

Laboratory diagnosis of invasive aspergillosis

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Invasive aspergillosis occurs in a wide range of clinical scenarios, is protean in its manifestations, and is still associated with an unacceptably high mortality rate. Early diagnosis is critical to a favourable outcome, but is difficult to achieve with current methods. Deep tissue diagnostic specimens are often difficult to obtain from critically ill patients. Newer antifungal agents exhibit differential mould activity, thus increasing the importance of establishing a specific diagnosis of invasive aspergillosis. For these reasons, a range of alternate diagnostic strategies have been investigated. Most investigative efforts have focused on molecular and serological diagnostic techniques. The detection of metabolites produced by *Aspergillus* spp and a range of aspergillus-specific antibodies represent additional, but relatively underused, diagnostic avenues. The detection of galactomannan has been incorporated into diagnostic criteria for invasive aspergillosis, reflecting an increased understanding of the performance, utility, and limitations of this technique. Measurement of (1,3)- β -D glucan in blood may be useful as a preliminary screening tool for invasive aspergillosis, despite the fact that this antigen can be detected in a number of other fungi. There have been extensive efforts directed toward the detection of *Aspergillus* spp DNA, but a lack of technical standardisation and relatively poor understanding of DNA release and kinetics continues to hamper the broad applicability of this technique. This review considers the application, utility, and limitations of the currently available and investigational diagnostic modalities for invasive aspergillosis.

Introduction

Aspergillus spp are ubiquitous opportunistic moulds that cause both allergic and invasive syndromes. The genus comprises approximately 180 species, of which 33 have been associated with human disease. Most infections are caused by *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus niger*; less commonly, *Aspergillus nidulans* can be implicated as the causative pathogen, especially in the setting of chronic granulomatous disease.²

An accurate diagnosis of invasive aspergillosis is important for clinical reasons; an earlier diagnosis is associated with improved patient survival³ and tests with a high negative predictive value may allow expensive and potentially toxic antifungal drugs to be withheld. New drugs—eg, voriconazole—exhibit differential mould activity; the ability to specifically exploit their anti-aspergillus properties requires a rapid and accurate laboratory diagnosis. The epidemiology of invasive aspergillosis is changing; invasive disease is increasingly observed in the non-neutropenic phase of haematopoietic stem cell transplantation^{4–6} and in non-classic settings such as critically ill patients in intensive care units.⁷ *Aspergillus* spp other than *A fumigatus*—some of which demonstrate inherent resistance to antifungal drugs—are increasingly recognised.^{8–10} An international collaborative effort recently produced standardised definitions for invasive fungal infections.¹¹ Thus, a review of the diagnostic modalities and their use in establishing a diagnosis of invasive aspergillosis is timely.

Diagnostic tools

Direct techniques

The advantages of direct techniques over culture include superior sensitivity and a relatively rapid turn around time. The principal disadvantage is the inability to definitively distinguish other filamentous fungi (eg,

Penicillium spp and *Scedosporium* spp) or implicate *Aspergillus* spp as the causative pathogen in circumstances in which there are atypical or non-specific morphological features. This disadvantage may compromise diagnostic accuracy and hence estimates of therapeutic efficacy if patients are recruited to clinical trials solely on the basis of hyphae that resemble *Aspergillus* spp. Within tissue sections, *Aspergillus* spp typically appear as slender septate hyphae that exhibit angular dichotomous branching (figure 1).

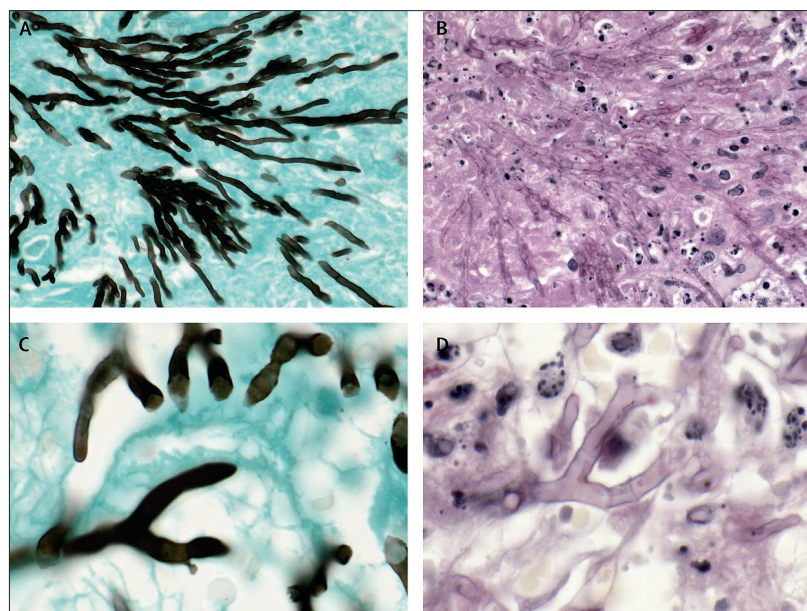


Figure 1: The appearance of *Aspergillus* spp in histological sections

(A) Gomori methanamine silver (GMS) stain of rabbit lung in experimental invasive pulmonary aspergillosis (magnification $\times 400$). (B) A similar section stained with periodic acid-Schiff (PAS) (magnification $\times 400$). (C) and (D) show acute angle dichotomous branching, which is typical of *Aspergillus* spp (magnification $\times 630$). The GMS sections demonstrate the prominent staining and stark appearance of hyphae. By contrast, with PAS there is preservation of background histological detail and hyphal morphology, but hyphae are less conspicuous against the background.

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Wet mounts, potassium hydroxide preparations, and use of routine stains

All specimens obtained in scenarios in which fungi are possible aetiological agents should be subject to a series of routine direct procedures; these procedures may vary according to the specimen, degree of clinical urgency, and the individual laboratory. Specimens may be examined as a wet mount preparation with or without the addition of 10% potassium hydroxide, which aids in the visualisation of hyphal elements through the partial digestion and clearing of proteinaceous material while leaving the fungal cell wall intact.¹² Subsequently, a smear is made on a slide, fixed and subjected to a variety of staining procedures. A Gram stain should be done as a matter of routine, but cytological stains (eg, Papanicolaou stain), fungal stains, and fluorescent stains may improve sensitivity.

