AZERBAIJAN REPUBLIC

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GENETIC ASPECTS OF AZOLE RESISTANT ASPERGILLUS FUMIGATUS STRAINS IN AZERBAIJAN

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ABSTRACT

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GENERAL DESCRIPTION OF THE WORK

Relevance of the problem and degree of elaboration. *Aspergillus fumigatus* is a ubiquitous mould causing a wide spectrum of conditions including colonisation, allergic bronchopulmonary aspergillosis (ABPA), severe asthma with fungal sensitization (SAFS), chronic pulmonary aspergillosis (CPA), and invasive aspergillosis (IA) and produces large amounts of spores. Inhaled spores are eliminated by the immune system of a healthy individual. 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[1]. Patients with immunosuppression and chronic pulmonary diseases (chronic obstructive pulmonary disease-COPD, pulmonary tuberculosis-PT, etc.) are the main risk groups for aspergillosis[2].

Triazoles (itraconazole - ITR, voriconazole - VOR, posaconazole - POS, and isavuconazole - ISA) are used in the treatment of diseases caused by fungi of genus *Aspergillus*. They act on enzyme 14α-demethylase coded by the *cyp51A* gene and block the synthesis of ergosterol, which is a component that contributes to the fluidity and permeability of the cell membrane. It results in altered membrane structure and permeability, leading to the accumulation of toxic methylated sterols in fungal cell.

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 consider VOR and ISA as first-line drugs in the treatment of IA. POS is recommended for the prevention of aspergillosis in patients with acute myeloid leukemia (AML) and myelodysplastic syndrome. *Infectious Diseases Society of America IDSA* recommends VOR for primary treatment (strong recommendation) and ISA/liposomal amphotericin B (AMB) as an alternative

[1] Dagenais, T. R., Keller, N. P. Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis // *Clin Microbiol Rev*, - 2009**,** *22* (3), p. 447-65

[2] Kosmidis, C., Denning, D. W. The clinical spectrum of pulmonary aspergillosis / *Thorax*, - 2015**,** *70* (3), p. 270-7.

therapy (moderate recommendation). Oral İTR and VOR are considered for the treatment of CPA[3].

Irrational use of azoles in treatment and agriculture resulted in the emergence of azole-resistant strains. The first resistant isolate was reported in 1997 and was associated with a point mutation in the *cyp51A* gene (M220R)[4]. Several mechanisms of azole resistance are known: 1) modification of 14α-demethylase; 2) increased expression of 14α-demethylase; 3) efflux mechanisms - increased expression of the efflux transporter Cdr1B; 4) alternative pathways for sterol synthesis; 5) extra- and intracellular breakdown of drugs.

Point mutations are commonly selected in patients by prolonged treatment with azoles[5]. 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 by the massive use of triazole fungicides in agriculture (TR_{34}/L98H, TR_{53}, and TR_{46}/Y121F/T289A) [6,7]. The mortality rate due to azole resistant isolates in patients with IA varies between 50-100%. Moreover, the mortality from azole-resistant isolates is 23%-31% higher compared to azole-susceptible individuals. As there is no specific risk factor that allows the prediction of azole resistance, both above mentioned guidelines (*ESCMID-ECMM-ERS* and *IDSA*) recommend an

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[3] Ullmann, A. J. Diagnosis and management of *Aspergillus* diseases: executive summary of the 2017 ESCMID-ECMM-ERS guideline/ Aguado, J. M., Arikan-Akdagli, S., Denning, D. W. [et al.] // *Clin Microbiol Infect*, - 2018, 24 Suppl 1, p. e1-e38.

[4] Denning, D. W. Itraconazole resistance in Aspergillus fumigatus / Venkateswarlu, K., Oakley, K. L., Anderson, M. J. [et al.] // *Antimicrob Agents Chemother* 1997, *41* (6), p. 1364-1368.

[5] Camps, S. M. Rapid induction of multiple resistance mechanisms in Aspergillus fumigatus during azole therapy: a case study and review of the literature / van der Linden, J. W., Li, Y., Kuijper, E. J. [et al.] // *Antimicrob Agents Chemother*, - 2012, *56* (1), p. 10-6.

[6] Snelders, E. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus* / Camps, S. M., Karawajczyk, A., Schaftenaar, G. [et al.] // *PLoS One*, - 2012, 7 (3):e31801, p. 1-11.

[7]Berger, S. Azole Resistance in *Aspergillus fumigatus*: A Consequence of Antifungal Use in Agriculture? / El Chazli, Y., Babu, A. F., Coste, A. T. [et al] // *Frontiers in Microbiology*, - 2017, 8:1024, p. 1-6.

epidemiological survey of azole resistance. In case of aspergillosis treatment failure, routine MIC (minimum inhibitory concentration) tests should be performed and if resistance to azole is detected, therapy should be adjusted. However, laboratories in low-resource settings and non-academic hospitals do not perform routinely antifungal susceptibility testing of moulds. Thus, empirical treatment regimens were recommended in regions with a prevalence of environmental azole resistance exceeding 10% threshold[3,8]. Large-scale studies have been carried out in European countries to identify resistance rates in clinical and environmental samples. Considering the relevance of the problem of azole resistance, conduction of a similar study in Azerbaijan Republic was necessary.

Object and subject of investigation. Epidemiological burden of fungal diseases in Azerbaijan Republic population (for year 2018) was estimated.

A prospective evaluation of the prevalence of resistant *A.fumigatus* strains in the environment of Azerbaijan Republic was carried out using 229 (218 soil and 11 air) environmental samples.

Prospective study was also conducted in 2 groups of patients: in a group of patients with all types (pulmonary diseases, otomycosis and onychomycosis) of diseases (sample n=163) and in a group of patients with only pulmonary diseases (sample n=1170).

Aim of investigation. Evaluation of epidemiology of aspergillosis and azole resistance in *A.fumigatus* and genetic analysis of *A.fumigatus* strains isolated from environment and clinical specimens.

