

MICROBIOLOGICAL AND MOLECULAR METHODS FOR RAPID DIAGNOSIS IN SYSTEMIC MYCOSES

Lubomira Boyanova

Received on February 28, 2024

Presented by B. Petrunov, Member of BAS, on May 28, 2024

Abstract

The most frequently employed methodologies for molecular, antigenic, and antibody detection in body fluids (serum, plasma, bronchoalveolar lavage (BAL)) are enzyme-linked immunosorbent assay (ELISA-Platelia), indirect immunofluorescence (IIF), latex agglutination, and polymerase chain reaction (PCR, real-time PCR). Additionally, the examination of clinical materials from a target site may contribute to the identification of medically significant fungi.

The objective of this study was to present the principal laboratory diagnostic methods for the detection of the most prevalent causative agents of invasive fungal diseases, including Candidosis, Aspergillosis, and Cryptococcosis.

More than 200 clinical serum samples were examined serologically from 2018 to 2022 in the National Reference Laboratory "Mycoses" at the National Center of Infectious and Parasitic Diseases in Sofia. Additionally, 20 whole blood samples were analyzed by real-time PCR. Twenty-four clinical samples, including sputum and bronchoalveolar lavage (BAL), were examined culturally on Saburo agar. A total of 10 strains of *Candida* spp. and 9 strains of *Aspergillus* spp. were isolated. The antifungal sensitivity of the isolated strains was tested against the most commonly used antifungals, including fluconazole, itraconazole, voriconazole, nystatin, isavuconazole, caspofungin, anidulafungin, and posaconazole.

Three tests for *Candida* spp. and one for *Cryptococcus* spp. were positive by latex agglutination. By indirect immunofluorescence (IIF) in 56 examined

The study was supported by the European Regional Development Fund through Operational Programme Science and Education for Smart Growth 2014–2020, Grant BG05M2OP001-1.002-0001-C04 "Fundamental Translational and Clinical Investigations on Infections and Immunity".

<https://doi.org/10.7546/CRABS.2024.11.10>

sera, antibodies were found in seven samples for *Candida* spp. with a titer greater than 1:160, and 54 samples were positive for antibodies to *Aspergillus* spp. out of 182 examined serum samples.

A total of 25 serum samples were examined using the ELISA-Platelia test for the detection of *Aspergillus* antigen. Of these, five samples were found to be positive.

A further 20 samples were tested using real-time PCR to detect the presence of DNA from the most frequently isolated fungal strains from blood cultures. These included *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Aspergillus fumigatus* and *Cryptococcus neoformans*. Of the over 200 serum samples tested for the presence of fungi, only a small number yielded positive results. Similarly, testing 20 tubes of whole blood yielded negative results for the presence of fungi using real-time PCR.

The application of microbiological and molecular methods for the rapid diagnosis of systemic mycoses represents a practical approach for routine use in microbiological laboratories.

Key words: systemic fungal disease, *Candida*, *Aspergillus*, *Cryptococcus*, real-time PCR

Introduction. Medically important fungi are eukaryotic microorganisms that are widely distributed in the environment. They can be dangerous for humans in the presence of predisposing factors, especially immune deficiency and the characteristics of the host. The diseases they cause are called “mycoses”. According to the body site they affect, they are classified into four categories: superficial, cutaneous, subcutaneous, and systemic mycoses [1].

Of particular interest are systemic mycoses, which affect internal organs and systems. These are also known as invasive fungal diseases (IFD). Patients are most often with immune deficiency or with accompanying disease, such as cancer and hematological patients, or patients undergone severe surgical interventions, including transplanted patients (bone marrow, organ), patients with AIDS, those who have suffered burns, sustained severe trauma, with diabetes, low birth weight newborns, etc.

A number of studies have confirmed the severe prognosis and high mortality associated with fungal infections. Consequently, accurate etiological diagnosis and etiotropic treatment of these diseases is of the utmost necessity, which presents a significant challenge in modern medicine.

Candidemia can occur in both immunocompromised and non-immunocompromised patients [2]. It most often occurs in immunocompromised patients with leukemia or another myeloproliferative disease (in 10–20% of cases), and over 70% of cases occur in patients with HIV-positive status [3]. Of the representatives of the genus *Candida*, *C. albicans* is the most commonly isolated species. In recent years, the number of non-albicans species has significantly increased, particularly from blood cultures, including *C. parapsilosis* and *C. glabrata*. *Aspergillus* genus, *A. fumigatus* and *A. flavus* are most often isolated as the causative agents of

invasive aspergillosis. A significant challenge in the field of medicine is the accurate diagnosis of invasive aspergillosis. This condition can manifest as an infection in the respiratory tract, including the lungs and sinuses [4].