Fungal stains

Fungal-specific stains should be applied in all cases in which invasive aspergillosis is considered a diagnostic possibility.¹³ Hyphal elements are stained with haematoxylin and eosin, although they may be difficult to visualise if sparse, fragmented, or present in the setting of substantial tissue necrosis. Fungal-specific stains—eg, Gomori's methenamine silver stain (GMS) and periodic acid-Schiff (PAS)—can be applied to histological sections and smears (figure 1). On occasion, GMS is referred to as Grocott's stain or the Grocott-Gomori silver stain—Robert Grocott demonstrated that GMS, which was initially designed as a stain for glycogen and mucin, also readily stained fungal elements.¹⁴ PAS has the advantage of providing a counter stain that reveals the background host cellular detail, tissue architecture, and inflammatory response. By contrast, the GMS counter stain removes the fine details of background host cells and tissues, but provides a more sensitive stain for detecting small fragments of cell wall that may be otherwise obscured by surrounding tissue elements. Thus, for detection of hyphal elements, the use of the GMS stain may be more sensitive; whereas PAS provides more of the cellular detail and architecture that may be of help in establishing relations between the fungus and other elements of tissue. This may be important in defining the individual aspergillus-related syndromes that vary according to the immunological status of the host. In this regard, GMS and PAS are complementary.

Fluorescent techniques

Fluorescent dyes—eg, Calcofluor white, Uvitex 2B, and Blankophor—are water-soluble colourless dyes that selectively bind to beta-glycosidically linked polysaccharides within fungal cell walls. They are not specific for *Aspergillus* spp, but have the advantages of relatively high sensitivity, rapid turnaround time, and broad applicability. They may be applied to frozen sections,

paraffin-embedded tissue, and other fresh clinical specimens—eg, bronchoalveolar lavage fluid (BAL) or corneal scrapings.^{15,16}

Immunohistochemistry, immunofluorescence, and in-situ hybridisation

Immunohistochemistry (using the monoclonal antibody WF-AF-1¹⁷ or EB-A1^{18,19}), immunofluorescence,²⁰ and in-situ hybridisation^{21,22} have been studied as diagnostic modalities. Collectively, these techniques have the potential to provide genus and species specific data, which may be important to improve diagnostic certainty when hyphae are seen invading tissue, but cultures or other adjunctive diagnostic data are negative. The availability of these modalities in routine clinical microbiology laboratories is variable.

Culture

A culture yielding *Aspergillus* spp, in addition to enabling a diagnosis of invasive aspergillosis, may further define therapeutic options via susceptibility testing or the isolation of a species possessing inherent antifungal resistance; examples of the latter include *A terreus* and *A nidulans*, which are both resistant to amphotericin B.^{10,23} The main disadvantage of culture is that it is relatively slow (the process takes days), is relatively insensitive,²⁴ and requires specialised expertise for species determination.

In common with other pathogenic fungi, the ability to grow at 37°C distinguishes *Aspergillus* spp from other non-pathogenic environmental moulds. *Aspergillus* spp can be recovered on most routine solid and liquid microbiological media (eg, blood agar, chocolate agar, brain heart infusion broth). A fungal-specific medium—eg, Sabouraud dextrose agar—should be included at the time of initial specimen set-up in clinical scenarios in which *Aspergillus* spp (or other moulds) are considered possible pathogens, because of superior yield.²⁵ The addition of antibiotics—eg, chloramphenicol and gentamicin—to the medium is required for the recovery of *Aspergillus* spp from specimens obtained from non-sterile sites, since they prevent bacterial overgrowth. Cycloheximide, a eukaryotic protein synthesis inhibitor, is frequently added to fungal media to inhibit the overgrowth of cultures by non-pathogenic environmental moulds; however, on occasion, cycloheximide may inhibit the growth of *Aspergillus* spp.²⁶

The identity of a laboratory isolate can often be inferred on the basis of colonial morphology and colour. Definitive identification, however, is dependent on a detailed inspection of conidial morphology and ontogeny and requires a microscopic examination of a simple teased preparation or a slide culture (a procedure in which sporulation is induced and the relevant diagnostic features are visualised on the under-surface of a cover-slip). The appearance and diagnostic features of individual species is beyond the scope of this review and readers are referred to definitive texts,²⁷ useful guides,²⁸ and excellent websites.

See
<http://www.aspergillus.man.ac.uk>
<http://www.mycology.adelaide.edu.au> and
<http://www.doctorfungus.org>

Several additional issues pertaining to culture require emphasis. First, the growth characteristics and morphological appearances of *Aspergillus* spp are protean and in some circumstances quite atypical; in this regard, *Aspergillus* spp are great mimics and should always be included in the list of diagnostic possibilities for an unidentified mould. Second, at least on occasion, sporulation may be difficult or impossible to induce,²⁹ and other modalities must be used for the purposes of identification. In this circumstance, molecular techniques are perhaps best placed to enable rapid and accurate identification.

Serological techniques

Galactomannan

Galactomannan is a heat-stable heteropolysaccharide present in the cell wall of most *Aspergillus* and *Penicillium* species.³⁰ The molecule is comprised of a non-immunogenic mannan core with immunoreactive side-chains of varying lengths containing galactofuranosyl units.³⁰ The composition of galactomannan varies between genera and strains, as well as the strain and conditions used for its production, extraction, and purification.³⁰ There are two commercial assays for the detection of galactomannan—the Pastorex kit (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) and Platelia ELISA (BioRad, Marnes-La-Coquette, France). Pastorex is now rarely used, while Platelia has been available in Europe for approximately 10 years and has recently been licensed in the USA. There has been a progressive increase in the understanding of the diagnostic utility of galactomannan to a point that has enabled its incorporation into diagnostic criteria.¹¹ However, galactomannan testing is not universally available to clinicians; the decision to offer galactomannan testing within a hospital microbiology laboratory depends on resources, the institutional incidence of invasive aspergillosis, and the hospital case-mix.

Details surrounding the release and kinetics of circulating galactomannan remain largely undefined. The growth phase, microenvironment, host immune status, and pathology may all influence galactomannan release.³¹ An abundance of data supports the notion that galactomannan production is proportional to fungal load in tissue;^{32–34} furthermore, galactomannan levels appear to have prognostic significance, with high unremitting levels in the face of antifungal therapy associated with an unfavourable outcome.^{10,33–37}

Assays to detect galactomannan have mostly used serum and BAL fluid. Galactomannan can also be detected in tissue and a number of bodily fluids including CSF, peritoneal fluid, urine, and pericardial fluid, although data to support its use at these sites is relatively scant, and is likely to remain that way.³⁸

Galactomannan assays use EB-A2, a monoclonal antibody derived from rats, which is directed towards the β (1,5)-linked galactofuranoside side-chain residues of the

galactomannan molecule.³⁹ Four or more epitopes are required for antibody binding.^{31,39} Detection is achieved using a sandwich ELISA format, which is made possible by multiple immunoreactive epitopes on a single galactomannan molecule.³⁹

There are a number of important determinants of analytical sensitivity of galactomannan assays. First, the binding of EB-A2 requires four or more galactofuranoside epitopes—sensitivity may be compromised by the inability to detect secreted antigens that bear fewer residues.³¹ Second, the Platelia assay is dependent on a pretreatment step, the goal of which is to remove complexing antibody that may block EB-A2 binding. However, the acid-sensitive galactofuranoside residues may be degraded by the edetic acid used in this step.³¹ Finally, the limit of detection using the sandwich ELISA format is lower (1 ng/L) than that achievable using latex agglutination (15 ng/L).⁴⁰ In terms of the analytical specificity, cross reactivity with other filamentous fungi, bacteria, drugs, and cotton swabs have been documented,^{41–45} but whether this is due to (exogenous) galactomannan or unrelated cross-reactive molecules is unclear.