Research objectives:

1. Retrospective assessment of burden of fungal pathologies, including aspergillosis, in Azerbaijan Republic;

2. Isolation of *A.fumigatus* strains from the environment and various clinical specimens and detection of resistant strains by broth microdilution (MIC) method;

3. Molecular analysis of *A.fumigatus* mutations responsible for azole resistance;

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[8] Verweij, P. E. International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus* / Ananda-Rajah, M., Andes, D., Arendrup, M. C. [et al] // *Drug Resist Updat*, - 2015**,** *21-22*, p. 30-40

4. Phylogenetic analysis of genetic relationship between environmental and clinical *A.fumigatus* strains.

Investigation methods: microscopic, cultural (mycological), molecular-genetic and **statistical** methods.

The key propositions brought forth for defense:

– according to the results of our calculations, the burden of fungal diseases in Azerbaijan Republic is quite high. Simultaneosuly, fungi of the genus *Aspergillus* are most often detected in patients with pulmonary pathologies. This indicates the significance of the problem of fungal pathologies in the country;

– the frequency of detection of resistant strains among clinical strains in Azerbaijan Republic is quite high, while the prevalence of resistant strains in the environment of Azerbaijan Republic is low;

– TR_{34}/L98H mutation are responsible for azole resistance in *A.fumigatus* strains in Azerbaijan. This mutations are selected under the pressure of azole fungicides used in the agricultural industry.

Scientific novelty of the investigation:

– for the first time in Azerbaijan the burden of fungal diseases, in particular aspergillosis, was estimated;

– for the first time in Azerbaijan azole resistance rates in environment and clinical specimens were evaluated;

– for the first time in Azerbaijan a genetic mutation responsible for resistance to azoles has been detetcted;

– for the first time in Azerbaijan epidemiological relationship between environmental and clinical *A.fumigatus* strains was studied.

Scientific and practical significance of the investigation:

– determining of resistance pattern will enable improvement of prevention and treatment programs. Obtained results will contribute to reduction of the mortality rate from infections caused by *Aspergillus* strains and reduction of patient treatment costs;

– comparative analysis of mutations of strains isolated from environment and clinic samples will enable detection of infection routes and causes of emergence of resistant strains;

– development of measures regarding the control of use of fungicidal agents will help to reduce the frequency rate of resistant strains.

Approbation and application of dissertation results:

The dissertation materials were discussed: in the scientific and practical conference "Təbabətin Aktual Problemləri", dedicated to the 100th anniversary of the Azerbaijan People's Republic (Baku, 2018), in the collection of scientific works of the Scientific Research Institute of Medical Prophylaxis, dedicated to the 100th anniversary of the Azerbaijan People's Republic (Baku, 2018), a collection of materials from the International Scientific Conference dedicated to the 85th anniversary of Rafig Ashraf oglu Askerov (Baku, 2018), a scientific conference "9th Trends in Medical Mycology" (Nice, 2019), materials of the international scientific and practical congress dedicated to the 90th anniversary of the Azerbaijan Medical University (Baku, 2020), at the online conference ASM Microbe Online (2020), scientific conference "9th Advances Against Aspergillosis" (Lugano, 2020), scientific conference "Integration of Education, Science and Business in Modern Environment: Winter Debates" - International Electronic Scientific and Practical Journal "WayScience" (2020), scientific conference «10th Trends in Medical Mycology» (Aberdeen, 2021), «1st international Azerbaijan Laboratory medicine congress & LAB EXPO» (Bakı, 2023).

Discussion of the dissertation work was performed at the inter-departmental meeting of "Medical Microbiology and Immunology" "Infectious Diseases", "Child and Adolescent Health, and Occupational Health" "Biological chemistry" departments of Azerbaijan Medical University (06.12.2021; Protocol No. 04). The dissertation was presented and discussed at scientific seminar of the Dissertation Defense Council BED 4.19, operating at the Azerbaijan Medical University (15.12.2023, Protocol No. 02)

Published scientific works: Based on the results of the dissertation work, 19 scientific works were published: 9 articles, 4 of which were published in foreign journals; 10 abstracts, 5 of which were presented at foreign conferences.

Application of the results. The main concepts of the dissertation are used in the educational process at the department of Medical Microbiology and Immunology of the Azerbaijan Medical University.

Organization in which the dissertation work was carried out: Scientific-Research Educational-Clinical Microbiological

Laboratory of the department of Medical Microbiology and Immunology of Azerbaijan Medical University.

Structure and volume of dissertation:

The dissertation is written in Russian in A4 format, Times New Roman 14 font with line spacing 1.5. Consists of Contents (2595 characters), Introduction (14939 characters), 4 chapters covering Literature Review (49408 characters), Materials and Methods (47480 characters), Research Results (32535), Discussion of the obtained results (41077 characters), Results (1364 characters), Practical recommendations (1311 characters), Used literature and a list of abbreviations (3086 characters). The volume of the dissertation is 177 pages (193795 characters). The dissertation is illustrated with 26 tables, 29 figures and 3 diagrams. The Used references consists of 207 sources, most of which are in English.

MATERIALS AND METHODS OF INVESTIGATION

***Estimation of the burden of fungal diseases*.** As part of the dissertation work, an assessment of the total burden of fungal diseases was performed and the proportion of *Aspergillus*-associated diseases was calculated. Epidemiological papers were searched in international databases (Pubmed, Google Scholar, and elibrary.ru). The search terms included "fungal infections and Azerbaijan", specific underlying conditions (e.g., "bronchial asthma", "chronic obstructive pulmonary disease", etc.), and specific fungal infections (e.g., "invasive aspergillosis"). Since no papers related burden of fungal infection were found, the LIFE (Leading International Fungal Education) model was utilized, which uses population at-risk to estimate the burden of fungal infections. The principle of the modeling used is calculation of the burden of fungal pathologies among high-risk group patients based on literature data providing the prevalence rates of fungal infections among different groups of patients[9].

