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# Invasive Aspergillosis: Diagnostic Strategies and Laboratory Tools

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**Abstract**

Invasive aspergillosis (IA) is a serious and fatal fungal infection caused by fungi of the genus *Aspergillus*. Their early diagnosis closely conditions the prognosis. On the other hand, they are often difficult to diagnose. Through this article, we intend to present the different microbiological techniques available, their sensitivities, their specificities and also their limits and to formulate recommendations. Current techniques are direct mycology with direct microscopic examination and fungal culture, the search for angioinvasion markers such as the Galactomannan antigen, the β-(1-3)-D-glucan antigen, the search for anti-*Aspergillus* antibodies, PCR *Aspergillus* and antifungigram. Given the multitude of these tests and the variabilities observed in terms of sensitivity and specificity, it is recommended to choose the most efficient tests, on compliant biological products and to interpret the biological results according to the patient's risk factors, clinical and radiological arguments. At the end of this work, we recommend for the biological diagnosis of IA in immunocompromised patients all detailed techniques except the search for anti-*Aspergillus* antibodies, and which must be requested and prioritized according to the clinical context of the patients.

**Keywords**: aspergillosis, invasive fungal infection, Laboratory Diagnoses, *Aspergillus.*

## 1. Introduction

Invasive aspergillosis (IA) is an opportunistic infection that primarily affects immunocompromised patients, particularly those with neutropenia [1]. Despite advances in management, it remains associated with significant morbidity and mortality [1,2]. The prognosis largely depends on the recovery of host immune function and the prompt initiation of targeted antifungal therapy. Such an outcome, however, requires an early and accurate biological diagnosis. Yet, establishing definitive evidence of invasive aspergillosis remains one of the most challenging diagnostic situations in clinical microbiology.

## 2. The Pathogen

*Aspergillus* species are ubiquitous, filamentous saprophytic fungi commonly found in soil and decaying vegetation. *Aspergillus fumigatus* is the species most frequently associated with invasive pulmonary aspergillosis, followed by species from the sections *Terrei* (e.g., *A. terreus*), *Flavi* (e.g., *A. flavus*), and *Nigri* (e.g., *A. niger* and *A. tubingensis*) [2]. Recently, the emergence of azole-resistant *A. fumigatus* strains has been reported, with an estimated prevalence of 3.2% in a prospective, multicenter international study [3].

## 3. Clinical Forms of Invasive Aspergillosis

### 3.1. Invasive Pulmonary Aspergillosis

Invasive pulmonary aspergillosis is by far the most common clinical manifestation, typically occurring in neutropenic patients [1,4]. Pulmonary involvement may be unilateral or bilateral, localized or rapidly progressive. Early diagnosis and treatment are critical to improving outcomes [1,2,4].

### 3.2. Extrapulmonary Aspergillosis

Extrapulmonary involvement occurs in approximately 25% of cases [4,5], with diagnosis often relying on tissue biopsy.

**Otorhinolaryngologic and upper airway involvement:**

Acute sinusitis primarily affects neutropenic or bone marrow transplant patients, whereas chronic sinusitis may occur in immunocompetent individuals [5]. Cerebral extension can be fatal. Diagnosis is based on CT, MRI, rhinoscopy, and biopsy. Laryngeal involvement is also possible. Tracheobronchial aspergillosis is more common after lung transplantation and has been reported in HIV infection [4,5].

**Cerebral aspergillosis:**

It is most commonly seen following allogeneic stem cell transplantation and is associated with a poor prognosis. It occurs in over 15% of disseminated aspergillosis cases [6]. Imaging (CT, MRI) can suggest the diagnosis, but definitive confirmation requires biopsy or abscess aspiration [4,6].

**Other sites:**

Spondylodiscitis is the most frequent osteoarticular manifestation, diagnosed by MRI and biopsy, and may occur in isolation or as part of disseminated disease [5]. Cutaneous lesions can arise at central venous catheter sites in neutropenic patients but are more common in burn patients [4]. Endocarditis generally follows cardiac surgery but may also occur in immunocompromised individuals. Renal, gastrointestinal, hepatic, splenic, and even thyroid involvement can be observed in 10–15% of disseminated aspergillosis cases [4,7].