There have been considerable efforts in establishing the appropriate galactomannan ELISA cut-off to maximise clinical sensitivity and specificity. The ELISA endpoint is a continuous variable and the optimal cut-off should be determined after defining the receiver–operator curve relation (ie, the relation between sensitivity and 1–specificity).⁴⁶ The cut-off level of 1.5 ng/L initially recommended by BioRad and used in many early studies has been progressively revised downwards; a cut-off of 0.5 ng/mL is now currently accepted by the US Food and Drug Administration (FDA), while a level of 0.7 ng/L is commonly used in Europe.⁴⁷

The clinical sensitivity of galactomannan ELISA is somewhat variable, with a range of 29–100%.³¹ There are a number of potential reasons for these disparate results. First, the performance of the assay may differ according to the host group and therefore the underlying pathological process. In studies of profoundly immunocompromised patients, sensitivity has been generally reported to be in excess of 90%,^{48,49} while in other settings—eg, chronic granulomatous disease⁵⁰ and solid organ transplantation—sensitivity appears to be somewhat lower.^{51–53} Second, accumulating evidence suggests that concomitant antifungal therapy leads to a decrease in the sensitivity of galactomannan.^{32,36,54} Finally, inadequate sampling strategies could conceivably compromise clinical sensitivity; the optimal sampling strategy for screening has not been rigorously defined, but the twice weekly determination of antigen levels has been generally used in patients deemed to be at risk of invasive aspergillosis. By contrast, galactomannan levels should be determined immediately in a host with a constellation of clinical features indicative of invasive aspergillosis to facilitate a definitive diagnosis.

The clinical specificity of galactomannan is generally estimated to be greater than 90%.^{32,36,47–49,55} The specificity of galactomannan in neonates and children appears to be lower, which is possibly due to the ingestion of extraneous galactomannan (in food and water) and translocation across a damaged or immature gut wall.^{45,47,56} Antibiotics represent an additional source of extraneous galactomannan that may compromise clinical specificity. The in-vitro reactivity of a range of antibiotics in galactomannan assays was originally reported in 1997.⁵⁷ More recently, positive galactomannan results in patients receiving piperacillin-tazobactam have been documented.^{58,59} This phenomenon has been further explored in vitro and in vivo and probably relates to the presence of galactomannan within the drug itself.^{60,61} This finding has forced some institutions to change their antibacterial protocols and the FDA to issue a warning.⁶²

(1,3)- β -D glucan

There has been an emergence of clinical data pertaining to the diagnostic utility of the cell wall component, (1,3)- β -D glucan.^{63–67} (1,3)- β -D glucan assays have been developed by Wako Pure Chemical Industries (Tokyo, Japan), Seikagaku Kogyo Corporation (Tokyo, Japan), Maruha Corporation (Tokyo, Japan) and Associates of Cape Code (Falmouth, USA); the assay developed by Associates of Cape Code—Fungitell—has been approved by the FDA in the USA for the diagnosis of invasive fungal infections. β -D glucan is present in the cell wall of most fungi; the notable exceptions are *Cryptococcus* spp and the zygomycetes.⁶⁷ The molecule is ubiquitous in the environment and has been used as a marker of fungal biomass.⁶⁸ The presence of (1,3)- β -D glucan in fungal species other than *Aspergillus* spp (eg, *Candida* spp, *Fusarium* spp, *Acremonium* spp, and *Pneumocystis jirovecii*) means that its role in establishing a specific diagnosis of invasive aspergillosis is not straightforward.

Assays to detect (1,3)- β -D glucan typically use serum. The common feature of all of the glucan assays is the ability of (1,3)- β -D glucan to activate a coagulation cascade within amoebocytes derived from the haemolymph of horseshoe crabs. Horseshoe crab lysate preparations were first used to detect endotoxin using the limulus test or limulus reaction (named after one type of horseshoe crab, *Limulus polyphemus*). Endotoxin induces clot formation via a serine protease zymogen named factor C (figure 2). Subsequently, evidence emerged that (1,3)- β -D glucan-induced clot formation independently of factor C, via a second serine protease zymogen, factor G, thus providing the impetus for the development of the current assays.

The analytical sensitivity of the Fungitell assay is in the order of 1 pg/mL, which is less than the cut-off of 60 pg/mL used in a recent clinical study.⁶⁷ A technical consideration pertinent to the analytical sensitivity of (1,3)- β -D glucan assays is that human plasma contains a number of inhibitors of serine proteases that need to be removed in a pretreatment step; this removal can be

achieved by an alkali reagent method (Fungitell), or by the addition of Triton X-100 and heating to 70°C for 10 minutes (Wako assay). The alkali pretreatment step in the Fungitell assay also converts triple-helix glucans into single-helix structures, which appear to be more reactive. Since both endotoxin and (1,3)- β -D glucan activate the horseshoe crab coagulation pathway, an assay that specifically detects (1,3)- β -D glucan requires removal of endotoxin from the specimen or the endotoxin-specific pathway from the lysate; correspondingly, endotoxin is inactivated by the addition of polymyxin in the pretreatment step in the Wako assay, while the Fungitell assay uses factor C to deplete limulus lysate. The pretreatment step also enhances analytical specificity via the removal of non-specific activators of serine proteases present in human serum.

There are no data that address the clinical sensitivity of the (1,3)- β -D glucan assays specifically for *Aspergillus* spp. The positive cut-off of 60 pg/mL was defined in a non-neutropenic group of patients with candidaemia.⁶⁹ The performance of (1,3)- β -D glucan in the context of antifungal therapy has not been rigorously studied. False-positive (1,3)- β -D glucan results have been documented in haemodialysis, cardiopulmonary bypass, treatment with immunoglobulin products, and exposure to glucan-containing gauze (eg, following major surgery).⁶⁹ Environmental (1,3)- β -D glucan contamination may also compromise specificity.

