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THE ROLE OF PATHOHISTOLOGICAL AND MICROBIOLOGICAL DIAGNOSIS IN LUNG DISEASES CAUSED BY *ASPERGILLUS SPECIES*

ULOGA PATOHISTOLOŠKE I MIKROBIOLOŠKE DIJAGNOZE U PLUĆNIM OBLICIMA BOLESTI IZAZVANIM GLJIVICAMA IZ RODA *ASPERGILLUS*

Željka Vrekić[1], Aleksandra Lovrenski[2,3], Dragana Tegeltija[2,3], Slađana Novković Ostojić[3], Bosiljka Krajnović[4], Anika Trudić[2,3]

[1] Center for Pathology and Histology, Clinical Center of Vojvodina, Novi Sad, Serbia

[2] Faculty of Medicine Novi Sad, University of Novi Sad, Serbia

[3] Institute for pulmonary diseases of Vojvodina, Sremska Kamenica, Serbia

[4] General Hospital of Kikinda, Serbia

Correspondence to:
Željka Vrekić, MD
Center for Pathology and Histology, Clinical Center of Vojvodina, Novi Sad, Serbia
Hajduk Veljkova 3, 21000 Novi Sad, Serbia
Mobile phone: +381 64 394 3768
e-mail: zeljkavrekic@hotmail.com

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Lung disease, laboratory diagnosis, aspergillosis, pathohistological finding, microbiological analysis

Ključne reči
plućna bolest, laboratorijska dijagnoza, aspergiloza, patohistološki nalaz, mikrobiološka analiza

Abstract

Introduction: Aspergillus species can cause a wide range of lung diseases, depending on the current state of immunity and the existing pulmonary diseases. The most common manifestations of lung diseases are aspergilloma, allergic bronchopulmonary aspergillosis, invasive pulmonary aspergillosis and chronic necrotizing pulmonary aspergillosis. **Material and Methods**: This prospective study included 26 patients with proven *Aspergillus species* infection during the period from January 2015. until December 2016. **Results**: Aspergilloma was diagnosed at 23 (88.5%) patients. At 2 (7.7%) patients chronic necrotizing aspergillosis was determined, and at 1 (3.8%) patient invasive aspergillosis was diagnosed. By pathohistological analysis, the presence of *Aspergillus* was detected at 16 (61.5%) patients, and at 10 (38.5%) patients by microbiological diagnosis. At two patients (7.7%) *Aspergillus* was confirmed both pathohistologically and microbiologically. **Conclusion**: Since the diagnosis of diseases caused by *Aspergillus species* is demanding, with characteristic clinical and radiological findings, a laboratory confirmation of the fungus is essential for the final diagnosis.

INTRODUCTION

Aspergillus is ubiquitous dimorphic fungus that is present worldwide, found in organic debris, dust, compost, foods, spices and rotted plants. More than 200 species of Aspergillus have been identified, however, only 19 are known to be pathogenic for humans. Aspergillosis was first described mycosis in man. The most common species is *Aspergillus fumigatus*, whereas *A. clavatus*, *A. flavus*, *A. nidulans*, *A. niger*, *A. oryzae* i *A. terreus* are infrequently responsible for human infection [(1)]. Infection occurs by inhalation of spores and can cause a variety of pulmonary diseases, depending on immune status and the presence of underlying lung disease. These manifestations range from aspergilloma and allergic bronchopulmonary aspergillosis (ABPA) to invasive pulmonary aspergillosis (IPA) and chronic necrotizing pulmonary aspergillosis (CNPA) [(2)].

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity reaction to Aspergillus antigen. It occurs in asthmatic patients or in patients with cystic fibrosis. ABPA is different from other hypersensitivity reactions, because fungi grow within the respiratory tract and can perform continuous antigenic stimulation. Histopathological examination of the lung shows eosinophilic infiltration of the parenchyma (eosinophilic pneumonia) and occlusion of the bronchus by mucous plug, which is made up of mucus, fib-

rin, eosinophils and mononuclear cells. Characteristically, there are no signs of invasion of the blood vessel or the lung parenchyma by hyphae [3].

