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Diagnostic Significance of *Aspergillus Species* Isolated from Clinical Specimens

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Abstract. Aspergillosis is an infection caused by *Aspergillus*, ubiquitous mold found in indoor and outdoor air. In patients with immune suppression and underlying lung diseases *Aspergillus* spores are able to cause various pathologic conditions. Annually 200000 new cases of invasive aspergillosis (IA) are registered. Mortality due to IA in chronic obstructive pulmonary diseases (COPD) patients varies within 72-95%. 3 million people suffer from different forms of chronic pulmonary aspergillosis (CPA). The aim of the investigation was to determine the incidence of *Aspergillus* species and their diagnostic significance in clinical samples. Specimens of 1170 patients were investigated for the presence of *Aspergillus spp.* for period from august 2017 to august 2018. The collected specimens were inoculated onto Sabouraud dextrose agar with chloramphenicol and incubated at 37 ˚C temperature for 10 days. In case of growth of mold colonies, further identification was performed based on macroscopic and microscopic features. Growth of *Aspergillus spp.* colonies were detected in 22 patients (1.88%). 16 of them were males, 6 – females. 7 patients had COPD, 7- bronchial asthma (BA), 4 – pulmonary tuberculosis (PT), 1 – acute respiratory insufficiency, 1 – bronchiectatic disease, 1 – exudative pleurisy and 1 – acute leucosis. Four different species were isolated: *Aspergillus niger*, *A. fumigatus*, *A. flavus,* and *A. terreus*. *A. niger* commonly was associated with BA, while in patients with COPD and PT prevailed *A. fumigatus*. We consider that in patients with COPD and PT risk of aspergillosis should be taken into account in order to provide them with appropriate treatment and reduce mortality rates

Key words. *Aspergillus spp.*, invasive aspergillosis, chronic pulmonary aspergillosis, chronic obstructive pulmonary disease, pulmonary tuberculosis

Klinik Örneklerden İzole edilen *Aspergillus* Türlerinin Tanısal Önemi

Öz: Aspergilloz, kapalı alanlarda və çevrede bulunan *Aspergillus* mantarı ile oluşan enfeksiyondur. İmmune sistemi baskılanmış kişilerde ve altta yatan akciğer hastalıkları olan hastalarda *Aspergillus* sporları çeşitli patolojik durumlara neden olabilir. Her yıl 200000 yeni invaziv aspergilloz (IA) vakası kayıt edilmektedir. Kronik obstrüktif akciğer hastalığı (KOAH) olan hastalarında IA'ya bağlı mortalite %72-95 arasında değişmektedir. 3 milyon insan farklı kronik pulmoner aspergilloz (KPA) formları ile hastadır. Araştırmanın amacı, *Aspergillus* türlerinin görülme sıklığını ve klinik örneklerde tanısal önemini belirlemektir.Ağustos 2017'den Ağustos 2018'e kadar olan periyotda 1170 hastanın örneği *Aspergillus spp*'in tespiti için incelenmiştir. Toplanan örnekler, kloramfenikollü Sabouraud dekstroz agarına inoküle edildi ve 10 gün boyunca 37°C'de inkübe edildi. Küf kolonilerinin üremesi durumunda, makroskobik ve mikroskobik özelliklere dayalı ileri tanımlama yapılmıştır. 22 hastada (1.88%) *Aspergillus* spp. kolonisinin ürediği tespit edildi. Onlardan 16'sı erkek, 6'sı kadındı. 7 hastada KOAH, 7 hastada bronşiyal astım (BA), 4 hastada akciğer tüberkulozu (AT), 1 hastada akut solunum yetmezliği, 1 hastada bronşektazi, 1 hastada eksüdatif plörezi, 1 hastada da akut lösemi vardı. Dört farkı *Aspergillus* türü izole edildi: *A. niger*, *A. fumigatus*, *A. flavus* ve *A. terreus*. *A. niger* genellikle BA ile ilişkiliyken, KOAH ve AT hastalarında *A. fumigatus* hakimdi. KOAH ve AT'lu hastalarda aspergilloz riskinin uygun tedavi sağlamak için ve mortalite oranlarını azaltmak maksadıyla dikkate alınması gerektiğini düşünüyoruz.