Antibodies directed toward *Aspergillus* spp

The demonstration of specific antibody is required to establish the diagnosis of chronic pulmonary aspergillosis.⁶⁹ Traditionally, antibody detection has not been considered useful for the diagnosis of acute invasive aspergillosis, following an early study that failed to document antibody formation in 15 patients with invasive aspergillosis.⁷⁰ Subsequently, antibody has been documented in approximately one-third of patients with invasive aspergillosis.^{47,71} The detection of antibody may

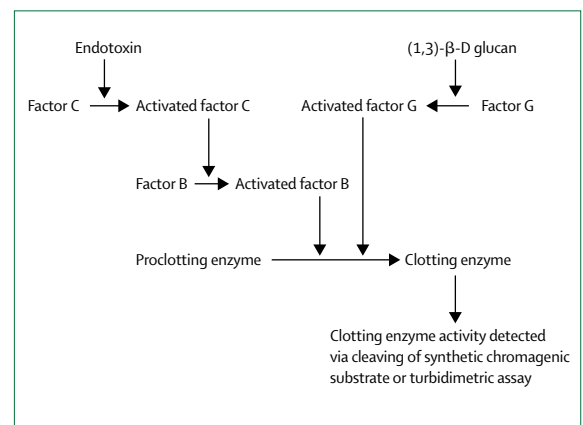


Figure 2: The pathways for the activation of the amoebocyte lysate by endotoxin and (1,3)- β -D glucan and the use of this pathway for the detection of (1,3)- β -D glucan

prove to be the best non-invasive means of establishing the diagnosis of subacute invasive aspergillosis in non-neutropenic patients with invasive aspergillosis, as illustrated by a recent case report describing invasive pulmonary aspergillosis in an individual with chronic granulomatous disease.⁷² Furthermore, antibody detection could be useful as a means of establishing a retrospective diagnosis of invasive aspergillosis in profoundly immunocompromised hosts who have undergone immunological reconstitution, although more work is required in this regard.

The detection of antibody

Many assay formats have been used to detect antibodies to *Aspergillus* spp, including immunodiffusion, counter immunoelectrophoresis, complement fixation, particle-haemagglutination, indirect-immunofluoresence, radio-immunoassay, and ELISA.^{73,74} The large number of epitopes in crude extracts may compromise specificity. The use of recombinant antigens—eg, dipeptidyl-peptidases,⁷⁵ superoxide dismutase,^{75,76} catalase,⁷⁵ metalloprotease,⁷⁵ mitogillin,⁷⁷ and galactomannoprotein^{71,78}—may rectify this situation. One potential advantage of using assays with a single antigen is the prospect of studying protective epitopes and thereby facilitating the generation of assays that may also confer prognostic information.

Metabolites

Aspergillus spp produce a range of extracellular enzymes (eg, metalloproteases, phospholipases) as well as primary (eg, mannitol)³³ and secondary metabolites (eg, gliotoxin),⁷⁹ all of which at least have the potential to serve as diagnostic markers for invasive aspergillosis. The ability of *Aspergillus* spp to produce D-mannitol has been known for many years⁸⁰ and its diagnostic potential examined in several experimental models of invasive aspergillosis,^{33,81} although it is limited in terms of its broad applicability as a diagnostic tool because of the complexity of measurements, which are done by gas liquid chromatography and mass spectroscopy. Recent work suggests that gliotoxin is produced by most *A fumigatus* strains and the possibility of using it as a diagnostic marker has been entertained.⁸² A comprehensive summary of the various secondary metabolites (mycotoxins) produced by *Aspergillus* spp can be found at <http://www.aspergillus.man.ac.uk>. The detection of metabolites represents an under-researched area in terms of their possible application as diagnostic modalities for invasive aspergillosis.

Nucleic acid tests

As far as the amplification of nucleic acid and diagnosis of invasive aspergillosis is concerned, PCR technology has dominated. A limited number of publications have used the isothermal technique nucleic acid sequence-based amplification.^{83,84} Only PCR will be discussed here. The lack of standardisation of technical issues has and

continues to represent a considerable barrier for the widespread application of PCR as a diagnostic modality for invasive aspergillosis and this is the focus of the following discussion.⁸⁵

Clinical specimens

Many studies have addressed the detection of nucleic acid from various fractions of blood (serum, plasma, whole blood) to establish a diagnosis of invasive aspergillosis, but PCR may also be applied to BAL specimens^{86,87} and tissue,⁸⁸ including paraffin-embedded sections.^{89,90} The optimal blood fraction for the detection of aspergillus DNA remains unknown. One study, using quantitative PCR (qPCR), suggested that the yield of DNA from serum, plasma, and white cell pellet was similar,⁹¹ while another demonstrated that the (qualitative) PCR signal from whole blood was superior to plasma.⁹² Serum has the advantage that it enables concomitant antigen testing⁹¹ and does not require the addition of anticoagulants (eg, sodium citrate, edetic acid, or heparin) that may inhibit PCR.⁹³

DNA extraction

There are a multitude of extraction techniques; the principal technical issues are summarised in table 1. The chosen extraction method represents a compromise between efficiency, freedom from exogenous contamination, and applicability to routine high-throughput laboratories. The fungal cell wall clearly represents the major hurdle to high-efficiency extraction of fungal DNA. DNA may be extracted using in-house methods, commercial kits (eg, Qiagen QIAmp Tissue Kit [Hilden, Germany]), and automated commercial techniques (eg, MagNA Pure LC [Roche Diagnostics, Basel, Switzerland]). Automated commercial techniques are probably required to make fungal DNA detection a viable option for routine clinical laboratories. The efficiency of extraction of fungal DNA may vary considerably between commercial kits.⁹⁴ High speed cell disruption incorporating chaotropic reagents and lysing matrices provide efficient and high yields of DNA from *Aspergillus* spp and other filamentous fungi.⁹⁵ Fungal contamination of extraction systems and reagents has been documented.⁹⁶ Considerable differences in DNA extraction protocols and performance is one aspect of molecular assays that hinders the comparison of studies.

Primer target

For clinical diagnostic purposes, the detection of a broad range of fungi is important, as is the ability to ultimately identify the specific pathogen(s). The optimal approach, in this regard, involves the application of broad-ranging panfungal primers with post-amplification analysis for species determination. Panfungal primers are directed toward conserved regions, usually within multicopy genes, which flank sequences containing species specific