From the genus *Cryptococcus*, *C. neoformans* is considered the most pathogenic for humans. *Cryptococcus neoformans* is more prevalent in immunocompromised individuals, particularly those infected with HIV. It can manifest in a variety of ways, ranging from localised (skin infection) to disseminated cryptococcosis. The infection typically begins after inhalation of basidiospores into the lung, serving as a portal of entry. It can range from asymptomatic colonisation to severe pneumonia. It can also spread via the bloodstream to the central nervous system (CNS), where it can cause meningoencephalitis or meningitis. This process begins with the invasion of the brain parenchyma and cerebral blood vessels, which is followed by infection of the subarachnoid space.

Although *Aspergillus* spores are often inhaled by healthy individuals without developing disease, they are capable of causing life-threatening infections in an increasing number of immunocompromised patients, including those undergoing chemotherapy or undergoing organ transplantation. Invasive fungal infections are characterized by high mortality, despite the use of antifungal therapy, so the earlier diagnoses with more sensitive methods is obligatory.

Materials and methods. Worldwide, methods for the detection of medically important fungi continue to be optimized. In our study, we tested the following clinical materials and diagnostic methods: microscopy, cultural isolation serological tests, and real-time PCR.

Clinical materials: We examined 24 clinical materials (sputum, BAL) – microscopically and culturally. More than 200 tubes of serum and 20 tubes of whole blood, taken in respective vacutainers, were examined.

Microscopy: Microscopic evaluation was done after a Gram stain to visualize bacteria and the presence of leukocytes.

Indirect immunofluorescence (IIF): The method we use to determine specific antibodies is indirect immunofluorescence (IIF) for *Candida*, *Aspergillus*, *Cryptococcus*. For the visualization of medically significant fungi in the NRL for mycoses at the National Center for Infectious and Parasitic Diseases (Sofia, Bulgaria), a method was developed for the detection of antibodies to *Candida*, *Aspergillus* and *Cryptococcus* called Indirect immunofluorescence (IIF). Sera of patients are used, and the staining is in two stages – in the first stage, serum is used for the antigens of the fungus, and in the second stage, anti-species serum (anti-antiserum) is used for the immunoglobulins of the antifungal antibodies. The anti-species serum is conjugated with a fluorescent dye. For this purpose, it is necessary to fix the antigen of the fungus (suspension corresponding to *Candida*, *Aspergillus* and *Cryptococcus*) on glass slides, which are stored in a refrigerator at -20°C . The reaction is carried out in two stages.

To detect antibodies against *C. albicans*, the serum of the patient diluted with saline solution from 1:10 to 1:640, after which the dilutions are dropped onto the slide and left in a humid chamber at 37°C for 30 min. Then it is washed in a weak stream of running water and allowed to dry. In the second stage, fluorescent serum against human globulins is instilled. The preparation is again left in a humid chamber at 37°C for 30 min, after which it is washed and dried. A fluorescence microscope visualization follows. The highest dilution at which there is clear fluorescence is taken as the serum titer. For *C. albicans*, a titer > 1:160 is considered indicative.

Indirect immunofluorescence for the detection of antibodies to *Aspergillus* spp. in the serum of patients is carried out in the same way as for *C. albicans*.

Latex agglutination (LA) test: Besides antibodies, another frequently used method for systemic mycoses is the proof of antigens – of *Candida*, *Aspergillus* and *Cryptococcus*.

Cryptococcus antigen detection method is the latex agglutination (LA) test. Because the blood culture may be negative, circulating antigens may be detected in immunocompromised patients [3, 4].