## 4. Diagnostic Approach

Diagnosing aspergillosis is challenging due to its heterogeneous clinical presentation [5,8]. **In addition to clinical evaluation, the diagnosis relies on radiological and microbiological evidence,** summarized in **Table 1.** Invasive forms are specifically classified according to the international consensus by the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) [9,10]. Three levels of diagnostic certainty are defined: **proven, probable, and possible invasive fungal infection (IFI)**:

- ✓ **Proven IFI** is established by positive histology or culture from a normally sterile site **(Table 2).**
- ✓ **Probable IFI** requires the presence of a host factor, a clinical criterion, and a mycological criterion **(Table 3).**
- ✓ **Possible IFI** is defined by a host factor and clinical features suggestive of IFI, but without mycological confirmation [10].

**Table 1:** Key Steps in the Biological Diagnosis of Invasive Aspergillosis.

| Step | Techniques |
|---|---|
| **Fungal detection** | - Microscopic examination of fresh specimens<br>- Microscopic examination of stained smears<br>- Microscopic examination of stained tissue sections |
| **Fungal identification** | - Macroscopic and microscopic culture characteristics<br>- MALDI-TOF mass spectrometry<br>- Molecular identification (species-specific probes or sequencing) |
| **In vitro antifungal susceptibility testing** | - Agar diffusion method (E test or antifungal impregnated discs)<br>- Broth microdilution (according to CLSI or EUCAST guidelines)<br>- Molecular resistance markers |
| **Detection of serum antibodies** | - Screening assays (ELISA, hemagglutination inhibition, etc.)<br>- Confirmatory tests (immunoelectrophoresis, Western blot) |
| **Detection of markers of angioinvasion** | - Galactomannan antigen detection in serum and BAL<br>- β-(1,3)-D-glucan antigen detection in serum<br>- Circulating fungal DNA detection (PCR) |

**Table 2 :** Diagnostic Criteria for Proven Invasive Aspergillosis [10].

| **Microscopic Analysis: Sterile Specimen** | **Culture: Sterile Specimen** | **Blood** | **Serology** | **Molecular Diagnosis: Tissue** |
|---|---|---|---|---|
| Histopathology, cytopathology, or direct microscopic examination of a specimen obtained by **needle aspiration** or **biopsy** showing hyphal **filaments** associated with tissue **damage.** | Recovery of a hyaline or pigmented mold by **culture** of a **specimen obtained by a sterile procedure** from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, **excluding BAL** fluid, a **paranasal or mastoid sinus** cavity specimen. | Not applicable | Not applicable | Detection of *Aspergillus* DNA by PCR combined with sequencing. |

### 4.1. Specimen Collection

**Blood samples:** Since blood cultures are rarely positive for *Aspergillus*, sampling is mainly used to detect the fungus indirectly through the release of cell wall antigens or DNA [11].

**Sputum or expectorated samples:** Non-invasive and easy to obtain, these samples carry a high risk of contamination by oropharyngeal flora (>50%), even when strict protocols are followed.

**Endotracheal and endobronchial aspiration:** Using an intubation tube to collect broncho-pulmonary secretions is the preferred method in ICU patients or when invasive procedures are contraindicated.

**Protected distal sampling (PDS) or protected bronchial brushing:** Performed during bronchoscopy, this technique allows targeted, protected sampling from the infectious site, reducing contamination from oropharyngeal flora.

**Bronchoalveolar lavage (BAL):** Conducted via two-step bronchoscopy, collecting both bronchial and alveolar fractions. Advantages include minimal contamination from oropharyngeal flora and the environment, sampling a larger alveolar territory than PDS, and recovering greater volumes of secretions, separated into bronchial and alveolar fractions.

**Transthoracic or endobronchial lung biopsy:** Involves fine-needle biopsy under ultrasound or CT guidance, or video-assisted surgical biopsy, and if necessary, open thoracic surgery. However, in neutropenic patients, the high risk of bleeding limits the feasibility of these invasive procedures.

### 4.2. Diagnostic Tests

**Histopathology:** Tissue samples are fixed in formalin and stained using hematoxylin-eosin-saffron (HES), periodic acid–Schiff (PAS), or Gomori Grocott. Visualization of septate hyphae measuring 2–5 µm in diameter with branching at 30–45° suggest *Aspergillus* infection. However, it does not allow differentiation between *Aspergillus* species, nor exclude other septate filamentous fungi (e.g., *Scedosporium* spp., *Fusarium* spp.) [2].

