Chapter

Molecular Diagnosis of Invasive Aspergillosis

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Abstract

Invasive aspergillosis (IA) is a disease that is difficult to manage and is associated with a significantly high morbidity and mortality, caused by different species of the genus *Aspergillus*, and closely related to immunocompromised patients; thus, it is important to understand the distribution and molecular epidemiology of the species causing this disease. Even though *Aspergillus fumigatus sensu stricto* is the most common species that cause IA, in recent years, there has been an increase in the number of species in the different sections which makes the diagnosis of this invasive fungal disease a great challenge. Conventional tests for the diagnosis of IA present limitations in sensitivity and specificity, while molecular tests have the potential to improve diagnosis by offering a more sensitive and rapid identification, but they are not yet standardized for reliable use in clinic. Nevertheless, there are some tests for the presumptive diagnosis of aspergillosis which, although are not specific for the identification of species, have been decisive in the case of IA. Among these are the Galactomannan test (GM), the Beta-D-glucan assay and volatile organic compounds (VOCs) testing. In this chapter, the recent advances and challenges in the molecular diagnosis of IA are revised.

Keywords: molecular diagnosis, invasive aspergillosis, PCR, epidemiology, molecular markers

1. Introduction

Aspergillosis is defined as tissue damage caused by fungi of the genus *Aspergillus* [1], which belongs to the class Ascomycetes. This genus consists of 8000 opportunistic and saprobic fungal species that have been reclassified as 250 species in nine primary sections: *Flavi*, *Fumigati*, *Nigri*, *Udagawae*, *Cricumdati*, *Versicolor*, *Usti*, *Terrei* and *Emericella* [2, 3]. Members of the genus *Aspergillus* are filamentous fungi that are ubiquitous in the environment [4, 5] and primarily develop close to decomposing plants, organic waste and soil, where they produce large numbers of conidia that are disseminated through the air [6].

Aspergillus is a cosmopolitan fungus that primarily infects immunocompromised hosts and individuals with the underlying lung disease. Some *Aspergillus* species are capable of causing a wide variety of diseases in humans and animals, collectively known as aspergillosis, which have increased significantly in recent years [7]. Approximately, 45 species have been reported to cause disease in humans [2, 8].

Aspergillosis can cause several clinical symptoms in humans, especially in immunocompromised individuals [8]. *A. fumigatus sensu stricto* is the most important opportunistic human pathogen, producing thousands of airborne conidia, which, due to their small size (2.5–3.5 μm in diameter), can be disseminated great distances by atmospheric disturbances such as wind convection currents and can survive under a wide range of environmental conditions. These airborne conidia can eventually be inhaled into the lungs of humans and animals, where an efficient innate immune response is required to prevent infection [8]. The primary diseases associated with this pathogen are allergic bronchopulmonary aspergillosis, chronic pulmonary aspergillosis, and IA, the latter of which is difficult to manage, leading to a significantly high morbidity and mortality. IA is the most serious disease caused by this fungus since it involves the invasion of fungal hyphae into tissue, and in some cases, haematogenously spread to other organs, particularly the brain [8]. The primary site of IA infection is the lungs. Infections of the skin and cornea may also occur, but fungal colonization of these sites is much less frequent. IA is rare in healthy individuals and almost exclusively affects patients with compromised immune systems [8–13]. The prognosis for these patients is often poor due to the scarcity of effective treatments combined with the already compromised state of health of these individuals, with mortality rates ranging between 30 and ≥90% depending on the immune status of the patient [14]. To reduce mortality in patients with IA, von Eiff et al. [15] administered empirical antifungal therapies to patients with severe neutropaenia (absolute granulocytosis of ≤500/μl) and observed a 90–41% reduction in mortality. However, this non-specific strategy has caused an increase in resistance to antifungals in patients undergoing long-term treatment [16]. In addition, some species belonging to the complex *A. fumigatus sensu latu* (*Fumigati* section) have been recognized as occasional causes of IA [17–20], and other IA-causing species belong to different *Aspergillus* sections or complexes (**Table 1**).

