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MODERN METHODS OF ASPERGILLOSIS DIAGNOSIS AND BIOTECHNOLOGICAL APPROACHES TO CREATION OF ALLERGY/ASPERGILLOSIS DIAGNOSTIC PREPARATIONS

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Abstract

Fungi of the *Aspergillus* genus are widely distributed in nature, including more than 300 species, but only a few of them are pathogenic for humans and animals. *Aspergillus* can grow on organic substrates in soil, on plants. Spores are constantly present in air, and with it food, household items, outer integument and mucous person causing the disease to cause aspergillosis. When aspergillosis usually affects the respiratory system, in some cases, skin, kidneys, brain, eyes. Invasive pulmonary aspergillosis is associated with high mortality. In recent years, the number of cases has increased. At risk are people with immune deficiencies. Crucial in the fight against this disease promptly started playing antifungal therapy and early diagnosis. When selecting tests are important technical and economic indicators of the

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complexity or simplicity the study, its reproducibility, the applicability of automated screening and cost analysis. In the present article we have analyzed and compared different methods for aspergillosis diagnostics. Biotechnological approaches for development of allergy/aspergillosis diagnostic preparations also have been discussed.

Introduction

Allergic diseases suffer from 10% to 30 % of the population. In Ukraine, these pathologies occur at an average of 30 % of the population depending on the state of the environment, the climate, the development of production in certain regions [1]. It is important to note that the frequency of aspergillosis cases increased about 14 times over the past 12 years. Aspergillosis is one of the most common allergic diseases caused by various species of fungi genus *Aspergillus* [2].

Aspergillus are widespread in the world, are ubiquitous in the environment. Fungal spores are always in the air in the form of inhaled bioaerosol and macro organisms. Spores can shatter mucosa of the respiratory tract, paranasal sinuses, and thanks to its small size (2.5-3.0 microns) reach the alveoli. However, most people, this infection is usually unnoticed or is localized [3]. It is now known for more than 600 species of *Aspergillus*, but only a few are pathogenic to humans [4]. Aspergillosis is a second frequency after mycotic *Candida* infection in most cases is caused by *Aspergillus fumigatus* (90 %), and *Asp. flavus*, *Asp. niger*, *Asp. terreus*, *Asp. nodulans* [1-4].

One of the main clinical methods of specific diagnostics of allergic disease is allergic skin testing. Prick test is one of the most effective skin allergy samples used in the diagnosis of allergic diseases. Test example technique characterized in that the allergens are histamine and the solvent are introduced into the epidermis of the skin with a special disposable prick lancet through [5-6].

The aim of the work was to analyze the data in the literature on modern methods of diagnosis of aspergillosis and approaches to the creation of diagnostic preparations for its detection.

Modern methods of aspergillosis diagnosis

Aspergillus fungi previously considered atypical pathogens are by far the cause of morbidity and mortality in patients with various immune deficiencies. Crucial in the fight against this disease is early diagnosis [7].

Although early diagnosis is extremely important in improving the prognosis of patients, there is still no proven technique that would be sufficiently sensitive and specific for routine clinical use.

When recognizing aspergillosis, the epidemiological background (profession, the diseases that weaken the immune system, etc.) is important. With the defeat of the bronchus and lung, diagnostic importance is the duration of the disease, formation of characteristic infiltrates with subsequent decay, the character of sputum, leukocytosis, eosinophilia. Confirmation of diagnosis is by biopsy (from sputum, material taken from the bronchi, biopsies of affected organs). In blood, *Aspergillus* is very rare, even in generalized forms of aspergillosis.

Diagnostic value has the appearance of antibodies to the pathogen, detectable serological reactions. Skin tests with specific *Aspergillus* antigen can be used only for relatively benign forms of aspergillosis in patients with normal immune systems. Note that at the stage of HIV-infection, the delayed hypersensitivity reaction sample becomes negative.

Clinical and radiological data on aspergillosis should be differentiated from other mycoses (nocardiosis, histoplasmosis, candidiasis), as well as pulmonary tuberculosis, lung abscesses, tumors, chronic bronchitis. As a result, the diagnosis of aspergillosis usually depends on the clinical manifestations and detection of fungi in tissue specimens obtained

from the alleged source of infection. Skin lesions, if they occur, do not pose any difficulties for biopsy; corneal scrapings are useful in the diagnosis of aspergillus keratitis, patients with suspected pulmonary aspergillosis may require lung biopsy [5, 8].

We should expect that prospective studies will shed light on the proper role of polymerase chain reaction (PCR) tests based on nucleic acid sequences and determination galactomannan antigen for the early diagnosis of aspergillosis [9, 10].

Microscopic examination of the affected tissue. The first method for detecting invasive disease is a microscopic examination of the affected tissue. However, the presence of *Aspergillus* species should be confirmed by culture, as microscopy only detects the presence of mycelium in the tissue, rather than the presence of a particular pathogen.