Feature	Definitions and goals	Specific considerations with respect to invasive aspergillosis
Sample	Sample type, volume, transport, and handling should be defined	Serum and white cell pellet equivalent and possibly superior to plasma as sample Heparin and citrate inhibitory to PCR Sample handling varies between studies (some have demonstrated stability at room temperature for 48 h, others recommend immediate freezing)
DNA extraction	Target is of an adequate concentration and quality for amplification. PCR inhibitors and DNA nucleases removed	Extraction efficiency for fungal DNA is low due to the requirement to break the cell wall Contamination rate 3-3% in one series, commercial reagents may be contaminated with fungal DNA
	Removal of red and white cells	Ideally negative and positive extraction controls should be used Red cell lysis buffer and white cell lysis buffer
	Disruption of cell wall	Enzymatic methods (eg, lyticase, zymolase) Chemical (eg, boiling in dilute alkali)
	Disruption of cell membrane	Physical (eg, glass bead milling, freeze-thawing, sonication, grinding in liquid nitrogen)
	Precipitation of protein and purification of DNA	Usually achieved with lysis buffer (sodium dodecyl sulphate, beta-mercaptoethanol, EDTA) Phenol-chloroform Silica fibres (eg, Qiagen Tissue Kit)
	Capture of DNA	Alcohol precipitation Magnetic beads (eg, MagNA Pure) Silica fibres (eg, Qiagen Tissue Kit)
Amplification	Nested PCR, real-time formats, PCR-ELISA represent the commonest formats	Nested formats potentially allow for optimal analytical sensitivity but are associated with contamination and are difficult to compare Real-time formats will probably dominate in the future
	Amplification controls	Negative and positive controls are required
Analytical sensitivity	The smallest number of target organisms reliably and reproducibly detected by the assay	Multicopy target preferable Assessed by serial dilution of <i>Aspergillus</i> spp (conidia or purified DNA) using the appropriate clinical specimen as the diluent Circulating DNA in invasive aspergillosis is typically less than 10 colony forming units per mL or less than 30 fg Extraction method, primer target, and detection method all influence analytical sensitivity
Analytical specificity	Does the test detect only what it purports to?	Specific primer and probe sequences initially identified from public databases Amplicon ideally should be sequenced and a BLAST search done Cross reactivity studies with a range of fungal and bacterial pathogens as well as human DNA are required Post-amplification detection probe(s) may enhance specificity
Inhibition controls	Inhibitors of DNA polymerase (eg, heparin)	Spiking with purified aspergillus DNA and analysing in a separate reaction Spiking with a plasmid construct containing different size and sequence or label to the target Amplifying a human housekeeping gene (eg, beta-globin, HLA2), which also allows some determination of specimen adequacy, although the relative dominance of human DNA in clinical samples may mask low levels of inhibitors which could interfere with target amplification
Contamination control	Uracil-D-glycolase, appropriate number of negative controls	

Table 1: Technical variables required for a robust and reproducible PCR assay

polymorphisms that can be exploited in post-amplification analysis.

The ribosomal DNA (rDNA) complex is the most common target. This complex contains both conserved and variable sequences and there is a large volume of data deposited in public databases for a wide range of genera and species. The recent genome sequencing of *A. fumigatus*, using strain Af293, revealed 35 repeating units;⁹⁷ the structure of the gene complex is illustrated in figure 3.⁹⁸ The mitochondrial genes encoding some of the tRNA genes⁹¹ and (apo)cytochrome b⁹⁹ have also been used as primer targets. Mitochondrial targets can be considered “multicopy” because of a multiple number of mitochondria per cell nucleus; in Af293, there were 12 copies of the mitochondrial genome present for every copy of the nuclear genome.⁹⁷

Amplification format

Nested PCR formats have been widely used for *Aspergillus* spp in an attempt to optimise analytical sensitivity, but the

requirement to open reaction tubes means that there is considerable risk of contamination and the subsequent generation of false-positive results. Real-time formats have been increasingly used and are likely to dominate in the near future.

Post-amplification analysis

Post-amplification detection techniques provide genus or species specific data but may also increase sensitivity and specificity.^{100,101} Real-time detection techniques (eg, TaqMan, LightCycler, molecular beacons) are automated, rapid, and reproducible, thus facilitating comparisons between studies. Southern blotting has had a valuable role in the evolution of PCR as a diagnostic modality, but is unlikely to have any substantial future role in routine clinical assays. Single-strand conformational polymorphism,^{102,103} restriction fragment length polymorphism digest pattern,¹⁰⁴ Line Probes,¹⁰⁵ fragment size determination,¹⁰⁶ and PCR-ELISA¹⁰⁷ may have a limited role

in specific instances, such as the identification of laboratory isolates.

Analytical sensitivity and specificity

The analytical sensitivity of a molecular assay is usually determined by serial dilution of the infectious agent in pooled non-infectious clinical material as the diluent.¹⁰⁸ Such a paradigm immediately presents a problem for *Aspergillus* spp or any other mould, since accurate and indeed meaningful dilution of hyphae is not possible. Two commonly used approaches include serial dilution of conidia or DNA (either purified genomic DNA or a plasmid construct), although neither are ideal; the former does not mimic a biologically valid scenario, since hyphae rather than conidia are the invasive form, while the latter does not control for issues in extraction efficiency. If it is intended that more than one species is detectable then DNA from those species should be included in the assessment of analytical sensitivity.¹⁰⁹ The analytical sensitivity of published assays varies by several orders of magnitude; however, most studies report detection limits in the order of 1–10 fg DNA; variability in the detection limit is yet another issue that compromises study comparability.

Studies differ considerably in terms of the methods and extent to which analytical specificity is determined; there

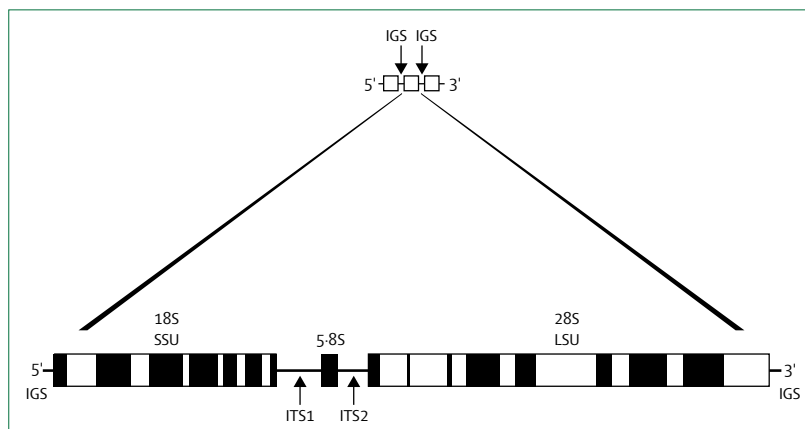


Figure 3: The structure of the ribosomal DNA complex

Shaded areas denote areas of variability that are present throughout the complex that can be exploited to design assays of varying levels of specificity. IGS=intergenic spacer; ITS=internal transcribed spacer; LSU=long subunit; SSU=short subunit. Adapted from reference 98.

are no standard techniques or criteria (table 2). Primer targets are generally identified by aligning sequences retrieved from public databases. This practice should be viewed as a necessary but insufficient step in establishing the analytical specificity of an assay and further validation procedures are required. Ideally, relatively early in assay

