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Detection and investigation of invasive mould disease

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A comprehensive review of diagnostic techniques for opportunistic systemic mycoses focused on invasive mould disease in immunocompromised patients is presented. We first analysed conventional diagnostic methods, such as microscopy examination, culture and radiology, underlining their limitations, which have led to the development of alternative methods, such as the detection of fungal components. Among these we highlight fungal antigen and DNA quantification, which make it possible to detect infections early and start appropriate treatment. We also briefly review the methods for carrying out susceptibility tests for antifungal drugs, including reference procedures, commercial techniques and their indications. Furthermore, we analyse the recommendations for therapeutic drug monitoring of antifungal agents in body fluids.

Keywords: galactomannan, β-D-glucan, PCR-based diagnosis, microscopic examination, cultures

Introduction

The objective of this review is to appraise the different techniques and procedures for detection and investigation of invasive fungal disease (IFD), specifically those utilized for diagnosis of invasive mould disease (IMD) in immunocompromised patients.

The conventional diagnostic methods, such as microscopy examination, culture and radiology, have important limitations, one being their low sensitivity. IMDs continue to have a high mortality rate, primarily due to the fact that diagnosis of these infections is often delayed, and consequently response to antifungal treatment is poor.[1,2] Classical microbiological methods cannot be considered early diagnostic techniques and their usefulness depends on the possibility of obtaining samples of deep tissues, which, in many cases, cannot be taken because of the condition of the patient. Furthermore, many fungal species take several days to grow in culture. It should also be noted that antibody detection techniques are useless in cases of opportunistic mycosis. These infections arise in patients who have alterations to their immune system, in whom antibody detection is not reliable for diagnosis of infection.[3,4] These limitations have led to the development of alternative methods based on the detection of fungal components, for example quantification of fungal antigens and DNA amplification. Most of the methods have been designed to diagnose aspergillosis and have been shown to be techniques that can detect infection early.

We review the strength of evidence that supports the utilization of the alternative methods to detect IMD in immunocompromised patients. We also briefly review the methods for carrying out susceptibility tests of antifungal drugs and recommendations for therapeutic drug monitoring of antifungal agents in body fluids to offer minimum requirements for laboratory investigation of IMD.

Conventional methods of microbiological diagnosis

The classical methods of microbiological diagnosis are those that are based on conventional techniques, such as microscopic examination, culture and identification of microorganisms. Microscopy and culture are of great help in identifying dermatophytoses and other superficial fungal infections,[3] but in the case of systemic fungal infections the usefulness of these techniques has always been limited.[5–7] We will review the most significant aspects of conventional methods of diagnosis.

Microscopic examination

Microscopy techniques include fresh and stained examination of microbiological samples, as well as histopathological studies. These diagnostic methods have important limitations, one

being their low sensitivity. It is possible to view fungal structures only when they are very abundant in the sample to be analysed, and this is usually when the infection is at an advanced stage. In addition, the sensitivity of microscopy is affected by the level of magnification and the number of fields examined. The second limitation is that, with a few exceptions, it is not possible to identify the species causing the infection by means of microscopy examination; this is fundamental, since there are therapeutic alternatives and the different antifungal drug susceptibility profiles of fungal species are known.[4,8]

Therefore, microscopy cannot be considered to be an early diagnostic technique, or one by means of which it is possible to classify the species causing the fungal infection. However, in many cases, it is the only technique that makes it possible to detect infection.[9,10] As for histopathological studies, both cytology and examination of histological sections help to detect fungal elements and diagnose infection. In the case of histological sections, haematoxylin and eosin, periodic acid–Schiff and silver stains, among others, may be the only methods capable of detecting the presence of a fungus in many cases of deep mycosis, such as zygomycosis.[6,8,9] In some infections, use of these stains to observe fungal structures can characterize the mycosis, such as the endosporulating spherules of *Coccidioides immitis*. It can also be determined if the infection is caused by yeasts or by mycelial fungi, and if one of the latter group is an agent of hyalohyphomycosis, phaeohyphomycosis or zygomycosis. Zygomycetes have wide, irregular, hyaline hyphae with no septa, and with open angle (45–90°) branches. Hyalohyphomycetes have fine, hyaline, septate hyphae with acute angle branches, while phaeohyphomycetes have thick-walled filaments with dark pigments.[11]