Prospective analysis. The prospective analysis aimed to estimate the rates of *Aspergillus spp*. isolation in different patient groups, including those with and without pulmonary diseases. This involved comparing

[9] Osmanov, A., Denning, D. W. Burden of serious fungal infections in Ukraine // *Mycoses,* - 2015**,** *58 Suppl 5*, p. 94-100.

isolation frequencies between patients with pulmonary and extra-pulmonary pathologies, and within specific pulmonary disease groups.

Chi-square ($\chi 2$) test/Fisher's exact test and binary logistic regression were employed. The Chi-square (or Fisher's exact test for small samples) determined the statistical significance of *Aspergillus spp*. and *A.fumigatus* isolation across different pathologies.

Binary logistic regression was used to identify the association between the independent variables (gender, age, and patient disease) and the dependent variable (growth or absence of growth *of A.fumigatus* strains). The regression analysis generated the odds ratio (OR) of *A.fumigatus* growth compared to absensce of growth, considering patient diagnoses, gender, and age. This method modeled the probability of *A.fumigatus* strains isolation depending on patient gender, age, and diseases.

Isolation of Aspergillus strains. Clinical samples from patients applied to the Scientific-Research Educational-Clinical Microbiological Laboratory, 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. Obtained specimens were inoculated on 2 plates with Sabouraud Dextrose Agar (SDA) agar (Pronadisa, Spain) with chloramphenicol (0.5 g/l) and incubated at 37°C for 7 days. Colonies resembling *Aspergillus spp.* were investigated using a lactophenol cotton blue mount (LCBM) preparation[10]. Morphological identification was performed in accordance with taxonomic keys and guides[11]. All isolates phenotypically identified as *A.fumigatus* complex were further incubated at 48 °C to exclude cryptic species[12] and processed for final identification using internal transcribed spacer regions 1 and 4 (ITS1 and ITS4)[13].

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[10] Leck, A. Preparation of lactophenol cotton blue slide mounts // *Community Eye Health*, - 1999**,** *12* (30), p. 24.

[11] McClenny, N. Laboratory detection and identification of *Aspergillus* species by microscopic observation and culture: the traditional approach // *Med Mycol*, - 2005, *43 Suppl 1*, p. S125-8.

[12] Riat, A. Azole Resistance of Environmental and Clinical *Aspergillus fumigatus* Isolates from Switzerland / Plojoux, J., Gindro, K., Schrenzel, J. [et al] // *Antimicrob Agents Chemother*, - 2018, 62 (4):e02088-17, p. 1-7

[13] Schoch, C. L. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi / Seifert, K. A., Huhndorf, S., Robert, V. [et al.] // *Proc Natl Acad Sci U S A*, - 2012**,** 109 (16), p. 6241-6.

Environmental samples were collected from eight regions of Azerbaijan Republic (Baku-Absheron, Daghlig Shirvan, Ganja-Qazakh, Shaki-Zaqatala, Lankaran, Quba-Khachmaz, Aran and Nakhchivan) during 2017-2019 (Figure 1). A total of 229 samples were collected, 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 SDA plates with chloramphenicol (0.5 g/l). Plates were incubated for 72 hours at 37° C[13,14.] Morphological and molecular genetic identification was conducted similarly to the procedures for clinical strains described above.

Molecular-genetic identification of strains. Molecular-genetic identification of strains was performed in Recombinant *DNA* and Recombinant Protein Center (REDPROM, Adnan Menderes University, Aydin, Turkey).

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 30 s, extension step at 72°C for 1 min, and final extension at 72°C for 8 min were performed. The amplicons obtained were separated by agarose gel electrophoresis. All amplified ITS fragments were sequenced (Macrogen, http://dna.macrogen.com/eng/)[14]. Sequences were identified by sequence homology using BLAST (Basic local Alignment Search Tool) which compares obtained sequences with the genebank at http://www.blast.ncbi.nlm.nih.gov.

Antifungal susceptibility testing. All *A.fumigatus* isolates were tested for susceptibility to ITR, VOR, and POS according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[15] method. Each antifungal was diluted in double strength RPMI1640 medium with 2% glucose and dispensed (100µl) in microdilution wells. Inoculum suspension was prepared by covering 2-5-day old culture on SDA with sterile water supplemented with 1% Tween 20. The suspensions of the spores were adjusted to 2 to 5 x 10^6 conidia/ml by counting in a hamocytometer 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.

Molecular-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[16].

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[14] Oryaşın, E. Antimicrobial susceptibility patterns of environmental and hospital isolations of enterococci in Aydın / Biyik, H. H., BaşbülbüL, G., BozdoğAn, B. // *Turkish Journal of Biology*, - 2013, 37, p. 514-519.

[15]Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds / EUCAST definitive document E.DEF9.3.2. - 2020.

[16] Snelders, E. Emergence of Azole Resistance in *Aspergillus fumigatus* and Spread of a Single Resistance Mechanism / van der Lee, H. A. L., Kuijpers, J., Rijs, A. [et al.] // *Plos Medicine,* - 2008**,** *5* (11), p. 1629-1637.

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 The obtained mycelial mats were used for DNA extraction. The complete coding sequences of the genes *cyp51A* and *cyp51B* were sequenced. To exclude the possibility of sequence alterations related to the PCR amplification process, each isolate was analyzed twice[17,18].

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.

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[17] Mellado, E. A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations / Garcia-Effron, G., Alcázar-Fuoli, L., Melchers, W. J. [et al.] // *Antimicrob Agents Chemother,* - 2007**,** *51* (6), p. 1897-1904.

[18] Diaz-Guerra, T. M. A point mutation in the 14alpha-sterol demethylase gene cyp51A contributes to itraconazole resistance in *Aspergillus fumigatus* / Mellado, E., Cuenca-Estrella, M., Rodriguez-Tudela, J. L. // *Antimicrob Agents Chemother,* - 2003**,** *47* (3), p. 1120-1124.