Country	*Total	Rate/100,000	Reference
Algeria	2865	7.1	[21]
Bangladesh	5166	3.2	[22]
Belgium	675	6.08	[23]
Brazil	8664	4.47	[24]
Burkina Faso	54[a]	0.3	[25]
Cameroon	1175	5.3	[26]
Canada	566	1.59	[27]
Chile	296	1.7	[28]
Colombia	2820	5.7	[29]
Czech Republic	297	2.8	[30]
Ecuador	594	1.3	[31]
Egypt	9001	10.7	[32]
France	1185	1.8	[33]
Greece	1125	10.4	[34]
Guatemala	671	4.4	[35]
Hungary	319[b]	3.2	[36]
Jordan	84	1.34	[37]
Kazakhstan	511	2.8	[38]
Korea	2150	4.48	[39]
Malaysia	1018	3.3	[40]

Country	*Total	Rate/100,000	Reference
Mexico	20[c]	0.017	[41]
	4[d]	0.0036	
Nepal	1119	4	[42]
Norway	278	5.3	[43]
Pakistan	10949	5.9	[44]
Peru	1621	5	[45]
Portugal	240	2.3	[46]
Philippines	3085	3	[47]
Qatar	11	0.6	[48]
Romania	1524	7.7	[49]
Russia	3238	2.27	[50]
Spain	4318	9.19	[51]
Thailand	941	1.4	[52]
Tanzania	20	0.05	[53]
Trinidad and Tobago	8	0.6	[54]
Ukraine	1233	2.7	[55]
Uruguay	773	22.4	[56]
United Kingdom	2001-2912[e]	4.59-4.6	[57]
Vietnam	14523	15.9	[58]

[a]*Haematological malignancies*

[b]*Chronic obstructive pulmonary disease.*

[c]*Renal, heart and liver transplant recipients.*

[d]*Allogenic- hematopoietic stem cell transplant.*

[e]*All risk groups except critical care patients.*

[*]*Total expected number of cases.*

Table 1.
Estimated burden of invasive aspergillosis.

2. Taxonomy of the genus *Aspergillus*

The genus *Aspergillus* belongs to the order Eurotiales and includes over 344 species, more than 40 of which are aetiological agents of opportunistic human infections, although some of them do so only occasionally [59].

Aspergillus is traditionally classified based on morphological characteristics, such as the size and arrangement of the aspergillary heads, the color of the conidia, the growth rate in different media and physiological characteristics. According to these morphological characteristics, Raper and Fennell [60] divided the genus *Aspergillus* into 18 groups. However, because this classification did not have any status in the nomenclature, Gams et al. [61] introduced the use of *Aspergillus* subgenera and sections. These studies showed that the groups organized by Raper and Fennell [60], which were based on phenotypic characteristics, largely coincide with the current classifications. However, because morphological variations in several sections resulted in controversial taxonomic groups, polyphasic identification was used, which involves the morphological, physiological, molecular and ecological characterization of a species [3]. Peterson [62] established the acceptance of five subgenera (*Aspergillus*, *Circumdati*, *Fumigati*, *Nidulantes* and *Ornati*) with 16 sections from a phylogenetic analysis rDNA region sequences. By contrast, Samson

and Varga [63], based on phylogenetic analysis using multilocus sequence typing (using calmodulin, RNA polymerase 2 and the rRNA gene), subdivided *Aspergillus* into eight subgenres: the subgenus *Aspergillus*, with the sections *Aspergillus* and *Restricti*; the subgenus *Fumigati*, with the sections *Fumigati*, *Clavati* and *Cervini*; the subgenus *Circumdati*, with the sections *Circumdati*, *Nigri*, *Flavi* and *Cremei*; the subgenus *Candidi*, with the section *Candidi*; the subgenus *Terrei*, with the sections *Terrei* and *Flavipedes*; the subgenus *Nidulantes*, with the sections *Nidulantes*, *Usti* and *Sparsi*; the subgenus *Warcupi*, with the sections *Warcupi* and *Zonati*; and the subgenus *Ornati*, with the section *Ornati*. Later, Varga et al. [64], based on multilocus sequence typing (using β-tubulin, calmodulin and the intergenic spacing regions [ITS] region), added the subgenus *Nidulantes* to the *Aenei* section.