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Anahtar kelimeler. *Aspergillus spp.*, invaziv aspergilloz, kronik pulmoner aspergilloz, kronik obstrüktif akciğer hastalığı, akciğer tüberkulozu

Introduction

Fungi of the genus *Aspergillus spp.* can cause a wide spectrum of pulmonary diseases including invasive aspergillosis (IA), chronic pulmonary aspergillosis (CPA), allergic bronchopulmonary aspergillosis (ABPA). Clinic manifestations of these diseases vary depending on the immune system condition and accompanying disease (Kosmidis and Denning, 2015; Latge, 1999).

Neutropenia, organ and hematopoietic stem cell transplantation (HSCT), immune insufficiency are the classic risk factors for IA. During the last decades, IA is more frequently encountered in other groups of patients (Bulpa et al., 2007; Guinea et al., 2010; Meersseman et al., 2004; Ribaud et al., 1999; Xu et al., 2012). In particular, patients with severe forms of chronic obstructive pulmonary diseases (COPD) have a high risk of IA. The number of COPD patients is continuing to grow which causes an increase in expenses on medicine (Mathers and Loncar, 2006; May and Li, 2015). According to the criteria of global initiative for obstructive lung diseases (GOLD) patients with III and IV stage COPD are in the risk group of IA. Underestimation of IA risk leads to high number of delayed IA diagnosis.

Pathophysiology

Aspergillus spores are enough small to penetrate the lung parenchyma. The majority of conidia are eradicated from the respiratory tract via mucociliary clearance of epithelial cells. In COPD patients the ciliary activity is decreased as a result of destruction of epithelial cells caused by smoking, frequent infection episodes. The second line of defense consists of alveolar macrophages (AM) phagocytizing conidia and neutrophils eliminating mycelium and germinating spores. The mechanism of conidia destruction has 4 stages: 1) phagocytosis of conidia; 2) swelling of conidia; 3) destruction of *Aspergillus* spores beginning after 6 hours from phagocytosis; 4) destruction of conidia via oxygen radicals. Application of steroids promotes growth of *Aspergillus spp.* and decreases activity of macrophages< neutrophils and T_1-helpers.

Histological investigations have revealed different mechanisms of disease development in animals with neutropenia in comparison with that of received steroids. In the second case infiltration of lung parenchyma by neutrophils, large areas of pneumonia with destruction of alveoli and bronchioles were observed. At the same time,

a very small amount of fungal conidia was seen in the parenchyma. Thus, the death of animal was caused by an excessive immune response of organism – not by fungal invasion. Moreover, autopsy results of COPD patients have revealed that cases of disseminated infections are rare, which is consistent with data of research conducted on animals (Bulpa et al., 2007).

Annually 200000 new cases of IA are registered. 50% of these cases are revealed in patients with blood malignancies (Bao et al., 2017). The incidence of IA in intensive care unit (ICU) patients varies within 6.1-57 cases (Schmiedel and Zimmerli, 2016). According to different data mortality due to IA in COPD patients varies within 72-95%. The difficulty of IA diagnosis is related to low frequency of classic signs and symptoms: fever, cough, chest pain, haemoptysis, halo sign or the red crescent in radiological investigations (Soubani et al., 2004). Radiological signs in patients with neutropenia are more specific than in COPD patients. The microbiological tests used in diagnostics possess low sensitivity due to high frequency of *Aspergillus spp.* colonization and rapid neutralization of spores by neutrophils of immune competent individuals. However, this group of patients have risk of aspergillosis associated with: use of steroids, selective pressure by broad-spectrum antibiotics, structural lung changes leading to formation of cavities, underlying diseases (Patel et al., 2011).