Histological sections can also be used to apply immunohistochemical techniques that make it possible to identify species present in tissue using a specific fluorescent antibody that binds to fungal elements in infected tissue. Furthermore, *in situ* hybridization and nucleic acid amplification methods can also be used, subsequent to the extraction of nucleic acid from tissue. Molecular techniques could become the techniques of choice for the identification of fungal species present in tissue samples since they appear reliable. However, there are no standardized methods, and all the studies published to date are of small case series, with non-representative numbers of samples.[9,12–14]

Microbiological culture

After microscopy examination, the samples must be inoculated onto a diverse range of culture media. It is important to point out that, in many cases, the samples taken are sent for histopathological and cytological evaluation only, and not for microbiological study. Clinical microbiologists have a responsibility to inform the appropriate clinical units to request both histopathology and microbiological/mycological investigations, since if the samples are not cultured, there is no possibility of identifying the species and carrying out susceptibility studies.

Although most fungi grow on standard culture media, such as blood agar and chocolate agar, media specific for fungal growth must also be used, including malt extract agar, cornmeal agar, Sabouraud glucose agar with cycloheximide, potato agar and brain heart infusion agar.[5,11] Cultures must be incubated at 30 and 37°C.

In samples in which there may be a polymicrobial infection or commensal bacterial flora, selective media are recommended, e.g. Sabouraud agar containing antibiotics such as chloramphenicol and gentamicin.[5,7,11] Some rare mould fungi, such as *Fusarium* spp. and *Scedosporium* spp., may be isolated from blood cultures and should not be considered as contaminants. The presence of filamentous fungi merits careful assessment. They are habitual laboratory contaminants and are part of the human saprophytic flora, and they decrease culture specificity. However, before ruling out fungi as contaminants or saprophytic flora, we must consider whether the patient has risk factors for an invasive mycosis and whether fungi have been cultured from other sites in the same patient.

As mentioned above, identification to species level is clinically useful since several alternative treatments are available. Characterization to species level is essential for all clinical isolates collected from deep tissue sites. Superficial and mucosal isolates should also be identified in cases of infection. For most clinical laboratories, characterization of fungal pathogens can be done using conventional methods of classification based on morphological, biochemical and physiological features. However, given the availability of nucleic acid sequencing techniques based on automated procedures, the identification of fungal species by molecular classification methods is closer to becoming routine clinical practice. Molecular identification, based on the sequencing of several DNA targets or fragments, has shown that classification by conventional techniques is not reliable for some moulds, especially the less common species.[15] Furthermore, it is apparent that the most frequently isolated species are, on occasion, groups of species, and that there are many cryptic species.[16–18] For clinical laboratories, the limitations of conventional identification techniques are important when a mistake in the classification of a species can lead to inappropriate antifungal treatment. This is not very frequent; therefore, in most cases, it is preferable to carry out an antifungal susceptibility test. Molecular identification should be considered, usually by referral to a reference laboratory, for cases that require confirmation or a more exhaustive analysis of the causative agent.

Radiology

Radiographic patterns can be useful for the detection of IMD. High-resolution CT and magnetic resonance imaging (MRI) scans make it possible to detect both pulmonary and extrapulmonary invasive aspergillosis and other IMDs with a degree of certainty, but they require the existence of macroscopic lesions, which are usually markers of poor prognosis. Most patients with IMD have macronodules and many also have halo signs. Other imaging findings are less common, and these include consolidation, wedge-shaped nodules, cavitary lesions and air-crescent signs. Patients presenting with a halo sign usually have a significantly better response to treatment. CT of the chest may be used to identify the halo sign, which is a macronodule surrounded by a perimeter of ground-glass opacity, and is an early sign of IMD.[19,20] It should be noted that the halo sign is not specific for invasive aspergillosis or even any IFD, as other infections and clinical entities can have similar radiographic patterns. The definitions of IFDs by the