A major component in the *Cryptococcus* capsule is glucuronoxylomannan. It acts as a circulating polysaccharide antigen in the serum. The principle of the method is based on latex particles loaded with monoclonal rabbit antibody. The particles react with the polysaccharide antigen from *Cryptococcus* to agglutination that can be visualized with the naked eye. LA tests for antigen detection in immunocompromised patients with CNS cryptococcosis are even more reliable. The test has a very high sensitivity and specificity [5, 6]. In our practice, this test is the tool of choice for this diagnosis, i.e. cryptococcal meningitis can be reliably diagnosed by antigen testing. In immunocompromised patients, CSF cryptococcal antigen is positive in more than 95% of cases. Culture testing was sometimes negative because the sample was of small volume (e.g. < 5 ml) and was not cultured for fungi (although *C. neoformans* also grows on ordinary nutrient media) or cultivation was short. Therefore, a longer time of cultural examination for cryptococci is necessary.

ELISA Platelia Enzyme-linked Immunosorbent Assay: Platelia (enzyme-linked immunosorbent assay) is an immunoenzymatic sandwich micro-test for the detection of circulating antigen (mannan in *Candida* or galactomannan in *Aspergillus*) in human serum and in bronchoalveolar lavage (BAL) fluid. Mannan and galactomannan antigen are polysaccharides non-covalently bound to the yeast cell wall and they appear to be the main biomarkers for the diagnosis of invasive fungal disease. A rat monoclonal antibody directed against *Candida* or *Aspergillus* antigen is used. The ELISA-sandwich technique is known as a commercial kit PLATELIA ELISA (BioRad, France) – respectively, for *Aspergillus* and *Candida*, and it is believed to be one of the most sensitive modern methods for the detection of mannan and galactomannan [7].

With ELISA tests, it is possible to test more serums once, latex agglutination tests can be used with less daily workload of the microbiological laboratory with such tests. The relatively high price of these tests should not be overlooked.

Aspergillus ELISA PLATELIA is a “sandwich type” immunoenzymatic microplate method that detects galactomannan in human serum. The method uses rat monoclonal antibodies directed against galactomannan. Serum samples are heat treated in the presence of EDTA to release immune complexes; then added to the wells of the plate loaded with monoclonal antibodies and incubated. In the presence of antigen, an antibody-antigen-antibody/peroxidase complex is formed. A chromogenic peroxidase substrate solution is added, which will react with the complexes bound to the wells to a colour reaction. This is an enzymatic reaction that is stopped by the addition of acid, which changes the colour of the well from blue to yellow. This is followed by spectrophotometric determination of the optical density of the ELISA – Rider samples set at a wavelength of 450 and 620 nm. Results are interpreted as positive or negative for the respective antigen.

The principle of the ELISA – *Candida* – PLATELIA method is the same as that for *Aspergillus*.

Cultural isolation: The identification of the isolated fungal strains was done using biochemical commercial kits Api 20 C Aux (BioRad) and Auxacolor according to the manufacturer’s instructions.

Species identification: The identification of the isolated fungal strains was done using biochemical commercial kits Api 20 C Aux (BioRad) and Auxacolor according to the manufacturer’s instructions.

MALDI TOF MS: MaldiTof (Bruker), in which a special well plate is used, on which a small portion of the pure culture is placed, 1 µl of formic acid is added. It is allowed to dry and 1 µl of “matrix” is dripped on top until dry. The plate is inserted into the device and the identification response is awaited.

Antimicrobial drug sensitivity test: The antimycograms were made using the so-called E-test, according to the recommendations of the European Committee for Antibiotic Susceptibility Testing (EUCAST). A 24-hour culture was suspended in 5 ml of 0.85% saline (0.5 McFarland) and Petri dishes were inoculated with Sabouraud dextrose agar. E-test strips with a concentration gradient are placed and on the 24th and 48th hours the Minimal Inhibitory Concentration (MIC) is read for the following antifungals: Fluconazole, Itraconazole, Miconazole, Nystatine, Voriconazole, Anidulafungin, Caspofungin, Isavuconazole, and Posaconazole.

DNA isolation and PCR analysis: At the National Center for Infectious and Parasitic Diseases (NCIPD, Sofia, Bulgaria) we isolated DNA from sera and from fungal strains in pure cultures. The following pure culture strains were used, after confirmatory identification: *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90050, *Candida parapsilosis* INSTAND 22019, *Cryptococcus neoformans* CIP 38, *Aspergillus fumigatus*.