PCR was carried out in a volume of 50 μL containing 10 mM (NH4) 2SO4, 10 mM KCl, 20 mM Tris-Cl (pH 8.8), 2 mM MgSO4, 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). PCR products were analyzed by electrophoresis on 0.8 or 1.3% agarose gels and visualized by transillumination after staining with ethidium bromide.

Detection of mutants was performed through comparison of obtained sequences with *cyp51A* sequence under accession number AF338659 of GenBank[17,18].

Phylogenetic analysis of strains. Phylogenetic analysis of strains was performed to detect genetic similarities between isolates. The ClustalW algorithm was used to determine similar DNA sequences[19]. For this purpose, the neighbor joining method was applied[20]. Phylogenetic distances were calculated using the maximum likelihood method[21]. The above mentioned analyzes were carried out in the MEGA X software package[22].

RESULTS OF INVESTIGATION AND DISCUSSION

The burden of fungal infections in Azerbaijan. The estimated burden of fungal infections was 225974 (2.3%) (table 1)[23].

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[19] Thompson, J. D., Higgins, D. G., Gibson, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice // *Nucleic Acids Res,* - 1994**,** *22* (22), p. 4673-80.

[20] Gascuel, O., Steel, M. Neighbor-joining revealed // *Mol Biol Evol,* - 2006**,** *23* (11), p. 1997-2000.

[21] Kannan, L., Wheeler, W. C. Maximum Parsimony on Phylogenetic networks // *Algorithms for Molecular Biology,* - 2012**,** *7* (1), p. 9.

[22] Kumar, S. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms / Stecher, G., Li, M., Knyaz, C. [et al.] // *Molecular Biology and Evolution,* - 2018**,** *35* (6), p. 1547-1549.

[23] Huseyov R.M. The burden of serious fungal infections in Azerbaijan / Javadov S., Osmanov A., Khasiyev S [et al.] // *Therapeutic Advances in Infectious Disease*, 2021, 8:20499361211043969 doi:10.1177/20499361211043969

Aspergilloses accounted for approximately 3.5% of the total number of fungal pathologies. The most common pathology was recurrent vulvovaginal candidiasis (rVVC), comprising approximately **70%** of all fungal pathologies.

Table 1

The burden of fungal infections in Azerbaijan

	Number of patients with underlying disorder					Rate	Total burden
	None	Respiratory	HIV/ AIDS	Cancer/ Tx	ICU		
Oesophageal candidiasis	–	–	579	–	–	5.8	579
Oral candidiasis	–	–	808	–	–	8.1	808
Candidemia	–	–	–	–	499	5	499
Candida peritonitis	–	–	–	–	75	0.75	75
RVVC	159489	–	–	–	–	3196	159489
ABPA	–	4927	–	–	–	49.4	4927
SAFS	–	6504	–	–	–	65.2	6504
CPA after TB	–	577	–	–	–	5.8	-
CPA total	–	2307	–	–	–	86.8	2307
IA	–	36	–	81	577	7.0	693
Cryptococcal meningitis	–	–	5	–	–	0.05	5
Pneumocystis pneumonia	–	–	55	–	–	0.55	55
Mucormycosis	–	–	–	20	–	0.2	20
Fungal keratitis	?	-	-	?	-	0.12	12
Allergic fungal sinusitis	50000					50	50000
Total burden	209489	14,351	1,447	101	1151	–	225974

Abbreviations. None – population at risk without underlying condition, HIV – human immune deficiency virus, AIDS – acquired immune deficiency syndrome, ICU – Intensive care unit, ABPA – allergic bronchopulmonary aspergillosis, SAFS – severe asthma with fungal sensitization, IA – invasive aspergillosis, CPA – chronic pulmonary aspergillosis, RVVC – recurrent vulvovaginal candidiasis

If exclude the proportion of rVVC, the proportion of aspergilloses among other fungal pathologies was **12%**, which is a notably high figure.

The burden of infections is underestimated due to insufficient epidemiological data on this problem from the Caucasus region.

Another issue is the complexity of differential diagnosis between fungal pathologies and underlying conditions related to their similar clinical manifestations. Thus, often a complex of diagnostic investigations should be performed for correct and timely diagnosis.

Observation of tissue invasion by filamentous fungi in biopsy/autopsy specimens provides a definitive (proven) diagnosis of invasive fungal infection. When it is impossible to identify fungi by microscopy, confirmation using culture or molecular methods is required.

However, culture methods have low reported sensitivity for detection of fungi in specimens. On the other hand, tissue biopsy and histopathological examination is not possible in immunosuppressed patients with low platelet counts. Moreover, patients sometimes refuse invasive procedures. Serological diagnostic methods like galactomannan and β-D-glucan are very helpful in this situation.

Better recognition of the pervasive problem of fungal infections would help to develop preventive and therapeutic measures and establishment of mycological reference laboratory in Azerbaijan.

Results of prospective analysis. The prevalence of resistant *A.fumigatus* strains in the environment of Azerbaijan Republic was assessed. Geographical distribution of strains is shown in Figure 1. Thirty-one strains were isolated from 229 environmental (218 soil and 11 air) samples (table 2).

The prospective study encompassed also two patient groups: one comprising individuals with all concomitant diseases (sample n=163), while the other focused solely on patients with pulmonary pathologies (sample n=1170).