Detection in tissues narrow septate mycelium with ramifications at an acute angle usually suggests the presence of aspergillosis. Other fungi such as *Pseudallescheria boydii* (also known as *Scedosporium apiospermum*, which are a form of asexual *Pseud. boydii*), fungi of the *Fusarium* and *Penicillium* genera histological study presented identical. *Mucorales* sometimes confused with *Aspergillus*, but the first is usually represented in the fabric wide aseptate, branching at right angles mycelium, unlike thin, branching at an acute angle septate mycelium observed in lesions caused by fungi of the *Aspergillus* genus. These differences are very important because treatment can vary depending on the pathogen.

Fungi of the *Aspergillus* genus are rapidly growing mushrooms and culture generally can be identified within three days of incubation. Sometimes, however, to identify culture and requires a longer period, especially when the amount of agent in the material is very small, so that the laboratory should be instructed to keep the cup with crops for at least four weeks. Culture will be sterile if for sowing was taken unlesioned tissue site.

Histologic confirmation is not always possible to obtain in critically ill patients with immunosuppression or those who have thrombocytopenia or other contraindications to

biopsy. In those patients who have pulmonary symptoms or new pulmonary infiltrates, *Aspergillus* selection of bronchoalveolar lavage (BAL) – a sufficient criterion to assign therapy [7]. Of the 15 surveyed HIV-infected patients who were diagnosed in this way, aspergillosis was later confirmed histologically or at autopsy in 14 patients [11].

Determination of galactomannan antigen. Galactomannan, which is released during mycelial growth, is the main component of the cell walls of fungi of the *Aspergillus* genus. Early detection of galactomannan in the serum by enzyme-linked immunosorbent assay (“sandwich” ELISA) investigated a variety of groups in Europe and the U.S. as a diagnostic test for invasive aspergillosis. Other methods for detecting the antigen were not sensitive enough for use in the diagnosis [9].

Determination of serum galactomannan can detect antigenemia in some patients on average 5-8 days before the first clinical symptoms, changes on chest radiographs or obtaining positive cultures of the fungus. On the other hand, a negative test suggests alternative diagnosis. Studies of this test yielded generally favorable results [12, 13-20], but not so good for this test completely replaced thorough clinical and microbiological assessment of the patient [16]. However, it can be an important adjunct to diagnosis. In one study, for example, galactomannan in the presence of blood for 8-9 days preceded the appearance radiographic changes and excretion of *Aspergillus* more than 80% of patients [12].

To evaluate the accuracy of galactomannan test for the diagnosis of invasive aspergillosis were included in a meta-analysis [21]. Were selected 27 studies that used for the diagnosis of aspergillosis standard criteria of the European Organization for the Research and Treatment of Cancer/Mycoses Study Group, and this meta-analysis has affected approximately 4,000 patients. When analyzed only proven cases or a combination of proven and probable cases of invasive aspergillosis, sensitivity ranged between 61 to 71 % with a

specificity of 89 to 93 %. Negative predictive value of the test corresponded to 95-98 %, while the positive predictive value of only 26-53 %.

These data suggest that galactomannan test is useful to exclude the diagnosis of invasive aspergillosis, but for confirmation of the disease has a sensitivity of only moderate to low [22]. Subgroup analyzes suggested that the test works best in patients with malignant hematological malignancies or hematological transplant recipients, and significantly worse than working in transplant recipients of solid organs [21]. This may be associated with higher pre-test probability of developing invasive aspergillosis in the first subgroup of patients than in the latter [22].

Sensitivity and specificity of the test galactomannan may vary depending on the destination of other drugs. False-positive reactions were demonstrated in patients who received piperacillin/tazobactam and amoxicillin/clavulanate, due to the presence of these antibiotics as part of galactomannan [23-25]. False positive tests pointed to five days after discontinuation of beta-lactam antibiotics [26]. The sensitivity of this test is also reduced during antifungal therapy [27].

The criterion for evaluation of the test is compared with a threshold value. The manufacturer recommends that the optical density index of 1.5 or higher, but some authors suggest to consider lower thresholds: 0.5 and above [16, 19], from 0.6 and above [20], 0.7 and above [15, 17] or 0.8 and above in one single measurement or 0.5 or higher in two consecutive tests in patients at high risk for invasive aspergillosis [18]. In one study, obtaining the index of above 0.8 and statistically confirmed the need to start antifungal therapy while receiving two tests dynamics from 0.5 and up to two consecutive sera increased specificity of 98.6 % [18]. Test approved for use in the U.S., suggests to consider a threshold optical density index of 0.5.

Final test to determine the role of serum galactomannan will depend on the results of more studies and more clinical observations of patients at risk for invasive aspergillosis [28]. Until such data are not available, so if the test is applied, required careful correlation of clinical data with the results. Proposals were presented on the use of this test in combination with PCR.

Galactomannan sample until approved only for studies of blood serum. Given that the galactomannan is soluble carbohydrate, it can be detected in samples of other fluids, including urine, cerebrospinal fluid and bronchoalveolar lavage [29]. Yes order to evaluate the usefulness of the detection of antigen in other samples besides sera required dalneyshnie study.

Polymerase chain reaction. Investigated tests for the detection of DNA gave a more mixed results than by definition galactomannan antigen [20, 30-32]. In one report, e.g., galactomannan test (ELISA) was more sensitive than the PCR test or other fungal components – beta-D-glucans [19]. In addition, there is a significant problem – the lack of standard PCR tests and the absence of these tests and commercially available [33]. It should be noted also that the result of PCR test may affect anticoagulation [34].