European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) include several radiological signs among clinical criteria for probable IFD (Table 1).[21]

Non-culture-based diagnostic methods

Because of the limitations of conventional methods, alternatives to culture techniques have been developed to try to diagnose invasive fungal infections (IFIs) earlier, by detection of fungal cell components. In the 1970s, a method to detect the capsular antigen of *Cryptococcus* was developed, and this is one of the alternative methods that has been most useful for the diagnosis of an opportunistic fungal infection.[22] However, with other fungal infections it has not been possible to develop such effective antigen detection methods, although there have been significant advances in the last few years, with the commercial availability of tests for detection of galactomannan (GM) and β-(1,3)-D-glucan (BDG).

Galactomannan detection

GM is a component of the cell wall of *Aspergillus* spp. Many other filamentous fungi, such as *Paecilomyces* spp. and *Penicillium* spp., may have GM in their cell wall, although in lower amounts than *Aspergillus*; therefore, the quantification of this fungal component is considered to be a method for the specific diagnosis of aspergillosis.[21]

There is a sandwich ELISA test (Platelia *Aspergillus* EIA; Bio-Rad Laboratories, Hemel Hempstead, UK) and, although it has limitations, it is one of the main diagnostic advances of the last few years. For this reason it has been included as a mycological criterion of probable invasive aspergillosis. Its main contribution has been that it has shown usefulness, when combined with high-resolution CT, in the early diagnosis of invasive aspergillosis in high-risk patients with onco-haematological diseases. Determinations are carried out in serum or plasma, and it is advisable to do serial quantifications to increase specificity and early diagnosis. A diagnostic-driven strategy that incorporates GM monitoring should be combined with high-resolution CT and appropriate clinical and microbiological evaluation to make an early diagnosis of invasive aspergillosis. A single positive GM index of ≥0.8, or two consecutive samples with an index of ≥0.5, should prompt a diagnostic work-up (Table 2).[23–25]

A meta-analysis undertaken in 2006 estimated the mean sensitivity and specificity to be 71% and 89%, respectively.[26] It must be pointed out that the mean sensitivity and specificity are 71% and 89%, respectively. Sensitivity increases to 82% if the analysis includes only patients that have undergone haematopoietic stem cell transplants (HSCTs), and it is even greater in the case of allogeneic transplants. However, sensitivity declines to 22% in solid organ transplant (SOT) recipients, calling the usefulness of the test into question. Another meta-analysis has reported comparable results when analysing publications by index of positivity, i.e. a cut-off of 0.5, 1 and 1.5.[27] In children, the technique also seems to be reliable, though fewer data are available.[28] It has also been shown to be reliable for treatment follow-up. In patients with leukaemia who were diagnosed as having invasive aspergillosis and who were receiving antifungal treatment, a GM index value of >1 was considered a sign of therapeutic failure and experts recommend an alternative salvage therapy. Other studies found predictive GM index levels of >2 or lack of 1 week GM decay.[29–31]

In other groups of patients, GM quantification does not have the same diagnostic and prognostic value. In non-neutropenic patients with aspergillosis, the sensitivity of serum GM detection is no greater than 50%,[23] and this limits the use of this method as a diagnostic technique in critical care patients, in whom aspergillosis seems to be an emerging infection. However, several studies have shown that GM determination in bronchoalveolar lavage (BAL) may be useful in both critically ill patients and SOT recipients.[32–34] Recently, other studies have analysed the use of GM quantification in BAL in patients with haematological diseases.[35] With a cut-off level of 1, the technique had around 90% sensitivity and a positive predictive value (PPV) >75%.[36] There are no data regarding the accuracy of the detection of GM in other clinical samples, but positive quantification of GM in CSF supports the diagnosis of CNS aspergillosis.[37] Pending a recommended cut-off by the manufacturer, a cut-off of 0.5 is recommended (Table 2).

