equal volume of phenol–chloroform was added to this mixture to form an emulsion. The resulting emulsion was transferred to microtubes and centrifuged at 15,000xg for 15 min. The liquid part above the resulting precipitate was extracted twice: phenol-chloroform and chloroform. DNA was obtained by precipitation of ethanol and centrifugation. The pellet was resuspended in 10 mM Tris-HCI pH 7.6. 1 mM EDTA (TE) with 50 μg/ml RNase A (Tang et al. 1992).

The complete sequences of the *cyp51A* were amplified using primers P450–A1 (5′-ATGGTGCCGATGCTATGG-3′) and P450-A2 (5′-CTGTC-TCACTTGGATGTG-3′) for *cyp51A* and P450–ATCGTC (5′–ATGTC 3') and P450-B2 (5'-TCAGGCTTTGGTAGCGG–3') for the *cyp51B* gene. To exclude the possibility of changes in the sequences associated with the PCR amplification process errors, each isolate was analyzed twice (Diaz–Guerra et al. 2003).

PCR was carried out in a volume of 50 μL containing 10 mM $(NH4)_2SO_4$, 10 mM KCl, 20 mM Tris–Cl (pH 8.8), 2 mM $MgSO_4$, 10 ng bovine serum albumin, 0.1% Triton X–100, 250 μM each dATP, dGTP, dCTP and dTTP, 1 μM of each primer, 2.5 U of Pfu DNA polymerase and 50 ng of genomic DNA. Amplification was performed in a thermal cycler (Perkin-Elmer Cetus). The first cycle is 5 minutes at 94°C, 45 seconds at 58° C and 2 minutes at 72°C, followed by 30 cycles – 30 seconds at 94°C, 45 seconds at 58°C and 2 minutes at 72°C, followed by a final cycle similar to the previous one, but 10 minutes at 72°C. PCR products were analyzed by electrophoresis on 0.8 or 1.3% agarose gels and visualized by transillumination after staining with ethidium bromide (Mellado et al. 2001).

Detection of mutants was performed through comparison of obtained sequences with *cyp51A* sequence under accession number AF338659 of GenBank (Snelders et al. 2009).

Results

A study was conducted on 50 *A.fumigatus* isolates, comprising 19 clinical and 31 environmental strains, collected from various regions in Azerbaijan. All clinical isolates were obtained from patients admitted to hospitals in Baku, the capital of Azerbaijan. The geographic distribution of the environmental isolates is illustrated in Figure 1 and detailed in Table 1.

Table 1 Distribution of 31 environmental *A.fumigatus* isolates obtained from soil (n=218) and air (n=11) samples in eight regions of Azerbaijan Republic.

Region	Samples (n)	*A.fumigatus* isolates (n)	Azole resistant isolates (n)
Eastern Region (Baku-Absheron)	131	26	1
Central region (Daghlig Shirvan)	21	2	0
Western region (Ganja-Qazakh)	18	1	0
Northern region (Shaki-Zaqatala)	9	0	0
Southern region (Lankaran)	21	1	0
Central region (Aran)	18	0	0
Northern region (Quba-Khachmaz)	6	0	0
Western region (Nakhchivan)	5	1	0
Total	229	31	1

The majority of environmental isolates (26) were obtained from the Baku-Absheron region, encompassing Baku and Sumgayit cities. Notably, only one environmental isolate (118e) exhibited resistance to all azoles (MIC range 1 to ≥8 mg/l) (table 2). A resistant isolate was found in the Baku-Absheron (eastern) region, resulting in a resistance prevalence of 0.4% in the environment.