**Direct Mycological Examination:** This involves visualization of the fungus or its asexual forms on fresh or stained specimens (Gomori Grocott, May-Grünwald-Giemsa, or fluorescent marking (calcofluor or Uvitex2B®). The septate and hyaline nature of the mycelial filaments, measuring between 2-4 µm in width and branching at an acute angle of 45°, is characteristic of the genus *Aspergillus*. This morphological examination is an essential element of diagnosis, particularly if *Aspergillus* heads are found.

**Culture:** Samples are cultured on Sabouraud agar medium with antibiotics, incubated 3–5 days at 25–37°C. Colony color varies by species: white (*A. candidus*), black (*A. niger*), shades of blue/green (*A. fumigatus*, *A. flavus*, *A. nidulans*), or brown-yellow (*A. terreus*). Isolation from a sterile site remains the key EORTC-MSG criterion for proven IA [10].

**Detection of *Aspergillus* Antigens:**

- ✓ ***Galactomannan (GM):*** present in the wall of *Aspergillus* is common to the genus. The GM detection technique currently used for *Aspergillus* is immunoenzymatic. With high analytical sensitivity, this test is mainly used as a screening test in patients at high risk of IA. In this context, serial galactomannan testing (at least twice a week during the risk period) has shown a sensitivity of 50 to 95% and a specificity of 60 to 98% for the detection of invasive aspergillosis. There are a number of causes of false-positive results (food absorption in children, contamination of certain beta-lactam antibiotics) and also false negatives. This test has also been used successfully in BAL and CSF samples. Currently available data suggest that monitoring the dynamic evolution of levels is more useful for diagnosis than a fixed cut-off level. A decrease in circulating galactomannan levels appears to be associated with a good prognosis, while its persistence or increase during treatment is associated with a poor prognosis [12].
- ✓ ***β-(1,3)-D-glucan (BG):*** is one of the glucose multimeric polysaccharides found in fungal cell walls. This molecule, which is not specific to the Aspergillus genus, is expressed by several fungi except *Cryptococcus* and Mucorales [11]. Quantitative measurement of BG is based on a modification of the mechanism of limulus amebocyte lysate. The principle is based on the property of limulus amebocytes to release coagulation factors when in the presence of BG. The measurement of serum (1-3)-ß-D-glucan for the diagnosis of invasive aspergillosis has a sensitivity of 80% and a specificity of 77% [13]. BG may be useful in hematology for the diagnosis of IA, but its specificity is limited and its clearance kinetics are slow [2]. BG is also useful because a negative result allows invasive fungal disease to be excluded (high negative predictive value) [11].

**Serological Detection of *Aspergillus* IgG Antibodies:** Different techniques exist to detect *Aspergillus* IgG antibodies, and they generally use antigens from *A. fumigatus* [11]. Some detection techniques visually identify the reaction with precipitating antibodies or precipitins (double immunodiffusion) and immunoelectrophoresis, while others use the sensitized hemagglutination or immunoenzymology (ELISA) techniques, which are faster and can be automated, allowing for the detection of different classes of immunoglobulins [8, 11]. Furthermore, *Aspergillus* serology in immunocompromised patients is of limited value, and their detection is a good marker of chronic infection and immunoallergic damage [4, 8].

**In Vitro Antifungal Susceptibility:** In cases of suspected invasive aspergillosis, it is recommended to assess the antifungal susceptibility of the isolate through in vitro testing or by identifying molecular resistance markers [8]. Reference methods established by the American Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) determine minimum inhibitory concentrations (MICs) using liquid dilution in tubes or microplates for yeasts and filamentous fungi. For filamentous fungi, MICs are determined at 100% inhibition, except for echinocandins, for which the minimal effective concentration (MEC) is used due to their mechanism of action, which allows residual in vitro growth. These reference techniques are labor-intensive and not suitable for routine testing, and are therefore limited to specialized laboratories. Commercial routine methods validated with the reference techniques include Sensititre YeastOne®, E-test®, Neo-Sensitabs™, and disc diffusion [14].