Nevertheless, three successive trials demonstrated high sensitivity PCR tests [11, 35, 36]. In the study of consecutive samples from 56 patients with invasive fungal infections who were treated from 1982 to 2000. The diagnosis was made in 52 cases, including confirmation of the causal organism in all cases in which the mold was isolated culturally from the tissue sample [35].

A potential benefit from the PCR in the study of the cerebrospinal fluid is presented in a small case series of 6 patients with suspected central nervous system aspergillosis [37]. It was suggested that a negative PCR test may be used to avoid invasive aspergillosis [33, 36]. However, only a large, prospective, multicenter study to answer the question of whether

routine screening to help assign antifungal therapy for those patients who need it, and not spend it to those who do not need it [33].

The combination of PCR and galactomannan determination. Combination use of the PCR and determining the galactomannan antigen was proposed as a potential strategy for the experts in this field. In one prospective study of 201 patients with hematological malignancies sensitivity and specificity of PCR tests for proven and probable cases of invasive aspergillosis were 64 and 90 %, respectively, when we used two consecutive positive results. Using PCR test galactomannan with increased sensitivity to 83 % but decreased the specificity of 70 %. And the PCR and determining the galactomannan demonstrated high negative predictive value (90 and 96 %, respectively), which increased to 98 % when a combination of methods [36-37].

Nucleic Acids Sequence Based Amplification (NASBA). It differs from PCR in that it defines a messenger RNA and not DNA. Direct detection of messenger RNA suggests that an infectious agent active copied and not the tissue is asymptomatic. Use of NASBA for the detection of invasive aspergillosis was evaluated in only one study of 128 patients with febrile neutropenia [38]. Invasive aspergillosis using a combination of galactomannan enzyme immunosorbent test and NASBA was diagnosed in 14 subjects. NASBA index was greater than 5.0. Evaluation of the usefulness of this method for the detection of invasive aspergillosis requires additional research.

Precipitation reaction. Detection of precipitating antibodies may be useful for the diagnosis of allergy to various mold fungi, including members of the *Aspergillus* genus, but has no role in the diagnosis of invasive aspergillosis.

Biotechnological approaches to creation of allergy/aspergillosis diagnostic preparations

For accurate diagnosis of allergy testing should be used based on antigens of the fungus – the pathogen, in this case – the fungus of the *Aspergillus* genus.

The most effective method is the destruction of the fungal cell by ultrasonic disintegration. Use of a disintegrant to the impact of ultrasonic waves in the range of 10-20 kHz, 10-60 min exposure, depending upon the volume of culture fluid is not permitted to destroy at least 90 % of the cells of *Candida* and 50-60 % – *Aspergillus* and *Mucor*. After the disruption of fungi for exemption from the damaged walls and whole cells using membrane plate type “Vladipor- MFA No. 3” [14].

Biological scheme for the *Aspergillus* antigens comprises the following steps.

1. Use of the fungus strain *Asp. fumigatus* No. 6 , which first 3 days was inoculated into tubes containing Sabouraud agar and cultured at 37 °C, and then flasks for 6-9 days until the “harvest” 300,000,000 spores cells/cm³.

2. Isolated antigens using an ultrasonic disintegrator. Impact disintegrator performed at a wavelength of 20 kHz and 90 minutes of exposure. Exemption from the destroyed and intact vegetative and spore cells accomplished by filtration through a membrane “Vladipor MFA-MA No. 3” using the vacuum pump. Filtered dezintegrat contains antigens fungus.

3. Assess antigens derived index agglomeration leukocyte reaction (specific agglomeration leukocytes RSAL). In the presence of agglomerated leucocytes at least 20 % and above are considered to be allergenic antigens.

4. To antigens with 20 % coefficient in RSAL and above as making a solution of phenol preservative 2.5 % final concentration.

5. At the last stage assess the allergenic properties of the formulation produced in laboratory animals. To this end, two days after intraperitoneal infection with a virulent strain albino mice *Asp. fumigatus* in a dose of 1×10^6 spor/sm³ 0.5 ml of the allergen is administered intradermally in an amount of 0.05 ml. Accounting for the reaction is carried out

in 24-48 and 76 hours. As a control intact animals are used. In infected mice, the causative agent of a day observed a pronounced hyperemia, increased skin temperature at the site of allergen and an increase in skin fold at 1-3 mm, which is maintained for at least three days. In intact mice after 24 hours at the site of allergen no reaction is supervised [3-5, 9, 17].

Conclusions

At present there are a lot of specific methods for diagnosis of invasive aspergillosis. For the diagnosis of aspergillosis (especially associated with the allergy of the body) is the most appropriate serological group of tests based on the detection of galactomannan antigen, – prick test and enzyme immunoassay. Among the most sensitive test should also be noted polymerase chain reaction.

It is shown that the basic biotechnological approaches to obtain allergy/aspergillosis diagnostic preparations consist in their establishment on the basis of antigens fungi of *Aspergillus*.

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