However, other studies have cast doubt on the reliability of serum GM detection even in patients with haematological diseases. It has been shown that prior administration of antifungals, especially prophylaxis with itraconazole, decreases test sensitivity by 30%.[38] On the other hand, an article published in 2009 indicated that the mean sensitivity of the detection of GM is significantly lower in patients with invasive aspergillosis caused by *Aspergillus fumigatus* than in infections caused by other species of *Aspergillus* (13% versus 49%).[39]

There have been many reports of false-positive GM results associated with the use of β-lactam antibiotics, especially piperacillin/tazobactam.[40] False-positive results have also been described in children colonized by *Bifidobacterium* spp. and in neonates.[41] Other possible causes of false-positive results are cross-reactions with other fungal species, immunoglobulins, haemoderivates, GM-containing solutions such as Plasma-Lyte (an intravenous hydration fluid by Baxter, Deerfield, IL, USA) and immunosuppressive drugs such as cyclophosphamide.[42,43]

The Third European Conference on Infections in Leukaemia (ECIL3) consensus report was published in 2009 on the diagnosis and treatment of aspergillosis in patients with haematological

Table 1. Radiographic patterns included as clinical criteria for diagnosis of probable invasive fungal disease in definitions by the EORTC/MSG

Fungal disease	Radiographic sign
Lower respiratory tract disease[a]	dense, well-circumscribed lesions with or without halo sign air-crescent sign cavity
Sinonasal infection	imaging showing sinusitis (plus at least one additional clinical sign)
CNS infection	focal lesions on imaging meningeal enhancement on MRI or CT

MRI, magnetic resonance imaging.
[a]Radiographic criterion of lower respiratory tract disease is the presence of one of the three signs on CT.

Table 2. Summary of recommendations for utilization of microbiological alternative methods for laboratory investigation of invasive mould disease

Alternative method	Indication	Recommendations
Galactomannan	early detection of aspergillosis	Serum serial testing in conjunction with high-resolution tomography in adult neutropenic patients undergoing intensive chemotherapy for leukaemia, or who have received an allogeneic transplant of haematopoietic stem cells. Cut-off: a single positive index of >0.7 or two consecutive samples of >0.5. In non-neutropenic patients, serum GM quantification does not have the same diagnostic and prognostic value. Serum serial testing is also useful in neutropenic paediatric patients. Serum value of >1 is considered a sign of therapeutic failure in adults and paediatric patients. Quantification in BAL (cut-off >1) and CSF (cut-off >0.5) samples may be useful in neutropenic and non-neutropenic patients.
β-D-Glucan	diagnosis of invasive fungal disease	Serum serial testing in both neutropenic patients and non-neutropenic patients. Cut-off: >60–80 pg/mL for the Fungitell test and >7 pg/mL for the Wako test. Lower accuracy has been described in haematological patients, which could be a significant limitation to use as screening. Less widely used than the GM test. Less data available.
PCR detection	detection of aspergillosis. No data for other mycoses	Additional technique that is still in development and its availability is limited in many cases to reference mycology laboratories.

GM, galactomannan; BAL, bronchoalveolar lavage.

malignancies, including patients with HSCT.[44] The recommendations were that when this test is used the results should be evaluated in conjunction with high-resolution CT in adult neutropenic patients undergoing intensive chemotherapy for leukaemia or in those who have received an allogeneic transplant of haematopoietic stem cells. Serum determinations must be performed every 3 or 4 days. The experts also recommend, although with a lower degree of evidence, that GM quantifications be performed with BAL and CSF samples when these are available, in both neutropenic and non-neutropenic patients. GM quantification is considered to be a useful diagnostic tool in paediatric patients, and in follow-up of a patient.

β-D-glucan detection

BDG is also a component of the fungal cell wall, but it is not specific for *Aspergillus* since it is present in many fungal species. In *Cryptococcus* spp., other basidiomycetes and zygomycetes, BDG is often present in very low quantities, and this decreases the sensitivity of the quantification technique, although several studies published recently have indicated that this test could also be useful for detecting cases of zygomycosis and cryptococcosis.[45,46]

The BDG test is considered to be a panfungal diagnostic method and it has been included in EORTC/MSG diagnostic criteria for IFIs in 2008, for all types of patients (Table 2).[21] However, the experience with this assay is limited. There are several techniques on the market, most of them available in Japan only, for serum quantification of this compound. In Europe and America, the most frequently used method is Fungitell (Associates of Cape Cod, East Falmouth, MA, USA).