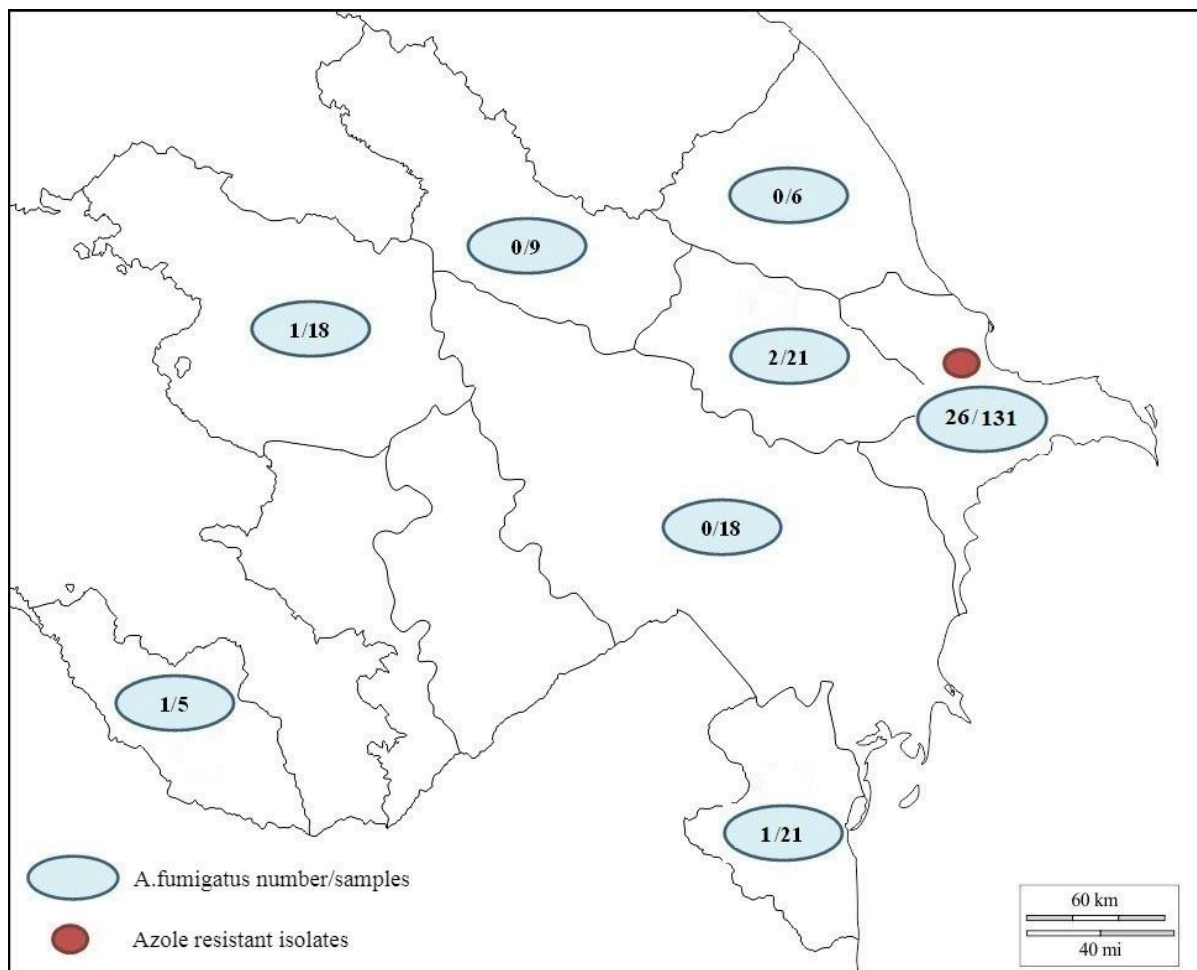

Fig. 1 – Distribution of 31 environmental *A.fumigatus* isolates obtained from soil (n=218) and air (n=11) samples in eight regions of Azerbaijan Republic. Blue circles: *A.fumigatus* isolates/samples investigated. Red circle: area with azole resistant isolate.

Table 2 Environmental origin of azole resistant *A.fumigatus* isolates.

Source	Examined samples (n)	Number of azole resistant isolates		
		ITR	VOR	POS
Public gardens	79	1	1	1
Hospital gardens	39	0	0	0
Vegetable fields	38	0	0	0
Private gardens	25	0	0	0
Hospital air	11	0	0	0
Others	37	0	0	0
Total	229	1	1	1

Among 19 tested clinical isolates two were (41°C and 68°C) resistant to all azoles (MIC range 2 to ≥16 mg/l) and two isolates (64°C and 94°C) were resistant only to POS (MIC=0.5 mg/l), accounting for an overall resistance rate of 21% among clinical isolates. However, it is important to note that these findings may be affected by the limited number of investigated isolates. Results of antifungal susceptibility, presented in Tables 3 and 4, indicate that among the 50 isolates, three (41°C, 68°C, and 118e) were resistant to three different medical azoles, while two (64°C and 94°C) exhibited resistance solely to POS.