Table 2

***A. fumigatus* strains, isolated from environment**

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

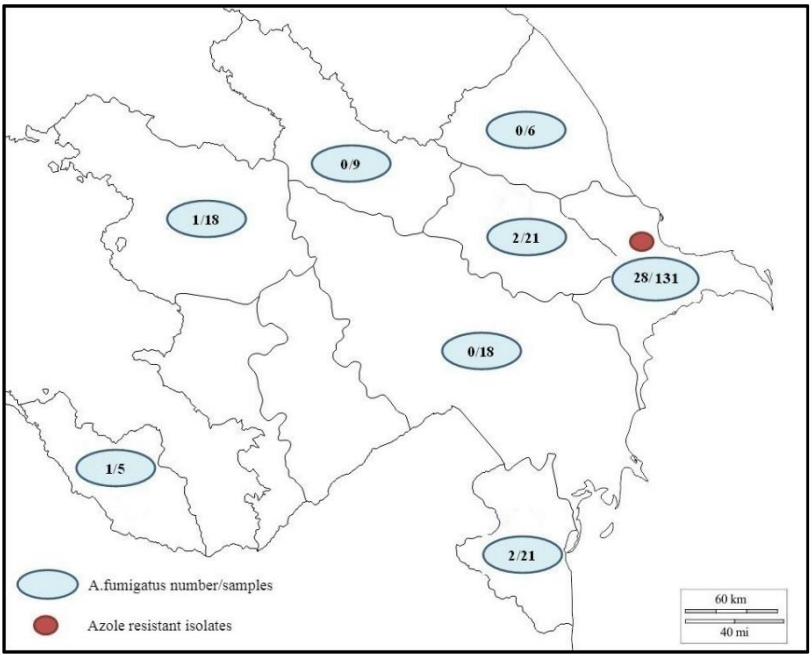

Figure 1. Distribution of 31 environmental *A.fumigatus* isolates obtained from environment of 8 regions of Azerbaijan Republic

Notes: blue circles: *A.fumigatus* isolates/samples investigated. Red circle: area with azole resistant isolates.

Within the first group, the average age of patients stood at 41.8 (±18.4) years. 165 *Aspergillus* strains were isolated: 87 *A.niger* strains, 28 *A. flavus* strains, 19 *A.fumigatus* strains, and 2 of *A. terreus* strains. 29 isolates were identified the genus level.

A graphical representation depicting the distribution of *Aspergillus* species across various patient groups is illustrated in Diagram 1.

A.niger was notably prevalent among patients with otitis externa (71%) and pulmonary diseases (56%). The second most frequently encountered species among patients with pulmonary disease and otitis externa were *A.fumigatus* (24.0%) and *A.flavus* (23.6%), respectively.

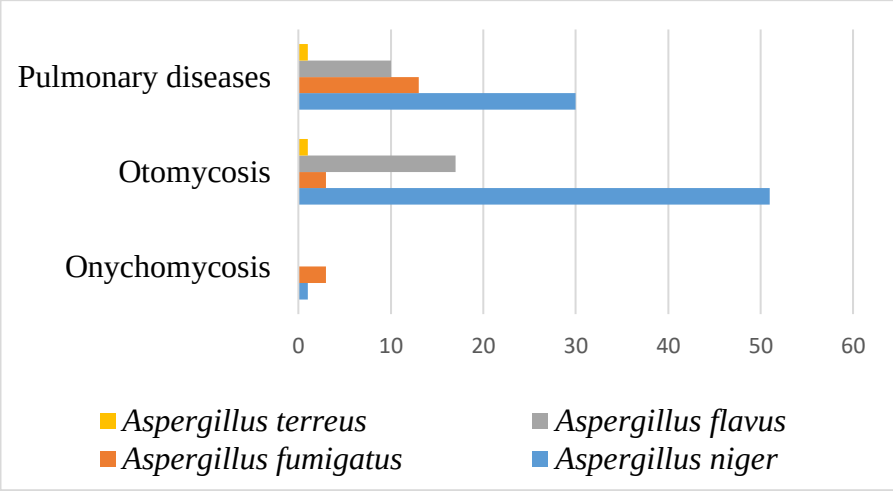

Diagram 1. Distribution of *Aspergillus* spp. in different groups of patients

Table 3 compares the statistical significance of differences in the prevalence of *A.fumigatus* and *Aspergillus spp*. in each patient group.

Statistically significant difference in prevalence of *A.fumigatus* and *Aspergillus spp.* were observed for PT ($p=0.01$), COPD ($p<0.01$) and otomycosis ($p<0.01$). In other words, the likelihood of detecting A. fumigatus compared to Aspergillus spp. high in patients with COPD and low in patients with otomycosis and PT.

Table 3

***A.fumigatus* and *Aspergillus spp.*, isolated from patients with different diseases**

Disease	*A.fumigatus (19)*	*Aspergillus spp. (146)*	*P*
COPD	5	3	<0.01
PT	4	5	0.02
BA	1	6	1.000
Otomycosis	3	88	<0.01
Other	6	44	1.000

Notes: COPD-chronic obstructive pulmonary disease, PT-pulmonary tuberculosis, BA-bronchial asthma

At the same time, in patients with other pulmonary diseases (BA) no significant difference in isolation rates of *A.fumigatus* and *Aspergillus spp*. has been observed ($p \geq 0.05$).

The results of binary regression analysis showed that a statistically significant risk factor for *A.fumigatus* isolation is COPD (Odds Ratio 14.056 95% 2.533-69.251, p=0.01). It is noteworthy that the results of logistic regression showed that the chances of isolation of *A.fumigatus* compared with other groups of patients is approximately 5 times lower in PT patients (OR 0.188 95% 0.043-0.816, p = 0.03), and 7 times lower in patients with otomycosis (0.137 95% 0.037-0.516, p=0.03).

Regression analysis has revealed that, indicators such as gender, age and the presence of other diseases did not have a significant effect on the outcome (isolation of *A. fumigatus*).

The second group consisted of 1170 patients diagnosed with various pulmonary diseases, with a mean age of 48.8 (±18.15). 22 *Aspergillus spp.* isolates were detected in 22 patients. The strains were isolated from patients with COPD (7), BA (7), PT (4) and other pulmonary conditions (acute respiratory failure, bronchiectasis, and exudative pleuritis).

Four *Aspergillus* species were isolated: *A.niger*, *A.fumigatus*, *A.flavus*, and *A.terreus*. The predominant isolates were *A.niger* (12 cases) and *A. fumigatus* (7 cases), as detailed in Table 4 and illustrated in Figure 2.