Subsequently, based on different studies [62, 64–66], a new classification was proposed for the genus *Aspergillus* that included four subgenres and 19 sections in which the subgenera *Ornati* and *Warcupi* were transferred to other genera, since they did not belong to the genus *Aspergillus*. Similarly, the *Cremei* section, which had been classified into the subgenus *Aspergillus* [62], was reclassified into the subgenus *Circumdati* by Houbraken and Samson [66], resulting in the following classification: the subgenus *Aspergillus*, with the sections *Aspergillus* (teleomorph *Eurotium*) and *Restricti* (teleomorph *Eurotium*); the subgenus *Circumdati*, with the sections *Candidi*, *Circumdati* (teleomorph *Neopetromyces*), *Flavi* (*Petromyces*), *Flavipedes* (*Fennellia*), *Nigri* and *Terrei*; the subgenus *Fumigati*, with the sections *Cervini*, *Clavati* (teleomorph *Neocarpenteles*, *Dichotomomyces*) and *Fumigati* (*Neosartorya*); and the subgenus *Nidulantes*, with sections *Aeni* (teleomorph *Emericella*), *Bispori*, *Cremei* (teleomorph *Chaetosartorya*), *Nidulantes* (teleomorph *Emericella*), *Ochraceorosei*, *Silvati*, *Sparsi* and *Usti* (teleomorph *Emericella*). Finally, the current classification consists of four subgenera (*Aspergillus*, *Circumdati*, *Fumigati* and *Nidulantes*) and 20 sections [67, 68], with 339 correctly identified species [59]. There are currently 45 species of *Aspergillus* described as human pathogens [2], although the number of clinically relevant fungal species has been steadily increasing in recent years and is likely to increase further in the future (**Table 1**).

Colonies of the genus *Aspergillus* are typically fast growing and can exhibit a range of colors, including white, yellow, yellow-brown, brown and black or exhibit shades of greenish-gray or blue-green. *Aspergillus* species are characterized by the production of specialized structure, called conidiophores, which in some cases can be dramatically different [59]. Although it is a unicellular structure, the conidiophore has three distinct parts: the vesicle (swollen apical end), the stipe (cylindrical section located below the vesicle) and the foot cell (final section, sometimes separated by a septum that joins the conidiophore with the mycelium). The vesicle is partially or entirely covered by an enclosure of phialides (uniseriate). In many species, other cells called metulae are located between the vesicle and the phialides that support a small number of compact phialides (biseriate). The set forms what is called the aspergilar head, which can be strictly uniseriate, biseriate or mixed. The conidia are unicellular, round, oval, elliptical, smooth or rough, hyaline or pigmented, with thick or thin walls, produced in long chains that can be divergent (radiated) or aggregated in compact (columnar) or lax columns (that tend to open). Few species produce other types of conidia besides the phialides and metulae on the vesicle, and most develop directly on the vegetative hyphae as round or ovoid forms called aleuroconidia. Some species can produce Hülle cells, which can be solitary or envelop the cleistothecia form of one of the associated teleomorphs. The types of known ascomata present great morphological variation, ranging from those surrounded by loose hyphae with a smooth pseudoparenchymatous appearance to those exhibiting compact sclerotium structures, with variations in size, ornamentation and type of ascospore surfaces also observed [69].

The classification of the genus *Aspergillus* into subgenres and sections is made based on four fundamental characteristics: the presence of a teleomorph, the presence or absence of metulae, the disposition of the metulae or phialides on the vesicle and the colouration of the colonies [69].

3. Epidemiology

The number of IA cases has increased in recent years. Recent data provided by Bongomin et al. [70] estimate approximately 250,000 cases that occur worldwide each year, with an associated increase in morbidity and mortality rates. This increase is of great importance for health-care systems, since the epidemiological surveillance systems in several countries are inefficient. When combined with the difficulties in diagnosing IA, the resulting delay in the application of timely treatment can lead to the death of patients (**Table 2**). Given the importance of this disease, several research groups have developed 'The *Aspergillus* guide', which includes diagnostic and therapeutic guidance, focusing on life-threatening diseases caused by *Aspergillus* spp., primarily in Europe [71].

The major risk factors for developing IA are neutropaenia, allogeneic transplantation of hematopoietic stem cells or solid organ transplantation (particularly lung), haematologic malignancy and chemotherapy with cytotoxic cancer. Patients with chronic granulomatous disease and advanced AIDS also have a high risk of developing IA, as do patients receiving treatment with chronic steroid therapies and tumor necrosis factor as well as those with long-term chronic

Species	Identification method	Reference
A. ustus (100)	Culture and molecular typing (RAPD)	[72]
Emericella quadrilineata (4)	Sequence-based analysis [ITS region, β-tubulin (*benA*) and calmodulin (*caM*)]	[73]
A. viridinutans	Sequence-based analysis (β -tubulin and rodlet A gene)	[74]
A. udagawae	Sequence-based analysis [β-tubulin (*benA*)]	[75]
*A. fumigatus (*32) *A. lentulus* (4) *A. calidoustus* (2) *A. tubingensis* (1) *A. sydowii* (1) *A. flavus* (1) *A. terreus* (1) *E. rugulosa* (1)	Sequence-based analysis [ITS region, β-tubulin (*benA*) and calmodulin (*caM*)]	[76]
Section *Usti*: *A. calidoustus* Section *Fumigati*: *A. novofumigatus* *A. viridinutans*	Sequence-based analysis [β-tubulin (*benA*)]	[77]
E. nidulans var. *echinulata*	Sequence-based analysis [ITS region, β-tubulin (*benA*) and calmodulin (*caM*)]	[78]
A. lentulus	Sequence-based analysis [ITS region, β-tubulin (*benA*) and calmodulin (*caM*)]	[79]
A. lentulus	Culture Thermotolerence Sequence-based analysis [ITS region, β-tubulin (*benA*)	[80]