***Aspergillus* DNA Detection:** The choice of primers for *Aspergillus* PCR determines the sensitivity and specificity of the assay. Studies report that PCR on serum has a sensitivity of 79.2% and a specificity of 79.6% [15,16]. When combined with serum galactomannan (GM), sensitivity increases to 90% [17]. Moreover, many experts, including the Fungal PCR Initiative (FPCRI, formerly the European *Aspergillus* PCR Initiative), have worked on standardizing *Aspergillus* PCR tests for the diagnosis of IA, with fungal DNA extraction being the most critical step. Using PCR techniques compliant with FPCRI recommendations can improve both sensitivity and, particularly, specificity [18]. Extraction from at least 1 mL of serum or, preferably, plasma, combined with targeting ribosomal DNA to compensate for low circulating DNA levels, has shown improved sensitivity and specificity [16,18]. Commercial kits also detect CYP51A gene mutations associated with azole resistance [16]. A bivariate meta-analysis including all studies involving hematology-oncology patients evaluated the performance of PCR for IA diagnosis in whole blood or serum according to EORTC/MSG criteria. This study recommends that two consecutive positive PCR results strongly suggest IA [19], a recommendation incorporated into the EORTC/MSG criteria **(Table 3)** [10].

**Table 3:** Diagnostic Criteria for Probable Invasive Aspergillosis [10].

| Host Factors | Clinical / Radiological Criteria | Mycological Criteria |
|---|---|---|
| -Recent neutropenia (<500 neutrophils/mm³) for >10 days<br><br>-Hematologic malignancy-Allogeneic stem cell transplantation<br><br>-Solid organ transplantation-Prolonged corticosteroid use ≥0.3 mg/kg for ≥3 weeks within the last 60 days<br><br>-T-cell immunosuppressive therapy within the last 90 days<br><br>-B-cell immunosuppressive therapy<br><br>-Severe inherited immunodeficiency<br><br>-Acute graft versus host disease | **Pulmonary aspergillosis:** Presence of ≥1 of the following on CT:<br>- Well-circumscribed dense lesion(s) with or without halo sign<br>- Air crescent sign<br>- Cavitary lesion- Segmental or lobar consolidation<br><br>**Tracheobronchitis:** Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or necrotic lesion seen on bronchoscopy<br><br>**Sinonasal disease:**<br>- Localized acute pain (including periorbital)<br>- Nasal ulcer with black eschar<br>- Paranasal sinus extension through bony barriers, including orbit<br><br>**Central nervous system infection:** Presence of ≥1:<br>- Focal lesion on imaging<br>- Meningeal enhancement on MRI or CT | - **Microscopic detection** of fungal elements in sputum, BAL, bronchial brush, or aspirate-Isolation of<br><br>-***Aspergillus* spp. Isolated in culture** of sputum, BAL, bronchial brush, aspirate, or sinus sample<br><br>- **Galactomannan antigen*** detected in plasma, serum, BAL, or CSF:<br>• Single serum/plasma: ≥1<br>• BAL: ≥1<br>• Single serum/plasma ≥0.7 and BAL ≥0.8<br>• CSF: ≥1<br><br>- ***Aspergillus* PCR:**<br>• Plasma, serum, or whole blood: ≥2 consecutive positive tests<br>• ≥2 positive PCRs in BAL or multiple double-positive tests<br>• ≥1 positive PCR in plasma, serum, or whole blood and ≥1 positive PCR in BAL |

***Kit used :** Platelia *Aspergillus* (Bio-Rad®) ; <0.5 = negative; ≥0.5 = positive

**Table 4** summarizes diagnostic sensitivities of IA tests according to specimen type.

**Table 4:** Sensitivity of common diagnostic tests for invasive pulmonary aspergillosis [17].

| Test | Sensitivity |
|---|---|
| **BAL** | |
| **Culture** | + |
| **Microscopy** | + |
| **Galactomannan antigen** | +++ |
| **β-(1,3)-D-glucan** | Not applicable |
| ***Aspergillus* PCR** | +++ |
| **Blood** | |
| **Culture** | - |
| **Galactomannan antigen** | ++ (highly patient-dependent) |
| **β-(1,3)-D-glucan** | ++ |
| ***Aspergillus* PCR** | ++ |

## 5. Conclusion

IA remains a major cause of mortality in neutropenic patients, particularly during periods of aplasia and following stem cell transplantation. Prognosis can be improved by optimizing the combination of diagnostic and therapeutic strategies. Given the challenges of biological diagnosis, it is essential to interpret laboratory results in the context of the patient's risk factors, clinical presentation, and radiological findings. Based on this work, the recommended biological tests for diagnosing IA in immunocompromised patients include mycological examination, galactomannan and β-(1,3)-D-glucan antigen detection, and *Aspergillus* PCR.

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