Table 4

***Aspergillus* strains associated with pulmonary diseases**

Disease	*Число больных*	*A.niger* (12)	*A.fumigatus* (7)	*A.flavus* (2)	*A.terreus* (1)
COPD	178	2	5	0	0
PT	195	1	2	0	1
BA	185	7	0	0	0
Other	8	2	0	1	0

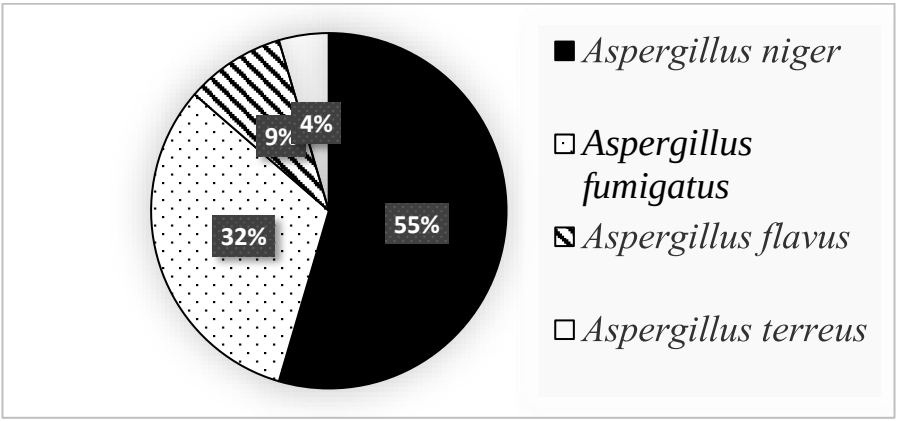

Figure 2. Proportion of isolated *Aspergillus* strains

A significant correlation was observed between *A.niger* and BA, while *A.fumigatus* prevailed in patients with COPD and PT (5 and 2 strains, respectively).

Aspergillus spp. isolation rate in patients with COPD was 3.9% (in 7 out of 178 patients), in patients with PT - 2.0% (in 4 out of 195 patients), in patients with BA - 3.8% (in 7 of 185 patients).

The results of the Chi-square ($\chi 2$) test/Fisher's exact test showed an association between COPD ($p=0.01$) and *A.fumigatus* isolation.

Both tests (Chi-square ($\chi 2$)/Fisher's exact test and binary logistic regression) showed absence of statistically significant difference in *A.fumigatus* isolation for patients with PT ($p=0.33$ and $p=0.2$, respectively).

In addition, the Chi-square ($\chi 2$)/Fisher's exact test showed an association between BA ($p<0.001$) and *A.niger* isolation. When using *A.niger* instead of *A.fumigatus* as the dependent variable, regression analysis also indicated a relationship between *A.niger* isolation and BA (OR 7.303 95% 2.238-23.839, $p<0.001$).

Prevalence of azole resistance in Azerbaijan. A total of 50 A. fumigatus strains were analysed, comprising 19 obtained from clinical samples and 31 sourced from the environment. The findings from the antimycotic susceptibility test are outlined in Table 5. All clinical isolates were obtained from patients admitted to hospitals in Baku, capital of Azerbaijan.

Table 5

Resistant strains from clinical specimens and environment

Source (n)	Antifungal agent	Resistant strains number
Clinical isolates (19)	İTR+VOR+POS	2
	POS	2
Environmental isolates (31)	İTR+VOR+POS	1

Among the 50 isolates, three (41c, 68c, and 118e) were resistant to three different medical azoles, while two (64c and 94c) exhibited resistance solely to POS. Notably, only one environmental isolate (118e) exhibited resistance to all azoles (MIC range 1 to ≥8 mg/l). A resistant isolate was found in the Baku-Absheron (eastern) region. Among 19 tested clinical isolates two were (41c and 68c) resistant to all azoles (MIC range 2 to ≥16 mg/l) and two isolates (64c and 94c) were resistant only to POS (MIC=0.5 mg/l).

The isolation rate of resistant strains among clinical strains was relatively high – 21 %. Possibly, the main reason for this is the small number of strains tested (19).

According to our data, the overall prevalence of azole resistant *A fumigatus* isolates in the environment of Azerbaijan Republic is 0.4%. The resistant *A. fumigatus* isolate was isolated from public garden. The environmental resistance is commonly associated with TR_{34}/L98H and TR_{46}/Y121F/T289A mutations which are

accompanied by resistance to itraconazole and cross resistance to posaconazole and voriconazole. In regions with a high environmental resistance rate (10%) a combination of echinocandin-azole+AMB is recommended as first-line therapy[3]. In regions with low rates of resistance, the main challenge is to prevent deaths from sporadic cases of azole-resistant isolates. In regions with low resistance rate (≤10%) the main problem is prevention of death from sporadic azole-resistant cases[24]. MIC testing of clinical isolates from patients with IA/treatment failure should also be performed.

Molecular-genetic analysis of resistant strains. The gene *cyp51A* was sequenced of five *A.fumigatus* strains exhibited phenotypical *in vitro* resistance to azole drugs: four strains - from clinical samples (41c, 64c, 68c, 94c), and one - from the environment (118e). Point mutations of the *cyp51A* gene were identified in four out of the five strains. Only strain 41c exhibited a combined **TR_{34}/L98H** mutation—a combination of tandem repeats in the promoter region along with the point mutation. Detection of this mutation marks the first evidence of azole-resistant *A.fumigatus* isolate in Azerbaijan. This mutation type has been observed in several European countries and is linked to environmental factors. It is believed that strains carrying this mutation are selected in the environment due to the pressure of azole drugs used in agriculture. In our study, the strain with this type of mutation was identified in a clinical sample.

Results of our investigation suggest that selection of resistant *A.fumigatus* strains can occur both during patient treatment and in the environment. This highlights the importance of a comprehensive approach to the problem of antimicrobial resistance, including both clinical practice and control strategies in agriculture and the environment. However, more thorough research is needed to fully understand the sources and mechanisms of antimicrobial resistance.