Species	Identification method	Reference
Culture: *A. fumigatus* (72.7) A.*flavus* (27.3) Nested PCR: *A. fumigatus* (30) A.*flavus* (20) Real-time PCR: *A. fumigatus* (6.25) A. *flavus* (12.5)	Culture (standard morphological procedures) Nested PCR Real-time PCR	[81]
Section *Circumdati*: *A. pallidofulvus* (6.66) *A. ochraceus* (2.22) Section *Flavi*: *A. tamari* (8.88) Section *Terrei*: *A. niveus* (6.66) Section *Versicolor*: *A. sydowii* (8.88) Section *Aspergillus*: *A. montevidensis* (6.66) Section *Nigri*: *A. brunneoviolaceus* (4.44)	Culture (standard morphological procedures) Sequence-based analysis [β-tubulin (*benA*) and calmodulin (*caM*)] MALDI-TOF MS	[82]
A. fumigatus (28.8) A. *flavus* (7.2)	Sequence-based analysis: [ITS region, β-tubulin (*benA*)]	[83]
A. flavus complex (15) *A. tubingensis* (3) *A. fumigatus (*2)	Sequence-based analysis: (region, β-tubulin (*benA*)	[84]
Section *Nidulantes*: *A. sublatus* (1)	Scanning electron microscopy of ascospores Sequencing of calmodulin gene	[85]

Table 2.
Species associated with invasive aspergillosis (IA).

diseases and other conditions, such as diabetes mellitus, rheumatological conditions, liver disease and chronic obstructive disease [8–13, 86–88]. It is also important to know the distribution and molecular epidemiology of *Aspergillus* species obtained from clinical and environmental sources in different geographical regions of the world, since different *Aspergillus* species can cause IA. In addition, although the *Fumigati* section (complex *A. fumigatus*) has been reported as the most frequent cause of IA, data suggest that IA can be caused by other species in immunocompromised hosts (**Table 2**), especially *A. niger*, *A. terreus* and species of the complex *A. flavus.* Therefore, the precise identification of *Aspergillus* species isolated from patients is of great importance for the selection of an effective antifungal therapy [84].

Because *Aspergillus* species are widely distributed fungi in the environment, and their conidia are dispersed primarily through air currents, their relationship with hosts in hospital environments is of great relevance. Standards have been established for hospital environments for adults and immunosuppressed children who require special attention. Ullmann et al. [71] recommend that patients should be separated from areas under construction or renovation and from potted plants and flowers in patient rooms and living quarters. In addition, they recommend placing patients in special rooms with positive air pressure and HEPA filters or laminar air flow and rooms with filters for water supplies, especially in showers.

4. Molecular diagnosis

Accurate and early diagnosis of an active *Aspergillus* infection is necessary to initiate effective antifungal therapy, particularly in critically ill patients [89]. The IA diagnosis is made based on the criteria defined for proven, probable or possible infection, implemented by the European Organization for Cancer Research and Treatment/Study Group on Mycoses (EORTC/MSG). These criteria depend on the clinical manifestations, host and fungal factors as well as the results of traditional laboratory methods (histopathology and culture) [90, 91]. However, the diagnosis of IA continues to be a challenge, since histopathology and cultures have limitations in their sensitivity and specificity as well as in the time required to obtain the results, which leads to significant delays in the initiation of treatment [89]. To achieve an accurate diagnosis and timely and effective treatment, molecular tests have been developed that overcome the limitations of conventional methods and can reduce the mortality rate associated with IA [92]. Thus, Samson et al. [59] suggested using a polyphasic approach (morphological characterization, physiological tests, ecological data, extrolite analysis and DNA sequencing) as a gold standard for the identification of *Aspergillus* species. However, because these methodologies are time and labour intensive, they are not practical in most clinical laboratories. Therefore, different PCR modalities have been developed through the use of specific molecular markers for the detection of *Aspergillus* species of medical importance to quickly identify the aetiological agent of aspergillosis [93, 94].