Primer target	Assay format	Intended specificity	BLAST search of primer and probe sequences; isolates with same probability match as intended target	Method by which analytical specificity determined and result	Reference
18S rRNA	PCR-ELISA	<i>Aspergillus</i> spp	<i>Aspergillus</i> spp, <i>Penicillium italicum</i> , <i>Penicillium commune</i> , <i>Penicillium chrysogenum</i> , <i>Penicillium brevicompactum</i> , <i>Penicillium phialosporum</i> , <i>Penicillium tardum</i> , <i>Penicillium allii</i> , <i>Penicillium expansum</i> , <i>Ajellomyces capsulatus</i> (teleomorph of <i>Histoplasma capsulatum</i>), <i>Paracoccidioides brasiliensis</i> , <i>Eupenicillium</i> spp, <i>Penicillioopsis</i> spp	Cross-reactivity studies: Amplification of <i>Aspergillus fumigatus</i> , <i>Aspergillus flavus</i> , <i>Aspergillus terreus</i> , <i>Aspergillus niger</i> , <i>Aspergillus nidulans</i> , <i>Aspergillus versicolor</i> , <i>Histoplasma capsulatum</i> No amplification of <i>Malassezia furfur</i> (3 strains), <i>Fusarium</i> spp (3 strains), <i>Trichosporon cutaneum</i> (2 strains), <i>Mucor</i> spp (3 strains), <i>Penicillium</i> spp (2 strains), <i>Pseudallescheria boydii</i> (1 strain), <i>Paecilomyces</i> spp (2 strains), <i>Saccharomyces cerevisiae</i> (2 strains)	Einsele et al ¹¹⁰
18S rRNA	TaqMan	<i>Aspergillus</i> spp	<i>Aspergillus</i> spp, <i>P italicum</i> , <i>Penicillium glabrum</i> , <i>P commune</i> , <i>P chrysogenum</i> , <i>P brevicompactum</i> , <i>P phialosporum</i> , <i>Penicillium purpurogenum</i> , <i>P tardum</i> , <i>Penicillium verrucosum</i> , <i>Penicillium hirsutum</i> , <i>Penicillium radicum</i> , <i>Penicillium funiculosum</i> , <i>Penicillium siamense</i> , <i>Penicillium pittii</i> , <i>Penicillium minioluteum</i> , <i>Penicillium pinophilum</i> , <i>Penicillium variabile</i> , <i>Penicillium rugulosum</i> , <i>Penicillium crateriforme</i> , <i>Penicillium variotii</i> , <i>Eupenicillium</i> spp, and others	Cross-reactivity studies: Amplification of <i>A fumigatus</i> , <i>A niger</i> , <i>A terreus</i> , <i>A flavus</i> , <i>Aspergillus oryzae</i> No amplification of <i>Candida albicans</i> , <i>Candida tropicalis</i> , <i>Candida krusei</i> , <i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Candida guilliermondii</i>	Kami et al ¹¹¹
Mitochondrial DNA (tRNA)	Competitive PCR with PCR-ELISA	<i>Aspergillus</i> spp	<i>A fumigatus</i>	Cross-reactivity studies: Amplification of 30 isolates of <i>A fumigatus</i> , <i>A niger</i> , <i>A terreus</i> , <i>A flavus</i> No amplification of <i>A nidulans</i> , <i>C albicans</i> , <i>C tropicalis</i> , <i>C krusei</i> , <i>C parapsilosis</i> , <i>C glabrata</i> , <i>Cryptococcus neoformans</i>	Bretagne et al ¹¹²
Mitochondrial DNA (tRNA)	Competitive PCR with PCR-ELISA	<i>A fumigatus</i> , <i>A flavus</i>	No database matches	Amplicon sequenced: revealing <i>A fumigatus</i> and <i>A flavus</i> Cross-reactivity studies: No amplification of <i>A niger</i> , <i>A terreus</i> , <i>A nidulans</i> , <i>Aspergillus ustus</i> , <i>Penicillium purpurogenum</i> , <i>Scopulariopsis brevicaulis</i>	Bretagne et al ¹¹³
Mitochondrial DNA (tRNA)	LightCycler	<i>A fumigatus</i>	<i>A fumigatus</i>	None, although clinical specificity assessed using 20 serum samples from healthy individuals	Costa et al ⁹¹
Mitochondrial DNA (cytochrome b)	LightCycler	<i>A fumigatus</i>	<i>Eupenicillium shearii</i> , <i>Neosartorya fischerii</i> , <i>A fumigatus</i>	Cross-reactivity studies: Amplification of <i>A fumigatus</i> , <i>Aspergillus clavatus</i> No amplification of <i>Candida</i> spp, other <i>Aspergillus</i> spp, <i>P chrysogenum</i> , <i>P expansum</i> , <i>P funiculosum</i> , <i>P variotii</i> , <i>Rhizopus oryzae</i> , <i>Fusarium proliferatum</i>	Spiess et al ⁹⁹

The BLAST searches were done at <http://www.ncbi.nlm.nih.gov>, using "search for short nearly exact matches". The primer and probe sequences were searched simultaneously and were separated by a string of at least ten nucleotides to ensure only the specified sequences were matched in the search algorithm. Only matches identical to those of the intended target are displayed.

Table 2: Selected examples of issues in establishing the analytical specificity of PCR assays to detect *Aspergillus* spp

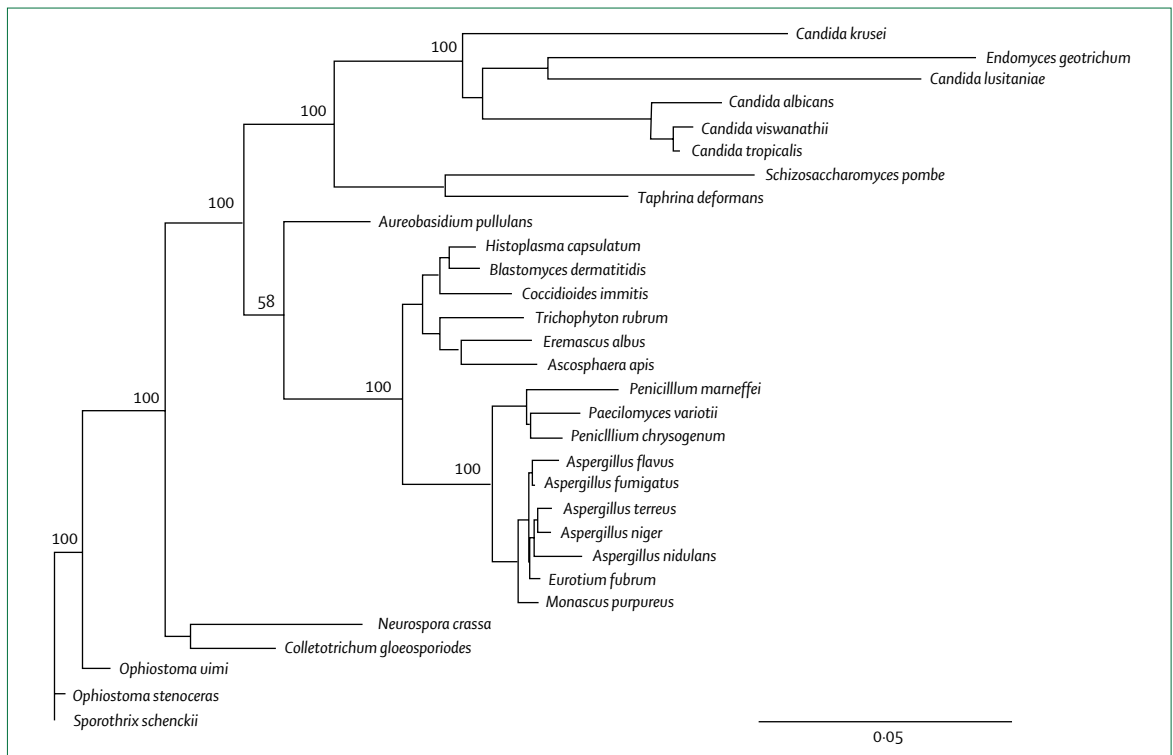


Figure 4: The phylogenetic relations between *Aspergillus* spp and other fungi based on the 18S rRNA complex. Adapted from reference 114.