[24] Lestrade, P. P. A. Triazole resistance in *Aspergillus fumigatus*: recent insights and challenges for patient management / Meis, J. F.. Melchers, W. J. G., Verweij, P. E. pet al.] // Clin Microbiol Infect, - 2019, 25 (7), p. 799-806.

The presence of resistant *A.fumigatus* strains in clinical specimens highlights the need for continuous monitoring and development of strategies to control the spread of antimicrobial resistance. It is important to note that the study provides primary data on azole-resistant isolates in Azerbaijan, and its results can serve as a starting point for a deeper analysis of the factors contributing to the development of resistance, as well as for the development of effective strategies to counter this phenomenon.

Phylogenetic analysis of Aspergillus strains. Analysis of the epidemiological relationship between clinical and environmental isolates was carried out using the MEGA X software package[23]. Eight clinical and 31 environmental strains were examined. Among the environmental isolates, 19 were sourced from public parks, 7 from soil and air within hospital area, and 4 from agricultural regions. The resulting phylogenetic tree from this analysis is depicted in Figure 2[25].

The analysis revealed the correlation between five clinical strains (22c, 39c, 41c, 52c, and 55c) and strains 54e and 128e from the environment, specifically public gardens. Strain 64c exhibited relationships with strains 57e, 91e, and 94e, while strain 42c displayed relationship with strains 43e and 144e sourced from public gardens. Notably, strain 73c significantly differed from the environmental strains. Particularly noteworthy was the isolation of strains 57e and 91e from soil samples within the hospital area, suggesting the possibility that patients were infected with environmental strains or, conversely, were the source of the strains themselves.

Our study has confirmed the presence of resistant strains and mutation responsible for resistance in Azerbaijan. This particular strain exhibited in vitro resistance to all tested azoles (ITR, VOR, POS). Considering azole drugs as first-line treatments for aspergillosis, the existence of this mutation poses a significant challenge in managing this pathology.

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[25] Гусейнов Р.М. Филогенетический анализ штаммов *Aspergillus fumigatus*, изолированных из клинических образцов и окружающей среды Азербайджана // *Azərbaycan Tibb Jurnalı*, - 2020, (специальный выпуск), с. 151-155.

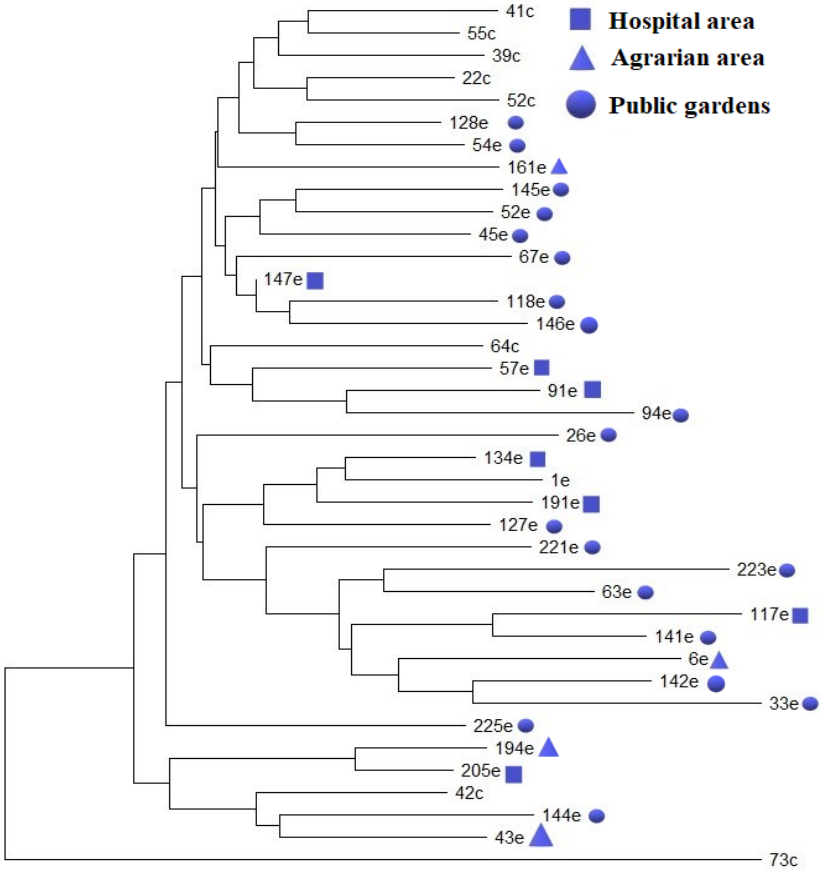

Figure 2. Phylogenetic tree of 39 *A.fumigatus* isolates
Notes: e- environmental isolates, c-clinic isolates.

From the clinical samples, we identified 19 strains, among which 4 showed in vitro resistance (21%), indicating a relatively high level of resistance. Only one strain (5%) among the 19 exhibited a mutation responsible for resistance to all tested azoles. The sample size of 19 clinical strains isn't sufficient for statistically significant conclusions regarding the prevalence of mutant strains. However, the mere existence of this mutation type suggests that such resistance is widespread in Azerbaijan's environment. To validate this assumption, a study involving a larger number of strains and patients is imperative.

Our research achieved several objectives outlined in this dissertation, including: 1) isolation of *A.fumigatus* strains; 2) detection of azole resistant *A.fumigatus* strains; 3) identification of mutations responsible for resistance; 4) evaluation of correlation between environmental and clinical samples.

Our findings are similar to research conducted in European countries, indicating that the predominant origin of resistance stems from the environmental selection of resistant strains influenced by azole fungicides. This process fosters the proliferation of tandem repeat-point mutation type mutations.

We advocate for the integration of these research outcomes into national medical practices. Moreover, the establishment of a reference mycological laboratory specialized in identification ofresistant fungal strains has become crucial.