Table 3 Antifungal susceptibility of 50 A.fumigatus isolates to 3 antifungal drugs.

Source (n)	**Antifungal agent**	**MIC (mg/l)**							
		≤0.12	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
Clinical isolates (19)	ITR	-	-	11	6	-	-	-	2
	VOR	-	-	10	7	-	-	2	-
	POS	3	12	2	-	1	1	-	-
Environmental isolates (31)	ITR	-	-	29	1	-	-	1	-
	VOR	-	3	17	10	-	-	1	-
	POS	5	25	-	1	-	-	-	-

Table 4 Resistant strains isolated from clinical and environmental samples.

Source (n)	**Antifungal agent**	**Number of resistant strains (n)**
Clinical isolates (19)	ITR+VOR+POS	2
	POS	2
Environmental isolates (31)	ITR+VOR+POS	1
Total (50)	-	5

Genetic analysis identified the TR34/L98H mutation in clinical isolate 41°C, while other isolates showed no mutation in *cyp51A* gene (Table 5).

Table 5 Results of *cyp*51A gene sequencing of 5 *A.fumigatus* isolates with azole resistance.

	Promoter	**Coding region *cyp51A***									
	cyp51A	**tat46ttt**	**gga89ggg**	**ctc98cac**	**gtga72atg**	**atc242gtc**	**act248aat**	**gag255gac**	**ttg358tta**	**aag427gag**	**tgc454tgt**
41c	TR34	Y46F	G89G	L98H	V172M		T248N	E255D	L358L	K427E	C454C
64c	Wt	Y46F	G89G		V172M		T248N	E255D	L358L	K427E	C454C
68c	Wt										
94c	Wt	Y46F	G89G		V172M	I242V	T248N	E255D	L358L	K427E	C454C
118e	Wt	Y46F	G89G		V172M		T248N	E255D	L358L	K427E	C454C

Note: Genetic analysis was performed in laboratory of Wageningen University & Research (by Eveline Snelders).

Discussion

Azole resistance in *A.fumigatus* poses a significant challenge, with two primary avenues of resistance development identified: prolonged azole treatment in patients and accumulation of azoles in the environment due to their use in agriculture (Hagiwara et al. 2016). This selective pressure results in the dominance of mutant strains adapted to azole-rich environment, ultimately overtaking susceptible strains and causing the rapid spread of azole-resistant *A.fumigatus* strains (Hof 2001).

Treatment with azoles leads to selection of point mutations (G54, P216, F219, M220, G138, Y431, G44) that modify the targets of these drugs (Howard et al. 2009). The environmental route of selection is characterized by the emergence of tandem repeats in promoter regions and point mutations in the *cyp51A* gene (Snelders et al. 2009, Chowdhary et al. 2012, Snelders et al. 2012 Hollomon 2017). TR34/L98H, TR46/Y121F/T289A, and TR53 are examples of this type of mutations (Meis et al. 2016).

First cases of environmental azole resistant *A.fumigatus* isolates were reported in the Netherlands and Denmark (Snelders et al. 2009, Mortensen et al. 2010). Intensive use of azoles in agriculture is attributed to their cost-effectiveness and broad antifungal spectrum. Additionally, their structural stability allows them to retain activity for extended periods across diverse ecological environments (Hof 2001). However, the latter feature is responsible for the selection of resistance in ubiquitously distributed isolates of *Aspergillus spp.* This route of resistance development has been documented since 2007 and a series of investigations have been conducted proving the link between clinical resistance to azoles and resistance found in environmental isolates (Verweij et al. 2009, Snelders et al. 2009, Chowdhary et al. 2012, Snelders et al. 2012, Hollomon 2017, Chowdhary & Meis 2018).

The hypothesis regarding the environmental origin of resistant isolates was first proposed in 2009 (Snelders et al. 2009). The presence of cross-resistance between environmental and clinical isolates, exhibiting the TR34/L98H mutation, against five agricultural azoles (propiconazole, tebuconazole, bromuconazole, difenconazole, and epoxiconazole), further supports this hypothesis (Snelders et al. 2012).

Population genetic analyses conducted on azole-resistant isolates, both from environmental and clinical settings in India, indicate a potential origin of resistant isolates from those that were already resistant to azoles and migrated from other countries. Additionally, it suggests that these resistant strains may have developed from azole-naïve isolates within the country (Chowdhary et al. 2012). Another research (Snelders et al. 2009) has revealed the dominance of a single resistance mechanism and genetic relatedness of environmental and clinical isolates.