Isolation of DNA was carried out by two methods – using a commercial INVITROGEN genomic DNA Mini kit (by Thermo Fisher Scientific, USA), and by CHELEEX 100 [8]. Cheleex 100 chelating material (BioRad) was used for ion exchange purification. Cheleex 100 resin was used for DNA isolation because it binds the Mg 2+ cation, which is the main cofactor of DNA-degrading enzymes (DNAses), and thus protects the sample from these enzymes. Granular polar resin binds cellular components after cell disruption, while DNA and RNA remain in the aqueous solution above Cheleex. INVITROGEN genomic DNA technology is made using a filtration column following the manufacturer’s instructions. The isolated DNA was subjected to real-time PCR for the detection of the most frequently isolated fungi that cause systemic mycoses.

Results. Fungal DNA detection tests were conducted at the National Reference Laboratory “Mycoses” at the National Center of Infectious and Parasitic Diseases (Sofia, Bulgaria). Clinical materials and patients were referred for examination from various sources, including hospitals in Sofia and the country, personal physicians, and infectious diseases units.

We analysed 24 samples (sputum and BAL). The fungal strains isolated by cultural examination were representatives of the genera *Candida* and *Aspergillus*. We identified 10 *Candida* spp. In addition to *Candida albicans*, non-albicans species were also identified such as *C. glabrata*, *C. krusei*, and *C. tropicalis*.

Among the strains of the *Aspergillus* genus, molds such as *A. fumigatus*, *A.flavus* and *A. niger* were identified (Table 1).

T a b l e 1
Distribution of fungal isolates from clinical material

Culturally Sputum BAL	An isolated strain		Negative
	Genus <i>Candida</i>	Genus <i>Aspergillus</i>	
	10	9	5

The “Minimum Inhibitory Concentration” (MIC) obtained determines whether the strain is sensitive (S), resistant (R), or with intermediate resistance (I). The results obtained as a total percentage (%) of susceptible strains (S) from the genera *Candida* and *Aspergillus* are presented in Fig. 1 and 2.

The results of the serological test for the detection of antigens and antibodies are presented in Table 2.

More than 200 tubes of serum and 20 tubes of whole blood, taken in respective vacutainers, were examined. DNA was isolated by InvitroGen DNA Blood Mini kit (QiAamp, 2003) from whole blood (clinical material), which was measured spectrophotometrically on a BioPhotometer spectrophotometer.

The obtained DNA from whole blood samples was run on real-time PCR targeting medically important fungi. The PCR test was negative for *Candida*

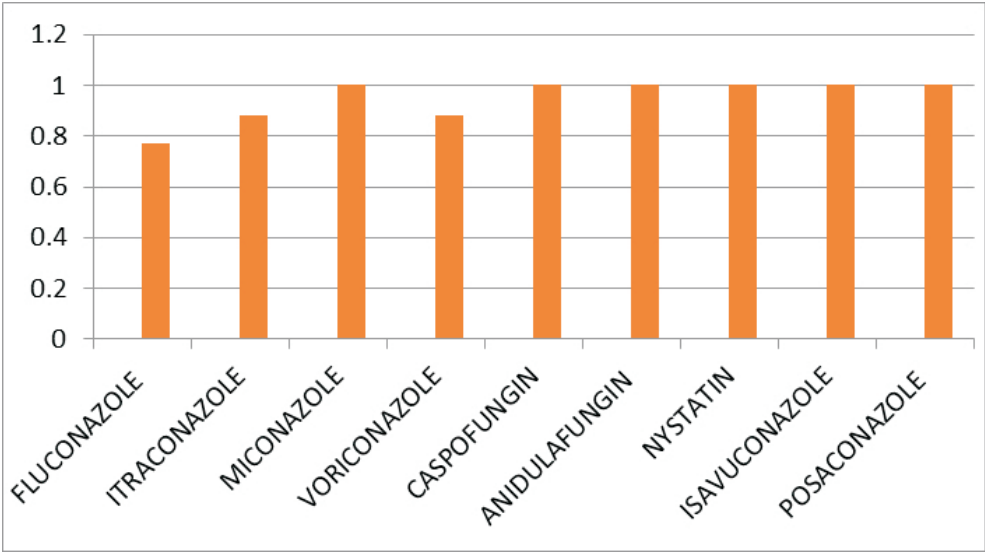


Fig. 1. Susceptibility (S) of *Candida* strains to antifungal agents (%)