3 million people suffer from different forms of CPA. CPA is divided into subacute invasive pulmonary aspergillosis (SAIA), chronic cavitary aspergillosis (CCPA), chronic fibrosing pulmonary aspergillosis (CFPA). CPA can follow pulmonary tuberculosis (PT) (1.74 mln), complicate ABPA (CPA incidence – 411000) and sarcoidosis (72000) (Denning et al., 2016).

The aim of our investigation was to determine the prevalence of *Aspergillus* species and their diagnostic significance in clinical samples of patients.

Material and Methods

The research was conducted on samples of patients applied to Scientific-Research Clinical Microbiological Laboratory, Educational-Therapeutic and Educational-Surgical Clinics of Azerbaijan Medical University and Scientific-Research Institute of Lung Diseases of Azerbaijan Republic. The respiratory samples including sputum and bronchoalveolar lavage fluid of 1170 patients were investigated for the presence of *Aspergillus spp.* for period from august 2017 to august 2018. 768 (65.6%) of investigated were males, 402 (34.4%) – females. The collected specimens were inoculated onto Sabouraud dextrose agar with chloramphenicol (0.5 g/l) (Pronadisa, Spain) and incubated at 37˚C temperature for 10 days. In case of growth of mold colonies, the further identification was performed based on macroscopic and microscopic features (McClenny, 2005; Samson et al., 2014). Color of the colonies, character of mycelium and microscopic features after coloring with lactophenol blue were investigated (Leck, 1999). In order to elicit the fact of contamination repeated collection of specimens from *Aspergillus spp.*-positive patients was performed. The growth of *Aspergillus spp*. colonies after repeated cultivation were evaluated as possibility of colonization/disease caused by fungi of this genus.

Results

Growth of *Aspergillus spp.* colonies was detected in 22 patients (prevalence – 1.88%). 16 of them were males, 6 – females. The mean age of patients was 53±18. 7 patients had COPD, 7 – asthma, 4 – PT, 1 – acute respiratory insufficiency, 1 – bronchiectasis, 1 - exudative pleurisy and 1 – acute leucosis. 4 species of the genus of *Aspergillus* were isolated: *A. niger*, *A. fumigatus*, *A. flavus,* and *A. terreus*. Among them, *A. niger* (12) and *A. fumigatus* (7) were the most frequent isolates. In our research *A. niger* commonly was associated with asthma, while in patients with COPD and PT prevailed *A. fumigatus* (5 and 2 strains respectively) (Table 1).

Table 1. *Aspergillus* species associated with diseases

Accompanying disease *(22)*	***A.fumigatus* (7)**	***A.niger* (12)**	***A.flavus* (2)**	***A.terreus* (1)**
COPD	5	2		
PT	2	1		1
Asthma		7		
Acute respiratory insufficiency			1	
Bronchiectasis		1		
Exudative pleurisy			1	
Acute leucosis		1		

Discussion

The genus *Aspergillus* consists of more than 300 species. A few of them can cause human diseases. The most frequent causative agent is *A. fumigatus*. Other frequent pathogens are *A. flavus*, *A. terreus*, *A. niger,* and *A. nidulans* (Samson et al., 2014).

Investigations of Tashiro et al. (2011) has shown that isolated *Aspergillus spp.* strains are not always etiological agents. In their research, 42% of *Aspergillus spp*. strains were isolated from patients without aspergillosis. The most frequent colonizing agents were *A. niger, A. versicolor, A. fumigatus, A. terreus, A. flavus, A. sydovii* and *A. nidulans*. Depending on analyzed population, the frequency of colonization varies within 36-91% range (Ascioglu et al., 2002; Horvath and Dummer, 1996; Khasawneh et al., 2006; Levy et al., 1992; Perfect et al., 2001; Soubani et al., 2004; Tashiro et al., 2011; Treger et al., 1985; Uffredi et al., 2003).