Azole resistance has been detected in most European countries. As per the Surveillance Collaboration on Aspergillus Resistance in Europe (SCARE), including 22 medical centres and 19 countries, the resistance rate was 3.2%. TR34/L98H and TR46/Y121F/A genotypes accounted for azole resistance in 26 cases. These resistance mechanisms were detected in Austria, Belgium, Denmark, France, Italy, Germany, Ireland, Poland, Portugal, Spain, Sweden, and Turkey (Lockhart et al. 2011, van der Linden et al. 2015). The prevalence of resistance significantly varies between countries, different medical institutions, and even among distinct patient groups within the same medical facility.

The Netherlands have reported 8-15% resistance prevalence between 2015-2018. Investigations in Italy, Denmark, and Spain have showed similar data - 6.1%, 6.25% and 6.6% respectively. Relatively low prevalences were documented in Switzerland, France, Portugal,

Poland, and Turkey – 1.1%, 1.8%, 2.6, 4.1, and 3.3% rates respectively. UK and Greece reported 6% and 1% prevalence of resistant ARAF strains in environment (Bosetti & Neofytos 2023).

First report of TR34/L98H mutation from USA was published in 2016 (Wiederhold et al. 2016). Results of passive surveillance study on 1356 *A.fumigatus* clinical isolates showed 1.4% prevalence of resistance (Berkow et al. 2018).

Azole resistance was also detected in South America (Argentina, Brazil, Colombia, and Peru). Studies in Argentina have revealed G54E, TR46/Y121F/T289A, TR34-L98H mutations from patients with corneal lesion, lymphoblatic leukemia and cystic fibrosis. M220K, G54E, and TR34-L98H mutations had been reported in a prospective study from hospital in Peru with overall 2.09% resistance rate (Macedo et al. 2021). Studies from Columbia have revealed TR_{34}/L98H, TR_{46}/Y121F/T289A and TR_{53} mutations detected in environmental *A.fumigatus* strains and 9.6 % resistance rate in environment (Alvarez-Moreno et al. 2017, Macedo et al. 2021). Azole resistance screening of 199 *A.fumigatus* strains in Brazil revealed resistance only in 2 strains (1%) (Pontes et al. 2020).

Studies from Asia have reported varying levels of azole resistance. Environmental samples from Vietnam and Iran demonstrated resistance rates of 65% and 18%, respectively. In contrast, clinical samples from Asian countries exhibited lower resistance rates, ranging from 2.9% to 7.5%. In Australia, 2% of clinical isolates were found to be resistant to azoles (Bosetti & Neofytos 2023). First case of TR34/L98H mutation in *CYP51A* gene in *A.fumigatus* in Korea was reported in 2018 (Lee et al. 2018).A recent genetic study that examined 2,026 isolates from 13 countries revealed a resistance rate of 6% (Ashu et al. 2017). The majority of isolates across Europe and Asia exhibited the same resistance mechanisms, particularly TR34/L98H and TR46/Y121F/T289A mutations, presumed to originate from the environment. Interestingly, these mechanisms were not commonly found in isolates from the United States. This discrepancy in resistance genotypes is thought to be linked to varying usage levels of azole fungicides in European and American agriculture industries. Notably, according to van der Linden et al. (van der Linden et al. 2011), isolates with the TR34/L98H mutation show a high fatality rate (89%) in aspergillosis. Similarly, a poor prognosis is associated with the TR46/Y121F/T289A mutation (van der Linden et al. 2013).

In our research, among 19 clinical isolates 4 have shown in vitro azole resistance (21%). Isolates 64c and 94c were resistant only to POS. Given that the type of mutation impacts the MIC values of VOR (G54 point mutations don't affect VOR susceptibility) (Riat et al. 2018, Rybak et al. 2019), it is likely that these clinical isolates primarily acquired resistance due to extended azole therapy. However, other two isolates (41°C and 68°C) exhibited resistance to all azole drugs, suggesting the possibility of an environmental origin for their resistance. The resistance rate in clinical isolates in Azerbaijan (21%) is similar to rates reported from Netherlands. However, there is need for studies on larger number of patients.