Whereas GM detection has been demonstrated to be a useful technique for early diagnosis of aspergillosis in patients with blood diseases, BDG quantification has been used for the diagnosis of aspergillosis and other mycoses, such as candidiasis, in critically ill patients and in cases of *Pneumocystis* pneumonia.[47–49]

As with the GM test, series of serum determinations two or three times a week are recommended while the risk of infection continues. The cut-off point to interpret a positive result is >60–80 pg/mL for the Fungitell test. Most studies published to date consider that it is an adequate technique to rule out *Candida* or *Aspergillus* infections, since it has a negative predictive value greater than 90%, and that it could be appropriate for early diagnosis of IFD.[46,49,50] However, another study has described a high number of false-positive results in patients with bacteraemia, and this seems to decrease test specificity, placing its PPV at only 52%.[51] Recently, a study has calculated the PPV of the BDG test to be between 10% and 12% in haematological patients, and this could be a significant limitation to the use of BDG quantification for screening fungal infection in haematological malignances.[52]

Koo *et al*.[45] have published the most complete study to date on the use of BDG detection. In their multicentre study, samples were analysed from 871 patients, of whom 228 had proven or probable fungal disease, as shown in Table 3. Technique sensitivity was 64%, with a cut-off point of 80 pg/mL, specificity was 84%, the positive likelihood ratio was 3.93 and the negative likelihood ratio was 0.43. The technique was of greater use in patients who did not have haematological diseases, although the use of antifungal prophylactic or empirical treatment did not significantly decrease test sensitivity. The use of albumin, immunoglobulins or haemodialysis was associated with false-positive results.

Finally, a study published in 2008 used a different BDG test: the β-glucan test (Wako Pure Chemical Industries, Osaka,

Table 3. Number of proven and probable fungal infections included in the study performed by Koo *et al.*[45] on the quantification of β-D-glucan

Fungal infection	Number of proven infections	Number of probable infections
Candidiasis	83	3
Aspergillosis	26	38
Pneumocystosis	0	28
Zygomycosis	7	1
Infections caused by other yeasts	16	0
Infections caused by other filamentous fungi	10	7

Japan), which is a colorimetric technique with a detection limit lower than that of Fungitell. With a cut-off point of 7 pg/mL, sensitivity for several fungal infections was 63%, specificity was 96%, PPV was 79% and negative predictive value was 91%.[53]

The BDG detection tests have been less widely used than the GM test, but their inclusion in the diagnostic criteria by EORTC/MSG may increase their use. The current trend is to recommend a combination of several diagnostic techniques to rule out fungal infection in patients at risk; therefore, the BDG and GM combination can become the strategy of reference. ECIL3 recommended screening for IFD using BDG for high-risk patients with prolonged neutropenia after chemotherapy for acute leukaemia or for allogeneic HSCT recipients, although with a level of evidence that was inferior to that of the GM quantification technique.

Nucleic acid detection

Due to the limitations of conventional techniques for the diagnosis of deep mycoses, fungal nucleic acid detection in clinical samples has always been considered to be an alternative method that has great potential, especially for those techniques based on PCR that make it possible to amplify small quantities of DNA. However, the high theoretical sensitivity of the PCR-based techniques has not been confirmed to date in clinical practice, although there are ever more data that seem to support the use of these techniques in the diagnosis of mycoses.