development, the amplicon should be sequenced and a BLAST search done to verify that the intended target has been amplified.¹⁰⁹ Subsequently, the assay should be challenged with organisms that have a high likelihood of cross-reacting with the target; in the case of *Aspergillus* spp, genera that are close phylogenetic relations—eg, *Penicillium* spp and *Paecilomyces* spp¹¹⁴ are especially important to consider (figure 4). A further consideration is that sequences are being continuously deposited in public databases; a unique sequence at the time of primer design may subsequently align with a sequence from an unrelated species or genus deposited at a later date. Some have suggested that BLAST searches are done on an annual basis to ensure there is no cross reactivity with recently submitted sequence.¹⁰⁹ A final consideration is that false-positive reactions due to carry-over contamination of amplicon from previous reactions may be prevented with the addition of uracil-D-glycolase.⁸⁵

Clinical sensitivity and specificity

There are a number of factors that potentially have an impact upon the clinical sensitivity of PCR. The magnitude of the quantitative PCR signal falls with antifungal therapy in both experimental models and in clinical contexts—this may account for false-negative PCR results.^{110,111,115,116} Patients at risk for invasive aspergillosis are also often prescribed a multitude of drugs and fluids, all of which may act as non-specific inhibitors of PCR; as a

result, inhibition controls are mandatory and may take the form of spiking the sample with aspergillus DNA, a plasmid construct, or amplification of a human gene such as betaglobin (table 1).

The application of diagnostic modalities

Laboratory isolates

Given the distinct differences in disease manifestations, prognosis, and antifungal susceptibility between different fungal genera and species, a rapid diagnosis will assume increasing importance. The inherent problems with identification using culture methods have been outlined. An increasing number of studies have examined the use of PCR to enable the accurate and rapid detection of laboratory isolates (table 3). The rapid identification of laboratory isolates using microarray technology with a panfungal chip is possible and no doubt the relevant studies will emerge in the near future.

Clinical specimens

The application of diagnostic modalities to tissue, respiratory tract secretions, and blood in the context of the pathophysiology of invasive pulmonary aspergillosis is illustrated in figure 5.

Tissue and sterile fluids

Histological and culture techniques applied to tissue form the reference diagnostic standard for invasive

aspergillosis¹¹ and have been, and continue to be, the standard tools by which tissue invasion and destruction by hyphae is documented. Within this context, the following points specifically deserve emphasis. First, difficulties in obtaining deep tissue specimens in patients who are least able to tolerate invasive procedures have been exhaustively documented and remain one of the principal factors driving the development of new diagnostic techniques. Second, the analytical sensitivity of both histology and culture is relatively low, meaning that invasive disease is well established by the time that culture and histology are positive. Third, the specificity of the reference standard for *Aspergillus* spp is optimised with the combination of histological and culture data and this rigorous standard has been used in some recent clinical trials.^{48,120} The problem, however, is that *Aspergillus* spp can only be recovered from tissue in the context of positive histology in 30–50% of cases.²⁴ Finally, the possibility of accepting a positive PCR result in tissue as the reference standard for invasive aspergillosis deserves increasing attention. Certainly, data from experimental models suggests that validated PCR is more sensitive than culture for the detection of *Aspergillus* spp in tissue, especially in the setting of substantial tissue necrosis,^{115,121} the key in this regard is assay validation.

Non-sterile sites

In the absence of tissue specimens, samples obtained from contiguous non-sterile sites—eg, the upper and lower respiratory tract—serve as a surrogate with which to establish the diagnosis of invasive aspergillosis. In the case of invasive pulmonary aspergillosis, viable hyphal elements or related serological or molecular markers are shed into the respiratory tract from infected parenchyma (figure 5). A body of data suggests this shedding occurs relatively late in the natural history, thus compromising attempts to establish an early diagnosis using this approach.^{25,122,123} The isolation of *Aspergillus* spp (or related serological, molecular, or biochemical markers) in the respiratory tract may

represent one of three scenarios: (1) evidence of current disease, (2) true colonisation, or (3) a marker for the future development of invasive disease. An example of the latter is provided by a study that demonstrated that a positive PCR result from BAL at the time of bone marrow transplant conditioning was predictive of the subsequent development of invasive pulmonary aspergillosis.¹²⁴

There are a number of points to make about using BAL specimens to secure a diagnosis of invasive pulmonary aspergillosis. First, although BAL is a safe procedure, even in patients with substantial immunological impairment, it is not a trivial undertaking and requires a dedicated and competent bronchoscopist and an adequate commitment of resources. Second, the overall sensitivity (using culture and microscopy) is relatively low and generally estimated to be in the order of 50%.^{122,123,125,126} Variations in BAL technique,¹²⁷ the location, size, and type of pulmonary lesions,^{128–130} and the timing of bronchoscopy¹²² are all important determinants of the overall estimate. The impact of antifungal therapy in terms of the recovery of aspergillus and related markers in the respiratory tract remains poorly defined. Third, the specificity of the isolation of *Aspergillus* spp from the respiratory tract in patients with substantial immunological impairment—eg, those with allogeneic haematopoietic stem cell transplantation or neutropenia—is very high,¹³¹ but for other patient groups, the likelihood of underlying invasive pulmonary aspergillosis varies enormously.^{25,131} Fourth, qPCR may prove to be especially useful in determining the relation between the fungal burden in the respiratory tract and the probability of underlying invasive disease;^{87,99,115} however, at the current time, the benefit of PCR over conventional culture remains to be further defined. Finally, the diagnostic yield from BAL fluid is potentially optimised with the application of more than one test; a recent study demonstrated sensitivity was improved with the concomitant application of galactomannan and PCR.¹³⁰