According to Perfect et al. (2001) data, 50% of all isolated strains colonized the respiratory tract and *A. fumigatus* constituted the majority among them (69%). These data differ from the results of Tashiro et al. (2011). Taking into account that investigation of the latter author conducted later than the research of Perfect et al. (2001) it can be concluded, that currently, *A.niger* strains have become the most common cause of colonization. It is consistent with data obtained by our investigation.

During our research 12 *A.niger* (52%), 7 *A. fumigatus* (33%), 2 *A. flavus* (10%) and 1 – *A. terreus* (5%) strains were isolated (Figure 1). The patient cohorts with the highest *Aspergillus*-colonization frequency were COPD and PT patients. Colonization can be the signal preceding severe *Aspergillus*-associated infection. IA often develops in hematological patients, hematopoietic stem cell transplantation (HSCT) and organs recipients, patients in the late stage of Acquired Immune Deficiency Syndrome (AIDS), granulomatosis (Chakrabarti et al., 2011; Marchetti et al., 2012; Meersseman et al., 2004). However, in recent years, cases of IA in patients with COPD have become more frequent (Bulpa et al., 2007; Guinea et al., 2010; Meersseman et al., 2004; Ribaud et al., 1999; Xu et al., 2012). Furthermore, COPD is one of the underlying conditions in CPA. 33.3-66.5% of patients with CPA have COPD as an underlying disease (Camuset et al., 2007; Denning et al., 2003; Smith and Denning, 2011).

Another condition caused by *Aspergillus spp.* is ABPA which can complicate asthma (Denning et al., 2013). According to estimations by Denning et al. (2013) the prevalence of ABPA in adults with asthma is 2.5% and annually 4.8 mln patients develop ABPA.

Investigations of Tutar et al. (2013) have revealed that 15.4% of *Aspergillus spp.* caused IA in COPD patients. It proves that this cohort of patients is at high risk of IA. The reasons are prolonged use of corticosteroids and broad-spectrum antibiotics (Bulpa et al., 2007; Guinea et al., 2010). Due to Guinea et al. (2010) data, 16.3% of COPD patients had *Aspergillus*-colonization. The diagnosis of probable IA was made to 22.1% of patients with colonization. In other research conducted by Shahi et al. (2015) out of 65 specimens, 16 were *Aspergillus*–positive. Colonization frequency was 24.6% (Shahi et al., 2015).

During our investigation specimens of 178 COPD patients were examined. *Aspergillus spp.* strains were detected in 7 (3.9%) sputum specimens among which 5 were *A. fumigatus*, 2 – *A. niger*. *Aspergillus*-colonization frequency was lower than in researches of the above-mentioned authors (3.9% versus 16.3% and 24.6% respectively). Diagnosis of probable IA was made in one case (14.3%) based on risk factors, clinical signs and results of microbiological examination (De Pauw et al., 2008).