Molecular tests for the diagnosis of IA have been developed for both home and commercial use to directly detect and identify *Aspergillus* spp. in different clinical specimens, including the following: whole blood, serum, plasma, bronchoalveolar lavage (BAL), sputum, bronchial aspirate, tissue, pleural effusion and cerebrospinal fluid (CSF), allowing for the PCR amplification of fungal DNA via nested, multiplex and real-time PCR [89, 93, 95–105] and with minor frequency via isothermal amplification (loop-mediated isothermal amplification, LAMP) [106]. The efficiency of these tests is variable and depends on many factors, including the DNA extraction method, clinical sample type, type of PCR, amplification target and detection method. The lack of standardization of these technical problems represents the most important barrier for the widespread application of PCR as a diagnostic modality for the diagnosis of IA [107].

4.1 DNA extraction methods

The quality and quantity of DNA available for amplification depends on the extraction method used. Home, commercial and automated methods for DNA extraction are available. In the home, enzymatic, chemical or physical agents are used to break the cell wall, while sodium dodecyl sulphate, beta-mercaptoethanol and ethylenediaminetetraacetic acid are used to lyse the membrane. The elimination of proteins and purification of the DNA is performed by an extraction with phenol-chloroform, after which the purified DNA is precipitated with alcohol [91]. DNA can also be extracted using commercial methods or kits, such as the Qiagen QIAmp Tissue Kit (Hilden, Germany), but they are disadvantageous in that the efficiency of fungal DNA extraction can vary considerably between different commercial brands. Furthermore, for both the home-based and commercial methods, contamination of the extraction systems and reagents has been reported, which contributes to variations in the sensitivity and specificity of the tests. The use of automated methods, such as MagNA Pure LC (Roche Diagnostics, Basel, Switzerland), is a viable and high-performance option for DNA extraction but may be cost-prohibitive in many in-hospital laboratories with limited resources [91, 108].

4.2 Types of clinical samples

The direct detection of *Aspergillus* DNA has primarily been performed in whole blood, serum, plasma, and BAL, and occasionally in other specimens, such as sputum, bronchial aspirate, tissue, pleural effusion, peritoneal fluid and cerebrospinal fluid. It has been observed that the interpretation of results is easier when sterile samples are used than when non-sterile samples are used, such as BAL, since the ubiquity of the fungus can promote its presence in the upper respiratory tract, making colonization, invasion or contamination difficult to determine. To date, the most appropriate clinical sample for the diagnosis of IA has not been defined. However, the use of total blood and its fractions (serum or plasma) are the most widely used, the ease at which both blood can be obtained and the interpretation of results. The optimal blood fraction for the detection of *Aspergillus* DNA is unknown. Studies have been conducted to identify the ideal hematological sample to detect *Aspergillus* DNA, taking into account that the processing of whole blood and plasma requires the use of an anticoagulant, such as EDTA, which could inhibit PCR. It has been observed that the greatest inhibition occurs when using heparin or sodium citrate as an anticoagulant [109]. Some authors consider that from a practical point of view, serum is the best hematological sample for the detection of *Aspergillus* DNA, since it is easier to process and allows for antigens to be detected at the same time [98]. Other authors have shown that the sensitivity of PCR in serum and blood is similar, since the levels of circulating *A. fumigatus* DNA are between 100 fg/ml and 1 ng/ml, both in serum and in whole blood. They also report that the sensitivity may increase when a combination of serum and blood is used, which can perhaps be explained by the fact that the performance of DNA detection improves when large volumes of samples are used, rather than by the combination of the two *per se* [95, 98, 109, 110]. Meanwhile, Springer et al. [111] reported that PCR performed using plasma showed a higher sensitivity (91%) than for serum (80%) and whole blood (55%). These observations contrast with previous reports, possibly due to differences in sample processing [107].

BAL has also been used to detect *Aspergillus* by PCR, showing promising and contradictory results. In clinical studies, the general ranges of sensitivity and specificity varied widely, from 73 to 100% and 80 to 100%, respectively, depending on the characteristics of the trial and the type of patients evaluated. The majority of PCR studies using BAL have been performed using patients with haemato-oncological disorders. While some studies demonstrated a high specificity [100], others have shown greater sensitivity [112] or high sensitivity and specificity [99].

Other types of samples that have been used are lung or other deep-tissue biopsies, CSF and pleural effusion, which provided acceptable values of sensitivity and specificity [102, 103, 113]. However, its applicability in routine laboratory diagnostics is limited by the difficulty in obtaining this type of specimen.