Aspergilloma is seen in patients with cavitary pulmonary disease. Colonization, mainly with A. fumigatus, occurring at already existing lung cavities (abscess, bronchiectasis, tuberculous cavities, cavities formed in pneumoconiosis, cancer, sarcoidosis, or lung infarction), and in all conditions where the cavity communicates with the environment and which can cause fungal colonization. The disease can be asymptomatic or accompanied by haemoptysis [3]. Invasive (disseminated) pulmonary aspergillosis (IPA) is a rare form of pulmonary mycoses where there is a spread of disease from lungs to other organs with appearance of granulomas, suppurating or necrotic lesions. IPA is typical for patients with extremely compromised immune response and it is a frequent cause of death in these patients. Risk factors can be hematological diseases (usually leukemia), HIV infection, bone marrow transplantation and treatment with broad-spectrum antibiotics [3].

Chronic necrotizing pulmonary aspergillosis (CNPA) occurs in patients with chronic lung disease and/or midly compromised immune systems. Chronic necrotizing pulmonary aspergillosis is a rare pathological entity and the available literature is based on case reports and small case series [4]. Disease has a slowly progressive course over weeks to months, and vascular invasion or dissemination to other organs is unusual. The clinical presentation is nonspecific, but, occasionally, patients may be asymptomatic [5,6].

Because the nonspecific clinical signs and symptoms, it is often difficult to set clinical diagnosis of aspergillosis, and the early laboratory diagnosis is critical for the selection of therapy, as well as for the survival of immunocompromised patients [7]. Diagnosis is made by microbiological examination of sputum, bronchial aspirate or bronchoalveolar lavage fluid, x-ray examination of the chest, and histopathological examination of material obtained at bronchoscopy, after an open lung biopsy or after lobectomy / pneumonectomy [3].

The aim of this study was to analyze the correlation of histopathological and microbiological diagnosis of aspergillosis in patients with lung diseases caused by Aspergillus.

MATERIAL AND METHODS

This prospective study included 26 patients with proven *Aspergillus species* infection at the Institute for Pulmonary Diseases in Sremska Kamenica during the period from January 2015. until December 2016. The material for pathohistological and microbiological analysis was processed at the Center for Pathology and Center for Microbiology, Virology and Immunology of the Institute.

Sampling and processing of material for histological analysis

Biopsy materials in patients with clinically and radiologically suspected infection induced by Aspergillus were obtained by bronchoscopy, open lung biopsy or thoracotomy. Samples were fixed in 10% neutral formalin, embedded in paraffin, and then cut on microtome to tissue slices of 4 microns thickness, and then stained with hematoxylin-eosin (HE). Microscopically, one can see septated fungal hyphae which show acute-angle dichotomous branching of 45 ° (Figure 1). In addition to standard HE staining, special stains were used periodic acid-Schiff stain (PAS) and staining with silver (Grocott method) that allow better visualization of fungi (Figure 2.) [8]. In cases when the hyphae can not be identified, the finding of calcium oxalate crystals visible in polarized light can be of great help in the diagnosis of infection with Aspergillus (they are especially characteristic for A. niger) [9].