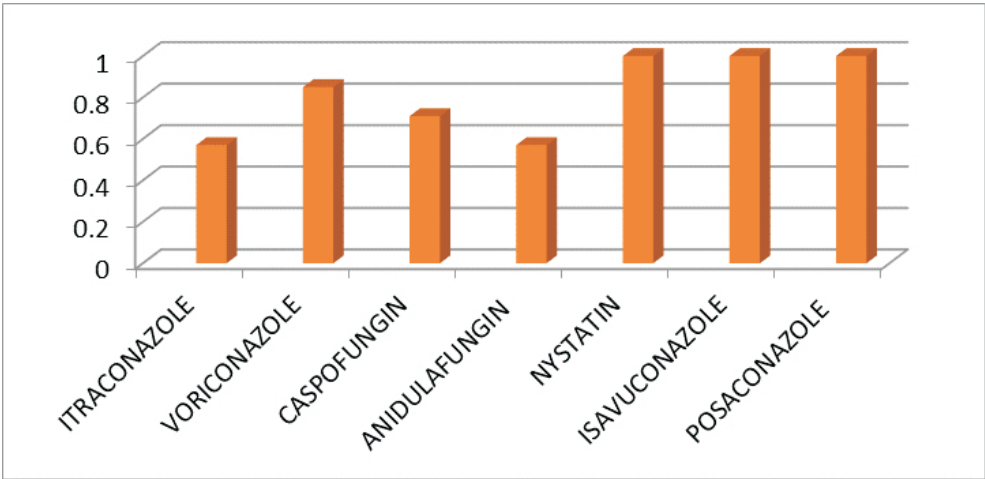


Fig. 2. Susceptibility (S) of *Aspergillus* strains to antifungals (%)

T a b l e 2

Results of the serological test (IIF) for antibody detection

IIF	Total number	Positive			Negative
		IgG (> 1:160)	IgM	IgA	
<i>Candida</i>	56	7	11	10	49 (IgG ≤ 1:160)
<i>Aspergillus</i>	182	54			128

albicans, *Candida glabrata*, *Candida parapsilosis*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*.

The serum samples tested by real-time PCR were positive for *Candida albicans* ($n = 5$), *Candida parapsilosis* ($n = 5$), *Candida glabrata* ($n = 2$) and *Aspergillus fumigatus* ($n = 6$).

Discussion. Invasive fungal diseases (IFD) have a severe prognosis and are characterized by high mortality, which necessitates accurate etiological diagnosis. In contrast to cultural methods, serological methods are faster. Standard cultural methods, despite their widespread use, do not have the speed and sensitivity of non-cultural methods for diagnosing systemic mycotic infection [9, 10].

Correct etiologic diagnosis will allow targeted therapy regarding the isolated etiologic fungal strain. Routine methods such as cultural isolation (including blood culture), species biochemical identification, imaging analysis of body sites delay the diagnosis of fungal infections. Diagnostic tests based on the detection of antigens (GM, mannan), antibodies and metabolites do not detect all fungal pathogens and do not meet the required sensitivity and specificity.

The shortcomings and limitations of the aforementioned methods have led to the development of molecular-genetic methods for the rapid and precise diagnosis of fungal infections, which is of particular importance for the diagnosis of probable systemic fungal infections and the initiation of timely antifungal therapy. These methods offer not only faster results, but also more specific identification of pathogenic microorganisms compared to traditional phenotypic methods.

A modern technique has been developed that combines PCR, hybridization of a specific probe and the detection of a light signal in one reaction step. This is fast DNA amplification and detection of the amplicon in “real-time” – “real-time PCR” [11].

Our analysis demonstrated that blood cultures from patients with histologically proven aspergillosis were negative by culture, while real-time PCR detected *Aspergillus flavus*. In principle, blood culture is considered an important tool for detecting systemic infection, but it has been shown to be positive in less than 50% of patients with chronic disseminated candidiasis.

Genetic methods exhibit greater specificity than serological methods. Molecular-genetic methods permit the proof and early detection of IFD with the objective of timely antimycotic therapy. Real-time PCR, in addition to early detection (within hours), also enables a quantitative assessment of the available DNA, which will allow for the distinction between infection and colonization and will provide information on the severity or progression of the disease. Real-time PCR is a method with high specificity and sensitivity and increases the reliability of the results [13–15].

Conclusions. We demonstrated that the need to introduce real-time PCR for the diagnosis of IFD for routine application in mycological laboratories is imperative [16].