4.3 Types of PCR

Nested PCR has been successfully used to detect *Aspergillus* spp. [102]. However, this technique is not the most suitable format, since although the process allows for great sensitivity, there is also the possibility of contamination and the generation of false-positive results. Multiplex PCR has also been used to detect *Aspergillus* spp., particularly in BAL samples. However, despite its adequate specificity and sensitivity (0.01 ng of DNA) [93], the detection method required (electrophoresis in an agarose gel) limits its application as a diagnostic tool, since results in this setting are expected to be obtained as quickly as possible. The primary limitation of these end-point PCR formats is the inability to differentiate between colonization or active infection. In 2006, the European *Aspergillus* PCR Initiative (EAPCRI) sought proposals for a technical consensus. This consensus was possible thanks to the generalization

of real-time quantitative PCR (qPCR). This technique drastically reduces the risk of contamination, allows quantitative management of the amplification reaction (quantification of the fungal load), differentiates between several pathogenic species when multiple probes are used in a single assay and allows for the result to be known; at the same time, the amplification is carried out, which helps establish an effective treatment in a timely manner [114]. Therefore, most of the PCR assays that have been developed for the diagnosis of IA are in a real-time format, primarily using hydrolysis probes (TaqMan) directed at the 18S, 28S and ITS regions [91].

Another alternative to detect *Aspergillus* is LAMP. This technique offers several potential advantages over PCR, among which is a more efficient amplification, the possibility of evaluating cell viability, a reduction in the contamination of the genetic material to be amplified and the possibility of using RNA as a target instead of DNA. The latter advantage allows for a greater sensitivity since highly expressed genes produce thousands of transcripts within a cell [106, 107].

4.4 Molecular marker or amplification targets

The selection of a molecular marker or a target region for PCR amplification to diagnose IA is of great importance, since it must favor a sensitive and specific amplification. The most commonly used markers to achieve high sensitivity in detecting *Aspergillus* spp., both in home and in commercial PCR assays, are multicopy genes, such as the ribosomal 18S and 28S genes, for which hundreds of copies are present in the genomes of *Aspergillus* spp. [89, 95, 97, 99, 102–105]. The disadvantage of using multicopy genes is that they involve highly conserved sequences in fungi, leading to a limited specificity (panfungal PCR). This type of amplification target does not always allow the differentiation of phylogenetically related species, such as species that are within the same section, which can often be important. For example, in the *Fumigati* section, although *A. fumigatus*, *A. lentulus* and *N. udagawe* are closely related, the latter two species are more resistant to antifungals than *A. fumigatus*. This PCR method may not even be able to distinguish between phylogenetically related genera (*Aspergillus*, *Penicillium* and *Paecilomyces* spp.), representing a problem in the selection of antifungal treatments [99]. Strategies have been designed to overcome the lack of specificity of multicopy genes, such as hybridization with species-specific probes [112]. Similarly, the variable regions of the rRNA gene, the ITS regions and D1/D2 are used [98, 100, 115]. ITS sequences may lack sufficient variation for the resolution of some *Aspergillus* species, and a bar code or a secondary identification marker is typically needed to identify an isolate at the species level. Based on these observations, the use of sequencing gene fragments, such as α-tubulin (*benA*) or calmodulin (*caM*), has been recommended for the identification of individual species within sections [107]. Single-copy genes have also been used as molecular markers in the diagnosis of IA, which provide a greater specificity to the assay but a lower sensitivity. The single-copy genes that have been used include *aspHS*, *SCW4* and *anxC4*, which have shown promising results for detecting *A. fumigatus*, *A. flavus*, *A. niger*, *A. nidulans*, *A. terreus* and *A. versicolour* in BAL samples [93, 101, 106]. Because the *aspHS* gene encodes a haemolysin that is overexpressed *in vivo* during infection, its detection provides specificity to differentiate an active infection (germinated conidia) from a non-active one (non-germinated conidia) [101].

4.5 Resistance markers

PCR has also been used to detect resistance to antifungals, since an increasing number of environmental and clinical *A. fumigatus* isolates with a lower susceptibility to azoles have been observed. Infections caused by azole-resistant fungi are associated with a very high mortality rate. The available evidence suggests that resistance may be emerging as a result of the widespread use of these compounds in

agricultural and clinical settings. The predominant resistance mechanism involves mutations in the *cyp51A* gene (L98H, Y121F, T289A and TR34), which encodes sterol demethylase, the target of azoles, and it has been shown that a variety of these mutations confer resistance to azoles. Molecular resistance tests have been used to assess fungal isolates as part of epidemiological surveillance studies of drug resistance and have been used to assess clinical isolates as a complement to phenotypic susceptibility testing. Real-time multiplex PCR has also been used to directly detect azole-resistant *Aspergillus* in BAL samples from patients at IA risk. However, more studies are needed to determine its potential utility in clinical care [107, 112].