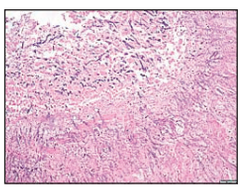

***Figure 1**. Septated fungal hyphae whit acute-angle dichotomous branching of 45 ° (HE x 20).*

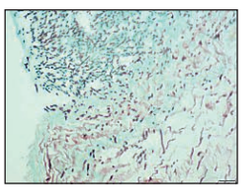

***Figure 2**. Special stains allow better visualization of fungi (Grocott x 20).*

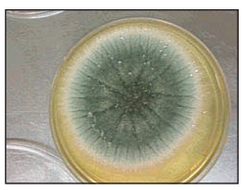

***Figure 3**. After the onset of spores, colonies can turn green, dark green or blue green, depending on the color of the spore.*

Microbiological diagnostic methods

Samples (sputum and broncholavage) sent for mycological examination to the Center for Mycrobiology, Immunology and Virology were seeded on Sabouraud agar. 2 substrates were inoculated for each sample. The substrates were incubated for up to 8 days at 37 ° C and 42 ° C with increasing humidity. Overgrown colonies are usually round and can reach up to 3 cm in diameter. In the beginning, colonies are white, cottony, and after the onset of spores they can turn green, dark green or blue green, depending on the color of the spore (Figure 3.). After recording the growth by macroscopic examination, a microscopic preparation was made. For the testing of fungi, a native preparation is made by applying a colony on a glass slide and adding 1-2 drops of lactophenol. The preparation is covered with a cover flake, after which microscopy is started. In the microscopic preparation fragments of thin, septate hyphae are seen, along which conidial heads can be seen [10]. *Aspregillus species* identification was performed by macroscopic and microscopic examination of mycologic growth.

RESULTS

Of the 26 patients included in this study there were 7 (26.93%) females and 19 (73.07%) men. Patients were aged 19 to 77 years, and the average age was 55.3 years. The disease in all patients were clinically manifest and was accompanied by cough in 16 (61.5%) cases. Patients have complained of hemoptysis in 11 (42.3%) cases, the fever in 9 (34.6%), chest pain in 8 (30.7%) and dyspnea in 6 cases (23.1%).

In 23 (88.5%) patients the disease presented in the form of aspergilloma, in 2 (7.7%) as CNPA, and only in 1 (3.8%) patient as IPA. ABPA was not diagnosed in our patients. In 23 (88.5%) patients with diagnosed aspergilloma, 11 (42.3%) had previously diagnosed tuberculosis with the for-

mation of caverns and 10 (38.5%) had bronchiectasis. In 2 (7,6%) patients with lung cancer (squamous cell and small cell carcinoma) aspergilloma was formed due to the excavation of the tumor mass. CNPA was diagnosed in 2 (7.7%) patients. One of them was previously diagnosed with chronic lymphocytic leukemia, while the other patient was diagnosed with squamous cell carcinoma of the lung. One (3.8%) patient (who was HIV positive) developed IPA due to the extremely compromised immune system.

In 13 (50%) patients aspergilloma was localized in the right upper lobe, in 7 (26.9%) in the left upper lobe, in 2 (7.7%) in the left lower lobe, and in 1 (3, 9%) patient in the middle lobe of the right lung. In 3 (11,5%) patients with CNPA and IBA, changes in lung parenchyma were bilateral and localized in the upper lobes.

In 16 patients hyphae of Aspergillus were detected in biopsy material, and in 10 patients in the materials taken for microbiological analysis. Diagnostic materials for histopathological examination were obtained by bronchobiopsy in 3 (18.75%) patients, catheter biopsy in 2 (12.5%) patients, transbronchial biopsy in 4 (25%) patients and surgical procedures in 7 (43.75%) patients. For microbiological analysis, sputum in 6 (60%) and bronchoalveolar lavage fluid in 4 (40%) patients were examined. In 6 patients both histopathological and microbiological analysis were performed, but only in 2 patients fungi were proven both by histopathological and microbiological analysis (Table 1.).

Of the 16 patients with histologically diagnosed aspergillosis in 13 (81.2%) patients the disease manifested as aspergilloma, in 2 (12.5%) patients as CNPA, while in 1 (6.3%) patient IPA developed. In 10 patients with microbiologically proven infection, in all cases disease manifested as aspergilloma. In 2 patients with histologically and microbiologically proven presence of fungal hyphae, disease presented as aspergilloma.