RESULTS

1. In 2018 in Azerbaijan 2.3% of population (225974 people) suffered from fungal diseases. 7927 people had aspergillosis, including 4927 patients with allergic bronchopulmonary aspergillosis (ABPA), 2307 patients with chronic pulmonary aspergillosis (CPA) and 36 patients with invasive aspergillosis (IA).
2. Analysis of clinical samples from 1170 patients with pulmonary diseases revealed 1.9% isolation rate of *Aspergillus spp*. in this group of patients. *Aspergillus* strains were detected in 3.9% of patients with chronic obstructive pulmonary disease (COPD), 2.0% of patients with pulmonary tuberculosis (PT) and 3.8% of patients with bronchial asthma (BA).
3. Frequency of resistant strains among clinical strains in Azerbaijan is relatively high and amounts to 21%. 19 clinical strains were isolated, of which 4 showed resistance to azoles (41c, 64c, 68c, 94c).
4. The prevalence of resistant strains in the environment of Azerbaijan is low and amounts to 0.4% (one out of 229 environmental samples).

5. For the first time in Azerbaijan Republic, the TR_{34}/L98H mutation, widespread in European countries and originating from the environment, was detected. It is believed that this "tandem repeat-point mutation" was selected by exposure to azoles used in the agricultural industry.
6. Phylogenetic analysis of 39 *A.fumigatus* strains in our study suggests the possibility of an epidemiological link between strains from the environment and clinical samples.

PRACTICAL RECOMMENDATIONS

1. The primary method used in diagnostics of fungal pathologies in Azerbaijan is cultural method. An important goal for our country is to enhance diagnostic capabilities, achievable through two approaches: i) establishing a reference mycological laboratory; ii) equipping major clinical centers with necessary diagnostic tests.stic tests.
2. In Azerbaijan, individuals at primary risk for aspergillosis are patients with pulmonary diseases such as COPD, PT, BA. Given this fact, systematic monitoring of these patients for the diagnosis of Aspergillus spp. infections is cruci
3. The prevalence of resistant strains in the environment of the Republic of Azerbaijan is low (0.4%). At this level of resistance, empirical treatment without routine antifungal susceptibility testing is recommended.
4. The TR_{34}/L98H mutation was isolated from a clinical sample of a patient with pulmonary tuberculosis (PT). Strains with this mutation do not respond to empirical treatment. Therefore, in cases of suscpected azole resistance (lack of response to empirical treatment), testing for antifungal susceptibility of *Aspergillus* strains is recommended.
5. The presence of an epidemiological link between strains from the environment and clinical samples dictates the need to study the extent of azole fungicide use in Azerbaijan.

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ABBREVIATIONS

1. ABPA – allergic bronchopulmonary aspergillosis;
2. ABPM – allergic bronchopulmonary mycosis;
3. AMB – Amphotericin B;
4. BA - Bronchial Asthma;
5. BAL - Bronchoalveolar Lavage
6. BAMS – bronchial asthma with mycotic sensitization;
7. HIV – Human Immunodeficiency Virus;
8. VOR - Voriconazole;
9. HSC - Hematopoietic Stem Cell;
10. IA - Invasive Aspergillosis;
11. IFI – invasive fungal infections;
12. ISA - Isavuconazole;
13. ITR - Itraconazole;
14. PDA – Potato Dextorse agar;
15. CT – computer tomography;
16. ETCP - Epithelial Cells of the Respiratory Tract;
17. PT - Pulmonary Tuberculosis;
18. MIC - Minimum Inhibitory Concentration;
19. MEC – minimum efficacy concentration;
20. NTM - Non-Tuberculous Mycobacteriosis;
21. AML - Acute Myeloid Leukemia;
22. ICU - Intensive Care Unit;
23. SAIA- Subacute Invasive Pulmonary Aspergillosis;
24. POS - Posaconazole;
25. PCP - Pneumocystis Pneumonia;
26. PCR - Polymerase Chain Reaction;
27. RVVC - Recurrent Vulvovaginal Candidiasis;
28. GVHD - Graft-versus-Host Disease;
29. SDA – Sabouraud Dextrose agar;
30. AIDS - Acquired Immunodeficiency Syndrome;
31. TAFS - Severe Asthma with Fungal Sensitization;
32. CGD - Chronic Granulomatous Disease;
33. CPA - Chronic Pulmonary Aspergillosis;
34. CNPA - Chronic Necrotizing Pulmonary Aspergillosis;

35. CCPA - Chronic Cavitary Pulmonary Aspergillosis;
36. CFPA - Chronic Fibrosing Pulmonary Aspergillosis;
37. FLU - Fluconazole;
38. BLAST – Basic Local Alignment Search Tool;
39. CDC – Centers for Disease Control and Prevention;
40. CORE – Chronic Obstructive Respiratory diseases in CIS countries;
41. E-тест – эпсилометрический градиент тест;
42. ECDC – European Centre for Disease Control;
43. ECOFF – epidemiological cut-off values;
44. EORTC/MSG -European Organization for Research and Treatment of Cancer and the Mycoses Study Group;
45. ESCMID-ECMM-ERS – European Society of Clinical Microbiology and Infectious Diseases/ European Confederation of Medical Mycology/European Respiratory Society;
46. EUCAST – European Committee on Antimicrobial Susceptibility Testing;
47. FISH – Fluorescent *in situ* hybridization;
48. GOLD – Global İnitiative for Obstructive Lung Diseases;
49. IDSA – Infectious Diseases Society of America;
50. ITS – internal transcribed rDNA spacer regions;
51. RPMI (Roswell Park Memorial Institute medium);
52. TR_{34}/L98H – сочетание тандемных повторов (tandem repeats) в промоторном регионе гена *Cyp51A* с точечной мутацией;
53. TRANSNET – Transplant-Associated Infection Surveillance Network

The defence will be held on "21" February 2024 at 16^{00} at the meeting of the Dissertation Council BED 4.19 of Supreme Attetation Comission under the Preesident ofnthe Rpublic of Azerbaijan operating at Azerbaijan Medical University

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