5. Other diagnostic methods

Nowadays, there are some tests for the presumptive diagnosis of aspergillosis which, although are not specific for the identification of species, have been decisive in the case of IA. Among these are the Galactomannan test (GM), the Beta-D-glucan (BDG) assay and volatile organic compounds (VOCs) testing.

5.1 Galactomannans

The GM is a polysaccharide, the main component of the *Aspergillus* cell wall, which binds to and is released from the hyphae during growth [116, 117]. The galactomannan is not only found in the walls of *Aspergillus* but also in the cell walls of other fungi such as *Fusarium* spp., *Histoplasma capsulatum*, *Penicillium* spp., *Paecilomyces* spp. and to a lesser extent in other fungi [109, 118–121].

One of the most widely used tests for its determination is Platelia™ *Aspergillus* EIA (immunoenzymatic sandwich microplate assay), which uses monoclonal antibodies that are directed against the GM of *Aspergillus* [117, 122–124]. The real interest of this test in the diagnosis of aspergillosis lies in the fact that the galactomannan represents a good indirect indicator of the fungus [119] once it is released into the bloodstream; furthermore, it can be detected in body fluids such as serum, bronchoalveolar lavage (BAL), cerebrospinal fluid or pleural fluid [118].

Currently, it is considered as a serological method that facilitates the diagnosis of IA [125], even though it presents a highly variable sensitivity which ranges from 40 to 100% and depends on the population to be evaluated. Hachem et al. [118] showed in a study with patients suffering from haematologic malignancies associated to IA produced by *Aspergillus* non-*fumigatus* a sensitivity of 49%, whereas patients that presented IA by *A. fumigatus sensu stricto* showed a sensitivity of 13%, and the specificity was of 99% in both groups. Another study conducted by Maschmeyer et al. [126] reported that in patients undergoing chemotherapy for cancer or patients with hematopoietic stem cell transplantation, the sensitivity of the GM was of 67–100% and the specificity was of 86–99%. The meta-analysis conducted by Leeflang et al. [117] in patients with neutropaenia showed a sensitivity and a specificity of 78 and 81%, respectively. Pfeiffer et al. [116] found a sensitivity and a specificity of 95% in patients with hematopoietic stem cell transplant and solid organ transplants, respectively. It is important to highlight that the detection of GM in patients on antibacterial treatment results in the reduction of the test's specificity, while in patients on antifungal treatment, the sensitivity decreases in most cases. However, this does not occur when using caspofungin which increases the sensitivity [109, 127]. It is necessary to consider that when using bronchoalveolar lavage (BAL) to determine galactomannans, the test increases its sensitivity and turns out to be a more useful mortality prediction test [128].

5.2 BDG

The ß-glucans are glucose polymers (polysaccharides) of high-molecular weight that are found naturally in the cell wall of various organisms, such as bacteria, yeasts, fungi and plants. The BDG is produced by most fungi of medical importance such as *Candida* spp., *Aspergillus* spp. and *Pneumocystis jirovecii*. Among the fungi that release little BDG in serum, there can be found Mucorales fungi and *Cryptococcus* [129]. This antigen is important in fungal infections as it is released during the infection and has a higher sensitivity for the diagnosis of IA compared to GM and can be detected in the plasma of patients with mycosis. Therefore, this antigen can be used as a marker of fungal infection although it does not allow the identification of species [130] and is included as a diagnostic criterion in the European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) [90, 131].

For the detection of BDG, the Fungitell assay (Cape Cod Associates, Inc.) is used, which was approved by the Food and Drug Administration in 2003 for the presumptive diagnosis of IFI [132]. For this test, the results are variable in different studies, with sensitivity values ranging from 80 to 90% and specificity values from 36 to 92%, according to the cut-off value used [133, 134]. In studies with patients suffering from haematologic malignancies who develop IA, the sensibility of the test varies from 55 to 95%, and the specificity from 77 to 96% [133, 135]. It is important to mention that data from existing clinical studies for the Fungitell assay, which is the most widely used test today, suggest that the use of a detection limit of 80 pg/ml is associated with a greater precision than those with a result of 60–80 pg/ml, which are considered as indefinite. The above indicates that higher cut-off values dramatically decrease the sensitivity of the test, whilst increasing its specificity [135, 136].