Table 1. *Histopathological and microbiological diagnosis of Aspergillus species.*

Analysis	**Diagnostic methods and materials**	**Number of patients with proven aspergilosis**
Histopathological	bronchobiopsy catheter biopsy transbronchial biopsy surgical procedures	3 2 4 7
Microbiological	sputum bronchoalveolar lavage fluid	6 4
Total		26
***Histopathological and microbiological**		*6 (but only 2 patients with histologically and microbiologically proven aspergilosis)*

DISCUSSION

Laboratory diagnosis of lung diseases caused by Aspergillus is challenging. Today used diagnostic criteria are EORTC / MSG (European Organisation for Research and Treatment of Cancer - Mycoses Study Group) criteria: "proven", "probable" and "possible". These terms indicate with what degree of certainty laboratory results correlate with clinical diagnosis[7,11,12]. Diagnosis of proven aspergillosis requires positive culture of fungi from the sample obtained from primarily sterile region and/or histopathological confirmation of the existence of fungal hyphae within lung tissue.

The setting of histopathological diagnosis of aspergillosis in lung biopsies is not always easy, and in the first place depends on the quality of the preparation, but also on the knowledge and experience of the pathologist. When analyzing bronchial biopsies, catheter biopsies and transbronchial biopsies (due to scanty material) hyphae are often sporadic and present in small number, and in addition to routine HE staining, a use of special stains such as periodic acid-Schiff method (PAS) and Grocott method which allow better visualization of fungi is highly recommended [8]. On material obtained after surgery, the pathologist first macroscopically evaluate lung tissue, and often Aspergillus may be suspected by the appearance of the tissue. Lesions are often excavated, filled with dark green, mushy material without smell. When samples are taken from the most suspected areas of lung parenchyma, then one can expect more representative histological picture. The histological finding of many fungal hyphae indicate the existence of diseases caused by Aspergillus [9].

Histologically, the main differential diagnostic problem in the first place is infection with fungi Mucor and Fusarium spp., which are by their morphology very similar of Aspergillus, but their hyphae branching at right angles, in contrast to Aspergilllus which hyphae branching at acute angles. In addition to these Aspergillus "look-alikes" fungi, the next most common diagnostic dilemma is infection with Candida spp. [13].

Microbiological identification of Aspergillus from clinical specimens is based on the cultivation with use of selective media for fungi. Aspergillus is a thermophilic species, grows well at high temperatures and can reach a size of 3 cm in a week [10]. Since the Aspergillus is morphologically highly variable, after macroscopic evaluation of microbial growth, microscopic examination is required. There is a possibility of obtaining false positives findings due to the presence of Aspergillus in respiratory samples of healthy individuals. Also, contamination of the samples can be caused by conidia present in the air [10]. Therefore, for the diagnosis of infections caused by Aspergillus indirect laboratory methods are applied. The most important early biomarkers are galactomannan and anti-Aspergillus antibodies. Galactomannan is the main component of the cell wall of fungi of the genus Asperillus which is liberated during the growth of fungi within the tissues, while the body produces specific antibodies during fungal infection. Both of these methods are based on the

principle of ELISA test, but the specificity and sensitivity of the assays are variable [7,14]. Infection by Aspergillus spp. demonstrated by direct or indirect laboratory tests should be interpreted along with clinical and radiological finding.

In our patients, the disease is usually manifested as aspergilloma, and in two cases as CNPA. The most severe clinical picture had a patient who was HIV positive and who developed IPA. In regard to clinical forms of the disease caused by Aspergillus, our results are consistent with other researches. It has been shown that the most common form of the disease is aspergilloma, while immunocompromised patients usually develop CNPA or IPA [3,15].