Most PCR-based methods have been developed for early diagnosis of aspergillosis. A noteworthy factor is that each study published has been carried out with different methodologies for nucleic acid extraction, different primers and probes, and different overall conditions of the PCR reaction, and this makes it difficult to come to any firm conclusions.[54,55]

A meta-analysis was performed of 16 studies carried out on 1618 patients, in which different PCR techniques were used to detect *Aspergillus* DNA in blood, serum and plasma samples. This meta-analysis showed that, even though it is very difficult to compare published studies, DNA detection can be of use. The final results of this analysis show that a positive result with a PCR technique has 88% sensitivity and 75% specificity for the diagnosis of invasive aspergillosis. Furthermore, when series of determinations are performed, two or more consecutive positive PCR results increase the likelihood of a diagnosis of invasive aspergillosis by 6-fold.[56]

Over the last few years great efforts have been made to resolve the limitations detailed above. In the first place, the problem of the lack of reproducibility is being overcome by standardization and quality control. A multicentre effort involving many laboratories worldwide is under way to standardize *Aspergillus* DNA detection using PCR-based techniques by a working group entitled European Aspergillus PCR Initiative (EAPCRI; www.eapcri.eu) of the International Society of Human and Animal Mycology (ISHAM).[57]

Work is being done to overcome the problem of low sensitivity of these techniques with blood samples; this may be due to the fact there is little *Aspergillus* DNA, or that blood components inhibit the PCR reaction. To rule out possible inhibition, it is necessary for these techniques to incorporate an internal control. Furthermore, in view of the possible scarcity of DNA in blood samples, it is advisable to increase the volume of the processed samples or to carry out series of tests. Several recent articles indicate that 1–3 mL volumes of blood or serum must be processed to improve detection of *Aspergillus* DNA.[57,58]

Another approach would be to carry out PCR tests with other types of samples, such as lung biopsies or other deep tissues. Some studies have shown that PCR can be very useful to detect *Aspergillus* in tissues and BAL, although this approach has the limitation that frequently these samples cannot be obtained.[9,10,55,59,60]

Many experts also believe that it is necessary to carry out serial testing for *Aspergillus* DNA in blood samples. This strategy is similar to the one followed for GM and BDG, with the purpose of increasing sensitivity and thereby early detection of infection.[61] A study performed by Barnes *et al.*[62] prospectively followed a cohort of 125 patients at risk of aspergillosis, carrying out series of DNA determinations by PCR. Sensitivity of a single determination with positive PCR was 87%, and that of two or more positive PCRs was 75%. Furthermore, this strategy made it possible to decrease the administration of antifungal drugs by reducing their empirical use.[62] Another study analysed the efficacy of series of determinations of *Aspergillus* DNA in blood and serum in 83 patients with blood disease by using two consecutive positive PCRs as the criterion for aspergillosis. Sensitivity was >90%, with a PPV of 73.3%.[63] Recently, a clinical trial has been published in which two strategies for managing patients with blood diseases were assessed: an empirical strategy and treatment based on series of DNA determinations by PCR. No clear differences were found between the strategies; however, in the PCR group mortality at day 30 of follow-up was significantly lower than in the group that received empirical treatment (1.5% versus 6.3%).[64]

Another aspect to bear in mind relative to the performance of PCR techniques is their combined use with quantification of other fungal components or with high-resolution CT. Studies that have analysed the combined use of GM and PCR have shown that 100% of aspergillosis cases can be detected, with a specificity close to 90% and a PPV of around 80%.[62,63] However, some studies have also been published that indicate that PCR techniques have a low diagnostic yield. Generally, these are analyses in series of patients in whom PCR was used as part of a strategy of patient management, and in which antifungal prophylaxis and empirical treatment were also included. These treatments have a direct effect on PCR results, and therefore must be taken into consideration when designing studies to assess the efficacy of these diagnostic techniques.[65,66]

To summarize, PCR methodology can be of use to diagnose invasive aspergillosis, although in the latest consensus of EORTC/MSG and ECIL3 it has not been included as a diagnostic criterion of infection.[21] It should be considered an additional technique that is in development, and its availability is limited in many cases to reference mycology laboratories (Table 2).