According to our data, the overall prevalence of azole resistant *A.fumigatus* isolates in the environment of Azerbaijan Republic is 0.4%. Our findings are lower than those of the United Kingdom (6,7%) (Sewell et al. 2019), Iran (7.6%) (Nabili et al. 2016), Switzerland (9.3%) (Riat et al. 2018), Italy (16.9%) (Prigitano et al. 2019) and the Netherlands (20.4%) (Snelders et al. 2009). The resistant *A.fumigatus* isolate was detected from public garden. Notably, here is a list of some triazoles approved for agricultural use in the Azerbaijan Republic: propiconazole, difenconazole, penconazole, hexaconazole, diniconazole, tebuconazole, epoxiconazole, and flusilazole.

The environmental resistance is commonly associated with TR_{34}/L98H and TR_{46}/Y121F/T289A mutations which are accompanied by resistance to ITR and cross resistance to POS and VOR. In regions with a high environmental resistance rate (10%) a combination of echinocandin-azole/amphotericin B is recommended as first-line therapy (Ullmann et al. 2018). In regions with a low resistance rate (≤10%) the main problem is prevention of death from sporadic azole-resistant cases (Lestrade et al. 2019). MIC testing of clinical isolates from patients with IA/treatment failure should also be performed.

Genetic analysis revealed TR34/L98H mutation responsible for resistance only in one clinic isolate - 41c. The TR34/L98H mechanism is common in environmental isolates and is selected as a result of the use of azole fungicides in agriculture. The mutant isolate has shown resistance to all tested azoles (ITR, VOR, and POS).

In Azerbaijan the diagnosis of fungal diseases is made by microscopic, cultural, and histopathological examinations. The laboratories do not perform MIC susceptibility testing for fungal isolates; therefore, there is no data-related prevalence of resistant fungal isolates. Currently, the empiric treatment regimen for aspergillosis in Azerbaijan is azole therapy.

The main limitation of the study is the low number (50) of isolates investigated. The ESCMID-ECMM-ERS guidelines recommend to test at least ≥100 *A.fumigatus* isolates to obtain reliable epidemiological data (Ullmann et al. 2018). Thus, it is difficult to conclude if there is a need to change the therapy regimen currently used. Another limitation is the sequencing of fungal ribosomal DNA [internal transcribed spacer region (ITS)]. This method provides good identification at the section level but not at the species level (Lamoth 2016). It is not possible to evaluate the prevalence of mutant isolates based on data on 19 clinical isolates, since the number of isolates in the sample is insufficient to draw any statistically significant conclusions. However, the very presence of this type of mutation allows us to conclude that this type of resistance is quite widespread in the environment of the Republic of Azerbaijan. To confirm this assumption, it is necessary to conduct a study on a large number of isolates with the participation of a larger number of patients.

Conclusion

Despite limitations in sample size and testing methodologies, our findings reveal a lower environmental resistance rate (0.4%) compared to a higher rate found in clinical isolates (21%) in Azerbaijan. Further comprehensive studies involving a larger number of patient isolates are necessary to gain a comprehensive understanding of the epidemiological landscape and to better guide therapeutic approaches. To our knowledge, this is the first report of TR_{34}/L98H mutation in Azerbaijan Republic.

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Conflicts of interest

RMH, SSJ, AO, AAK, SMA, BB, EO, TBT, IK, FA, MM, IH, NI, GM, IV declare no conflicts of interest related to this work. DWD and family hold Founder shares in F2G Ltd, a University of Manchester spin-out antifungal discovery company. He acts or has recently acted as a consultant to Scynexis, Cidara, Quintiles, Pulmatrix, Pulmocide, Zambon, iCo Therapeutics, Roivant, and Fujifilm. In the last 3 years, he has been paid for talks on behalf of Astellas, Dynamiker, Gilead, Merck, Mylan, and Pfizer. He is a longstanding member of the Infectious Disease Society of America Aspergillosis Guidelines group, the European Society for Clinical Microbiology, Infectious Diseases Aspergillosis Guidelines group, and the British Society for Medical Mycology Standards of Care committee

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Code availability

Not applicable

Ethics approval

Not applicable

Authors contribution

All authors contributed to the study conception and design. The first draft of the manuscript was written by Ravil Huseynov and Ali Osmanov and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Consent to participate

Not applicable

Consent for publication

Not applicable

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