The β-D-glucan assay is often useful in combination with culture, as it improves conditions for success [135]. Several factors that may increase the levels of BDG on IFI have been identified, such as thrombocyte infusions with leukocyte depletion filters, hemodialysis with cellulose membranes [137], the use of antibiotics such as amoxicillin-clavulanic acid or piperacillin-tazobactam [138], the use of surgical gauzes containing glucan, the administration of human blood products (immunoglobulins or albumin), severe mucositis and the presence of serious bacterial infections [129]. On the other hand, Pickering et al. [139] reported that high concentrations of bilirubin and triglycerides inhibit the levels of BDG and cause false-negative results, while hemolysis causes false-positive results.

5.3 Volatile organic compounds (VOCs)

The air exhaled by patients with invasive diseases contains a large number of VOCs, produced by different metabolic pathways, which can be used as biomarkers of pulmonary disease [109, 140]. Several techniques have been used to determine VOCs, like the gas chromatography and mass spectrometry which is impractical for use in the clinic [141–143]. One alternative seems to be the use of electronic noses and artificial olfactory systems that use a series of sensors that help discriminate each smell that represents a unique blend of VOCs, which function through pattern recognition algorithms called 'breathprints' [144, 145]. Several volatile organic compounds characteristic of *Aspergillus* spp. have been identified such as 3-octanone, isoamyl alcohol, ethanol, cyclohexanone, 2-methyl-2-propanol, 2-methylfuran, 2-ethyl-1-hexanol and 2-pentylfuran, among others [141–143, 146].

Among the advantages of these devices are their low cost, most of them are manual, easy to operate and provide results in few minutes. With regard to the benefits for the patient, they are non-invasive tests, safe, fast and easy to perform.

The sensitivity and specificity of this test is of 100 and 83.3%, respectively, which makes it one of the best options for the diagnosis of IA [140].

It is necessary to consider that in order to validate all these tests, more studies that provide additional advantages for their use must be conducted.

6. Discussion and conclusions

Currently, IA has become very important among fungal infections around the world since the number of cases has increased coupled with a high rate of morbidity and mortality, detected mainly in immunocompromised patients. The *A. fumigatus complex* has been reported as the most frequent cause of IA in immunocompromised hosts. During the last few years, new species have been described that belong to other complexes that also cause this disease, which represents a challenge to understand the epidemiology of this nosological entity. On the other hand, the appearance of new species causing IA generates problems to establish its diagnosis since conventional methods continue to be used, which fail to discriminate between closely related species, such as imaging, histopathology, microscopy and culture procedures. These last ones are still considered the gold standards even though they have a low sensitivity and are time-consuming [109]. Other methods that have been considered important are immunological methods to detect GM and BDG antigens in serum and other biological fluids. They also have limitations, such as cross reactions with other fungal species and interference with antibiotics such as b-lactams or with plasma infusion solutions [147]. In addition, these methods only identify the fungus at the complex level. It is important to identify the species, since recently, the presence of 'cryptic' *Aspergillus* species has been revealed in clinical samples of IA, which present differences in the susceptibility to antifungals, as is the case of voriconazole considered as the therapy of choice for invasive aspergillosis. The effectiveness of this drug is uncertain in the cryptic *Aspergillus* species, since it has been shown that resistance to multiple antifungal drugs is frequent, particularly in *A. lentulus*, *A. alliaceus*, *A. sydowii*, *A. calidoustus*, *A. keveii*, *A. insuetus* and *A. fumigatiaffinis* [19]. Currently, there is more awareness of the need to identify *Aspergillus* at the species level. This can be achieved through a polyphasic strategy [3]. In addition to the phenotypic characteristics, it includes the analysis of multilocus sequences, as well as PCR with specific probes for each species, such as multiplex qPCR, to identify clinically relevant *Aspergillus* species from the complex *A. fumigatus*, *A. terreus*, *A. flavus*, *A. niger and A. nidulans* [112]. However, molecular methods have not yet been recognized as diagnostic criteria for the identification of invasive fungal infection (IFI) by EORTC/MSG, due to the lack of standardization protocols, and the significant rates of false positives and false negatives. In addition, it should be considered that before its implementation in routine clinical practice, each diagnostic test must follow a long validation process, which involves various aspects such as limit of sensitivity, reproducibility and precision, so that the task is nothing simple; however, its use is paramount, so several recent studies have evaluated its application [108, 109, 148].

Therefore, it is clear that despite the efforts made so far to implement effective diagnostic methods, there is still no consensus about which is the ideal method. Therefore, as long as these methods are not standardized and their reliability is not guaranteed to improve the detection of *Aspergillus* spp. in an effective and timely manner, the diagnosis of IA will continue to represent a challenge.

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