There are citations that suggest an association between previous lung disease and the development of specific forms of aspergillosis. It was found that aspergilloma usually occurs in patients with tuberculous cavities, bronchiectasis, excavated tumors, abscesses, areas of the lung infarction and, rarely, within cysts in the lungs and in those changes that allow settlement of saprophytic fungi and development of the disease [3,16]. Dagenais and Keller investigated the risk factors that lead to the development of IPA. The most common reasons for a compromised immune response in these patients were neutropenia, prolonged corticosteroid therapy, HIV infection and hematologic diseases [12]. CNPA usually occurs in patients with chronic pulmonary disease (eg, chronic obstructive pulmonary disease), in patients after surgery of the chest, in patients with compromised immune response due to diabetes, in patients with chronic liver disease and in alcoholics [5]. The results of our study do not differ significantly from the data of other authors.

By now, in the literature has not been processed data on the localization of pulmonary changes. In 23 (88.5 %) patients the most common site of lung were the upper lobes. In our patients, we most commonly found aspergilloma which usually developed within preexisted tuberculous cavities that occur in the upper lobes of the right lung [17].

Histologically, the presence of the fungus was usually proven in biopsy material, and by microbiological methods fungus was most commonly detected in the sputum. The finding of Aspergillus in the sputum should always be interpreted along with clinical and radiological picture, because this sample is unsterile material in which Aspergillus can be found in healthy individuals in small numbers. In case when aspergillosis is suspected, as much as possible different samples should be sent to microbiological examination. In our patients, serologic tests were not carried out, due to technical limitations, although they are recommended in the diagnosis of diseases caused by Aspergillus.

CONCLUSION

Aspergillosis is still the leading cause of morbidity and mortality in immunocompromised patients and has becoming a growing problem due to the wide use of corticosteroids, immunosuppressive and antineoplastic drugs, as well as a result of increase organ transplantation. On the other hand, the clinical manifestations of infections caused by Aspergillus in immunocompetent individuals are often non-specific, which significantly compromise possibility to set the correct diagnosis.

While setting of histological diagnosis allows rapid diagnosis of fungal infections, there is no possibility of a final identification of pathogens. On the other hand, the microbiological diagnosis enable definite diagnosis with the ability to identify the cause. Disadvantages of microbiological analysis are that cultivation is time-consuming and there is the possibility of contamination of the samples with fungus in healthy people. Although the sampled material is often not sufficient to implement both analysis, if aspergillosis is suspected, samples should be sent both to histopathological and microbiological examination. The characteristic clinical and radiological findings along with Aspergillus isolated in samples taken from the respiratory tract, as well as the presence of histologically proven fungal hyphae, are highly indicative for setting the final diagnosis of lung diseases caused by Aspergillus.

Sažetak

Uvod: *Aspergillus species* može da izazove čitav spektar plućnih bolesti u zavisnosti od stanja imuniteta i postojećih plućnih oboljenja. Najčešće manifestacije plućnih bolesti su: aspergilom, alergijska bronhopulmonalna aspergiloza, invazivna plućna aspergiloza i hronična nekrotizirajuća plućna aspergiloza. **Materijal i metode:** Ova prospektivna studija obuhvatila je 26 pacijenata sa dokazanom infekcijom *Aspergillus species* u periodu od januara 2015. do decembra 2016. godine. **Rezultati:** Aspergilom je dijagnostikovan kod 23 (88,5%) pacijenata. Kod 2 (7,7%) pacijenta utvrđena je hronična nekrotizirajuća, a kod 1 (3,8%) invazivna aspergiloza. Patohistološkom analizom, prisustvo aspergilusa dokazano je kod 16 (61,5%) pacijenata, a mikrobiološkom kod 10 (38,5%) pacijenata. Kod dva (7,7%) pacijenta aspergilus je dokazan i patohistološki i mikrobiološki. **Zaključak:** S obzirom da je dijagnostika oboljenja izazvanih *Aspergillus species* zahtevna, uz karakterističan klinički i radiološki nalaz, neophodna je laboratorijska potvrda uzročnika za konačno postavljanje dijagnoze.

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