With respect to nucleic acid detection techniques for diagnosis of other IMDs, there are much more limited data to support their use. Work has been performed with PCR techniques with other mould species such as Zygomycetes, *Scedosporium* spp., *Fusarium* spp. and endemic fungi. There are published studies that have used animal models and small series of patients to assess the yield of different PCR-based DNA detection methods. These have been used in blood and serum samples and with tissue samples. In general, the results are promising, but larger studies should be performed with standardized methods.[12,67–72]

To summarize this overview on non-culture techniques of diagnosis, we must include a brief comment on commercial PCR platforms. The best known is the SeptiFast diagnostic kit (Roche Molecular Diagnostics, Pleasanton, CA, USA), which detects the DNA of bacteria and some fungal species, such as *Candida* and *Aspergillus*. There are very few data on the clinical usefulness of these commercial kits, although they are being used in several ongoing studies.

Antifungal susceptibility testing

Standardization of antifungal susceptibility testing has improved in recent years. Reference methods have been drawn up that make it possible to perform these tests in a reliable and standardized manner. Reference techniques established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and by the US Clinical Laboratory Standard Institute (CLSI) have helped to determine the susceptibility of the most prevalent fungal pathogens to different antifungal agents.[73–76]

The reference techniques have served to establish clinical breakpoints to interpret antifungal susceptibility studies. They are based on the idea that the strains that have an MIC for an antifungal above a certain value respond significantly less well to treatment with that drug, since it is impossible to achieve therapeutic concentrations *in vivo*.[77,78] To date, breakpoints have been established for infections by *Candida* spp. only, and for some of the antifungal compounds available. For infections with other species of yeasts and filamentous fungi, no breakpoints have yet been established, although it is advisable not to treat with drugs that are inactive *in vitro*, or with those that have a high MIC for the species causing the mycosis; this is known as an epidemiological cut-off.[79,80] In the case of *Aspergillus* spp., some experts have proposed epidemiological cut-offs and even tentative breakpoints to interpret the results of susceptibility testing of those species to azole agents. An MIC value for itraconazole and voriconazole of $\geq$2 mg/L, and $\geq$0.5 mg/L for posaconazole, should be taken as resistant *in vitro*.[81,82]

These reference methods are complex techniques based on drug dilution series. Furthermore, they are expensive and require experience in their performance and they are not applicable in most clinical laboratories. For this reason, agar diffusion methods and commercial methods have been developed to extend susceptibility studies, since these are more practical for use in clinical laboratories. Not all these methods show a great correlation with reference procedures; therefore, mycology laboratories are advised to use disc diffusion methods or commercial methods whose results are comparable to those of the reference procedures.[83–86] Reference techniques are only recommended in specialized laboratories that carry out susceptibility studies for other reasons, such as the validation of new techniques, and sensitivity studies with new antifungal drugs or with rare species of fungi. Furthermore, the establishment of breakpoints and surveillance studies to detect resistance must also be carried out using the reference procedures.[87,88]

It may seem surprising but there is no proper evidence to support recommending that susceptibility testing be done in the clinical setting. However, there are situations in which *in vitro* susceptibility studies can contribute information that is very useful for individual patient management, and undoubtedly these should be done routinely with strains from therapeutic failures, breakthrough fungaemias and patients who have previously received antifungal prophylaxis, and in cases with uncommon species, whose susceptibility profile is not known. In addition, epidemiological studies are of fundamental importance. Several studies have shown that epidemiological surveillance of fungal infections helps determine the susceptibility of different species and their prevalence, and this makes it possible to choose the most appropriate treatment.[78,87,88]

Antifungal therapeutic drug monitoring

Therapeutic drug monitoring (TDM) of antifungal concentrations in body fluids has emerged in the field of medical mycology in recent years. Routine performance of these determinations could reduce the rate of therapeutic failures, since many are due to the fact that appropriate concentrations of antifungal drugs are not achieved in biological fluids, and this may be related to their pharmacokinetic properties. TDM can also be used to check compliance and absorption and to avoid toxic levels. Amphotericin B, fluconazole and echinocandins have a predictable pharmacokinetic pattern, and therefore monitoring is not apparently necessary. But flucytosine, itraconazole, voriconazole and posaconazole show such notable pharmacokinetic variability that monitoring is indicated in some clinical situations (Table 4).[89–91]