Laboratory diagnosis of invasive fungal disease should be used together with other diagnostic procedures such as culture technique, histological examination of biopsy samples, CT imaging, and can be used as an aid in the diagnosis of invasive fungal infection [17].

Timely antifungal therapy is very important to reduce the risk of severe systemic fungal infection.

REFERENCES

- [1] KANTARDZHIEV T. (2012) Etiological diagnosis and etiotropic therapy of mycoses, (in Bulgarian).
- [2] DIMOPOULOS G., A. KARABINIS, G. SAMONIS, M. E. FALAGAS (2007) Candidemia in immunocompromised and immunocompetent critically ill patients: a prospective comparative study, *Eur. J. Clin. Microbiol. Infect. Dis.*, **26**(6), 377–384.
- [3] KANTARDZHIEV T. (1990) Systemic mycoses in patients with AIDS, *Epidemiology, Microbiology and Infectious Diseases*, **1**, 1–6, (in Bulgarian).
- [4] KANTARDZHIEV T., M. SAMNALIEV (1991) Chemotherapy of systemic mycoses, *Military Medicine*, **2**, 54–57, (in Bulgarian).
- [5] RIMEK D., K. REDETZKE, J. SINGH et al. (2004) Performance of the Candida mannan antigen detection in patients with fungemia, *Mycoses*, **47**(suppl. 1), 23–26.
- [6] VERON V., S. SIMON, D. BLANCHET, C. AZNAR (2009) Real-time polymerase chain reaction detection of *Cryptococcus neoformans* and *Cryptococcus gattii* in human samples, *Diagn. Microbiol. Infect. Dis.*, **65**(1), 69–72.
- [7] FUJITA S., T. TAKAMURA, M. NAGAHARA, T. HASHIMOTO (2006) Evaluation of a newly developed down-flow immunoassay for detection of serum mannan antigens in patients with candidemia, *J. Med. Microbiol.*, **55**, 537–543.
- [8] MALCHEVA M. (2020) Molecular methods for studying the serotype of Streptococcus pneumoniae colonizing the nasopharynx in children, Doctoral Dissertation – NCIPD.
- [9] DE REPENTIGNY L., L. KAUFMAN, G. T. COLE et al. (1994) Immunodiagnosis of invasive fungal infections, *J. Med. Vet. Mycol.*, **32**, 239–252.
- [10] WALSH T. J., S. J. CHANOCK (1998) Diagnosis of invasive fungal infections: advances in nonculture systems, *Curr. Clin. Top. Infect. Dis.*, **18**, 101–153.
- [11] EINSELE H., H. HEBART, G. ROLLER et al. (1997) Detection and identification of fungal pathogens in blood by using molecular probes, *J. Clin. Microbiol.*, **35**(6), 1353–1360.
- [12] KWIZERA R., A. KATENDE, A. TEU et al. (2020) Algorithm-aided diagnosis of chronic pulmonary aspergillosis in low- and middle-income countries by use of a lateral flow device, *Eur. J. Clin. Microbiol. Infect. Dis.*, **39**(1), 1–3.
- [13] ANANE S., F. KALFALLAH (2007) Biological diagnosis of systemic candidiasis: difficulties and future prospects, *Pathol. Biol. (Paris)*, **55**(5), 262–272.
- [14] HADRICH I., C. MARY, F. MAKNI et al. (2011) Comparison of PCR-Elisa and real-time PCR for invasive aspergillosis diagnosis in patients with hematological malignancies, *Medical Mycology*, **49**(5), 489–494.
- [15] KANTARDZHIEV T. (1990) Immunological diagnosis of systemic candidiasis, aspergillosis and cryptococcosis, *Epidemiology, Microbiology and Infectious Diseases*, **3**, 1–7, (in Bulgarian).

- [16] LETSCHER-BRU V., A. CAVALIER, E. PERNOT-MARINO et al. (1998) *Aspergillus* galactomannan antigen detection with Platelia *Aspergillus*: multiple positive antigenemia without *Aspergillus* infection, J. Mycol. Med., **8**(2), 112–113.
- [17] HOPFER R. L. (1997) Contemporary techniques for molecular diagnoses of mycoses, Clin. Microbiol. Newsletter, **19**(22), 169–173.

National Center of Infectious and Parasitic Diseases
26 Yanko Sakazov Blvd, 1504 Sofia, Bulgaria
e-mail: miraanbo@abv.bg