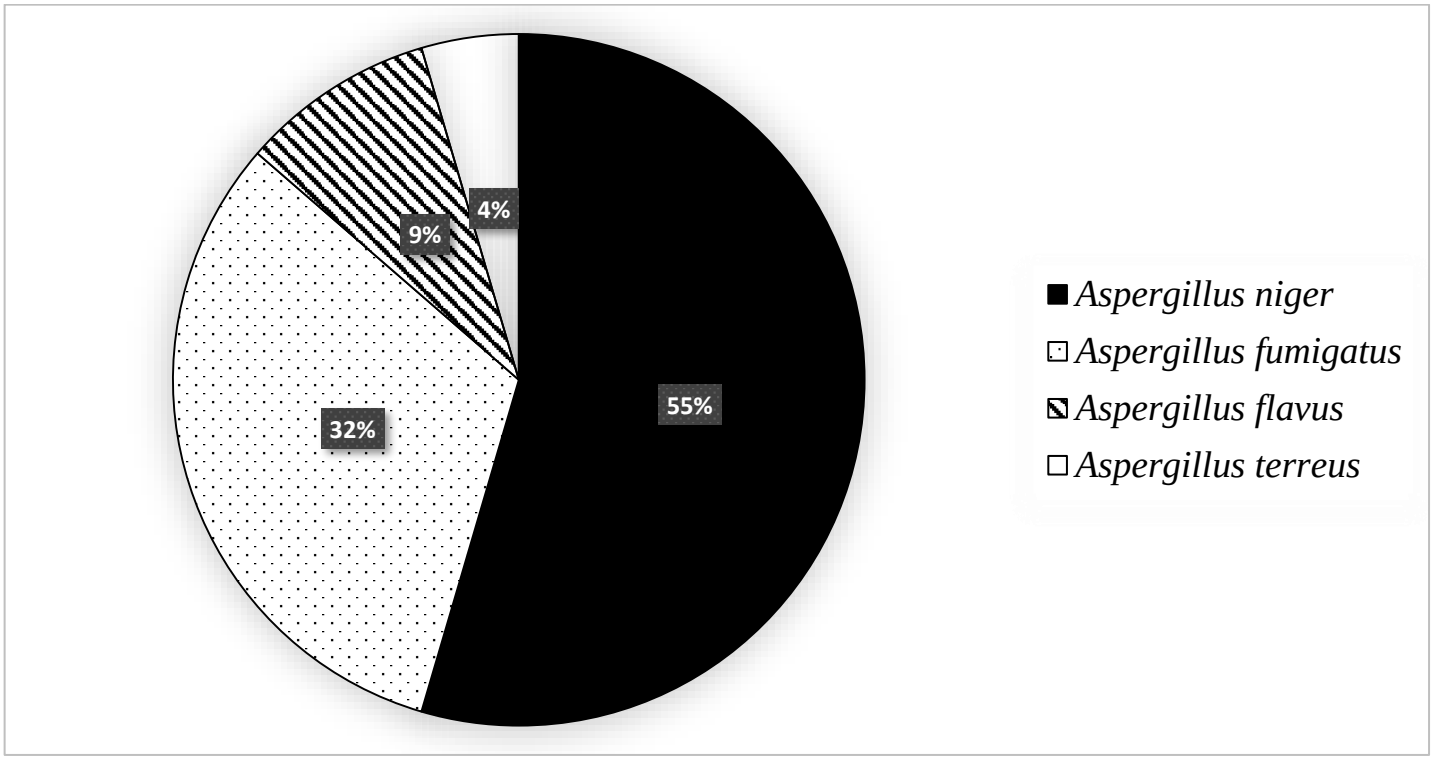

Figure 1. The proportion of isolated *Aspergillus* species

According to Denning et al (2011) data worldwide in 1.2 million people with preceding pulmonary tuberculosis develops CPA. Proportions of CPA patients with preceding PT varies within 15.3-93% range (Addrizzo-Harris et al., 1997; Camuset et al., 2007; Chen et al., 1997; Denning et al., 2011; Kosmidis and Denning, 2015; Nam et al., 2010; Shah et al., 2008; Smith and Denning, 2011).

Formation of cavities in PT can promote colonization and further development of CPA. During our investigation 3 (1.63%) *Aspergillus spp.* strains were isolated from 191 patients with PT (2 – *A.fumigatus*, 1 – *A. niger*). Risk of IA should be considered in cases of "smear-negative pulmonary tuberculosis", "progressing fibrosis of upper lobes of lungs" and "recurrent pulmonary tuberculosis" (Denning et al., 2011).

Conclusion

Prolonged use of corticosteroids and broad-spectrum antibiotics are the risk factors of IA in COPD patients. Our research has revealed a low prevalence of *Aspergillus*-colonization in COPD and PT patients (3.9% and 1.63% respectively). However, the development of aspergillosis in these patients can significantly worsen the patient's condition and cause a fatal outcome. The complexity of making the diagnosis of aspergillosis is related to the necessity of histopathological confirmation of the diagnosis of IA and nonspecificity of radiological signs of CPA. Thus, we consider that in patients with COPD and PT risk of aspergillosis should be taken into account in order to provide them with appropriate treatment and reduce mortality rates.

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