Itraconazole is not soluble in water and is absorbed irregularly by the digestive tract. It binds to plasma proteins (>98%), is not eliminated in urine, does not pass into CSF and is metabolized in the liver, thereby interacting with many drugs. It is available as oral and intravenous formulations. It is advisable to begin drug concentration determinations after 4–7 days of treatment and in the pharmacological trough. These determinations must be performed when oral treatment is begun, when there are gastrointestinal alterations, medication that causes interaction or signs of therapeutic failure.[89,92]

Voriconazole has a complex pharmacokinetic profile. The oral formulation sometimes has limited bioavailability; furthermore, metabolism of the drug in the liver is variable according to the genetic configuration of cytochrome P450 of the patient. On the other hand, it interacts with many drugs that are metabolized in the liver. For this reason, current treatment guidelines recommend the determination of plasma concentrations of this drug if there is a lack of clinical response, change in dosage, toxicity, liver/digestive

Table 4. Summary of serum or plasma therapeutic drug monitoring of antifungals

Antifungal	Optimal concentration (mg/L)		Toxic concentration (mg/L)	Monitoring indications
	prophylaxis	therapy		
Flucytosine	ND	10–50	>100	kidney failure toxicity
Amphotericin B	ND	ND	ND	none
Fluconazole	ND	ND	ND	none
Itraconazole	>0.5	>1	ND	start of oral therapy GI dysfunction interaction with drugs therapeutic failure
Voriconazole	>0.5	>1	>4	start of therapy GI or liver dysfunction interaction with drugs therapeutic failure change of dose toxicity
Posaconazole	>0.7	>0.7–1	ND	start of therapy GI or liver dysfunction interaction with drugs therapeutic failure
Echinocandins	ND	ND	ND	none

ND, insufficient data to recommend a given concentration; GI, gastrointestinal.

dysfunction or the presence of drugs that interact. Some experts recommend quantification of the drug in all patients, at the start of treatment. There are doubts as to when these determinations should be started, but there is a certain degree of consensus that they must be performed at least after 48 h of treatment and in the pharmacological trough.[93–95]

As to posaconazole, it is advisable to perform determinations of plasma concentrations of this drug, since it may have limitations in absorption, interactions with other drugs metabolized in the liver and, possibly, individual variations in its metabolism. Plasma concentrations should be determined after at least 2 days of treatment and in the pharmacological trough.[96,97]

Conclusions

After reviewing much of the literature, minimal requirements for the laboratory investigation of IMDs in immunocompromised patients have been proposed. The conventional methods of microbiological diagnosis, such as microscopic examination, culture and identification of microorganisms, have limited usefulness in detecting systemic fungal infections, but in many cases they are the only techniques available. Identification to species level is clinically useful since several alternative treatments are available. Characterization to species level is mandatory for all clinical isolates collected from deep tissue sites. Molecular identification should be considered, usually by referral to a reference laboratory, for cases that require confirmation or a more exhaustive study of the causal agent.

Alternatives to culture techniques have been developed to try to diagnose IFIs earlier. The serial quantification of serum GM can be used by clinical laboratories to detect invasive aspergillosis in patients with haematological malignancy who are at high risk of this infection. Quantification of GM in other clinical samples may also be useful in neutropenic and non-neutropenic patients.

There is less evidence to recommend BDG detection as a minimal requirement for the clinical laboratory, although it could be useful in diagnosing IFD in many groups of patients. PCR-based procedures should be considered as additional techniques for the early detection and confirmation of IMD, but their availability is limited in many cases to reference mycology laboratories.

In vitro susceptibility studies should be done routinely with strains from therapeutic failures, in breakthrough fungaemias, in patients who have previously received antifungal prophylaxis and in cases with uncommon species. Clinical laboratories are advised to use disc diffusion methods or commercial methods whose results are comparable to those of reference procedures. Periodic epidemiological studies should also be carried out. Finally, itraconazole, voriconazole and posaconazole show such notable pharmacokinetic variability that monitoring is indicated in some clinical situations.

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