Specimen	Target	Demonstrated specificity	PCR format	Detection method	Reference
Cultures	ITS1-5-8S rRNA-ITS2	<i>Aspergillus fumigatus</i> , <i>Aspergillus flavus</i> , <i>Aspergillus terreus</i> , <i>Aspergillus niger</i> , <i>Aspergillus ustus</i>	Conventional	Sequencing of amplicon	Henry et al ¹²⁷
Cultures	ITS1-5-8S rRNA-ITS2	<i>A fumigatus</i> , <i>A flavus</i> , <i>Aspergillus nidulans</i> , <i>Aspergillus versicolor</i>	Conventional	Line probe	Martin et al ¹⁰⁵
Cultures	ITS1-5-8S rRNA-ITS2	<i>A fumigatus</i> , <i>A flavus</i> , <i>A terreus</i> , <i>A niger</i> , <i>A nidulans</i>	Conventional	SSCP	Rath et al ⁹³
Cultures	ITS1-5-8S rRNA-ITS2	<i>A fumigatus</i>	Multiplex PCR	Ethidium bromide	Luo et al ¹¹⁸
Cultures	ITS1-5-8S rRNA-ITS2	<i>A fumigatus</i>	Nested	Ethidium bromide	Zhao et al ¹¹⁹
Cultures	5-8S rRNA-ITS2 region	<i>A fumigatus</i> , <i>A flavus</i> , <i>A terreus</i> , <i>A niger</i>	Conventional	Automated fluorescent capillary electrophoresis (detection of different length of amplicon)	Turenne et al ¹⁰⁶
Cultures	18S rRNA	<i>A fumigatus</i> , <i>A terreus</i>	Conventional	SSCP	Walsh et al ¹⁰²
Cultures and tissue	ITS2	<i>Aspergillus</i> spp and <i>Penicillium</i> spp, <i>A fumigatus</i> , <i>A flavus</i> , <i>A terreus</i> , <i>A niger</i> , <i>A nidulans</i> , <i>A ustus</i> , <i>A versicolor</i>	Conventional	PCR-ELISA	De Aguirre et al ¹⁰⁷

SSCP=single-strand conformational polymorphism

Table 3: The use of PCR in the identification of *Aspergillus* spp

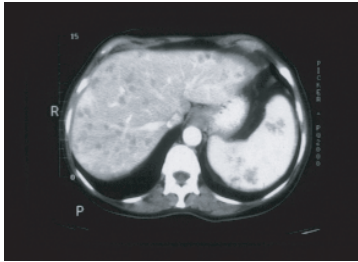
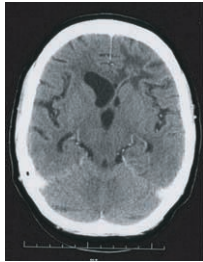
Pathogenesis of IPA	Compartmental characteristics and inter-compartmental relations	Clinical samples and sampling strategies
<p>Respiratory tract</p> <p>Inhalation of conidia, germination, mucosal surfaces breached</p> <p>Appearance of viable or non-viable hyphal elements or surrogate markers (galactomannan, DNA) in the contiguous respiratory tract</p>	<p>Appearance of <i>Aspergillus</i> spp and associated markers occurs late in the natural history of IA</p> <p>Quantitative relation between fungal load in tissue and respiratory tract difficult to determine with culture; quantitative PCR may be useful</p> <p>Positive predictive value depends on underlying disease</p>	<p>Bronchoalveolar lavage Sensitivity ~50%</p> <p>Sputum examination Positive sputum cultures occur relatively late in the natural history</p>
<p>Lung and sinus tissue</p> <p>Tissue invasion and damage</p>	<p>Destruction and invasion of tissue by hyphal elements represents the conceptual underpinning for IA; the demonstration of <i>Aspergillus</i> spp in tissue is the reference standard for IA</p> <p>Sensitivity of tissue sampling may be low due to sampling error, prior antifungal therapy, fungal tissue load beneath the analytical sensitivity of histology and culture</p> <p>Highest specificity achieved with combination of histology and culture</p>	<p>Fine needle aspiration May be more sensitive than bronchoscopy depending on the radiological pattern of disease</p> <p>Complications include pneumothorax, haemoptysis, haemorrhagic complications, seeding of needle tract</p> <p>Open biopsy Sensitivity compromised by infarction and necrosis Increased diagnostic certainty may not translate to improved patient outcome</p>
<p>Blood</p> <p>Haematogenous dissemination</p>	<p>Blood cultures typically negative</p> <p>Risk factors for haematogenous dissemination remain poorly defined</p>	<p>Blood sampling Blood cultures generally not helpful PCR and galactomannan from blood potentially useful Optimal sampling strategies yet to be rigorously defined</p>
<p>Dissemination to non-contiguous sites</p>	<p>A</p> 	<p>B</p> 

Figure 5: Compartmental characteristics, inter-compartmental relations, and sampling strategies as they relate to the pathogenesis of invasive pulmonary aspergillosis (A) Hepatosplenic aspergillosis (courtesy of Damon Eisen). (B) Cerebral abscess due to *Aspergillus fumigatus*. IA=invasive aspergillosis.

Blood

Blood sampling represents the optimal non-invasive diagnostic approach for invasive aspergillosis. Despite their propensity for vascular invasion, *Aspergillus* spp are only very infrequently isolated from blood using conventional culture techniques, hence the traditional dependence on tissue specimens to secure a definitive diagnosis of invasive aspergillosis. There is an extensive body of literature examining the diagnostic utility of molecular and serological techniques in blood. Galactomannan has been incorporated into diagnostic criteria for invasive aspergillosis and the technical issues required for PCR to be applied in the same manner have

been discussed. However, there remain some additional pertinent issues. First, specific sampling strategies are yet to be systematically studied—yield is almost certainly a function of the volume and frequency of sampling, as is the case with blood cultures. Second, the appropriate interpretation of a positive galactomannan or validated PCR result in a patient at risk of invasive aspergillosis, but without subsequent evidence of invasive disease, remains unclear and difficult to resolve; the most conservative interpretation in this context is that all single positive results are false-positive, but at least on occasion, such results may reflect true invasive disease that has aborted or is non-progressive. Third, a body of evidence suggests

Search strategy and selection criteria

PubMed was searched for English-language literature using the following terms: "Aspergillus", "aspergillosis", "diagnosis", "fungus", "fungal", "culture", "histology", "galactomannan", "glucan", "serology", "antibody", "PCR", "molecular", "metabolite", "mannitol", and "gliotoxin". Further relevant references, not identified by this strategy, were retrieved from the primary publications.

that both PCR and galactomannan may enable a specific diagnosis to be established earlier than is possible using a conventional approach.^{32,49,132} Fourth, the combination of different diagnostic modalities—eg, concomitant measurement of galactomannan and (1,3)- β -D glucan—is a strategy that may optimise diagnostic accuracy.⁶³ Finally, it seems likely that both PCR and galactomannan engender important prognostic information; a falling galactomannan titre or a positive-turning-negative PCR signal in the context of antifungal therapy is usually associated with a successful outcome. However, at the current time, galactomannan and PCR have not been systematically used to guide antifungal therapy.

The incorporation of diagnostic data into management strategies

Galactomannan (and validated PCR) applied to blood can be used as screening tools to further improve the identification of patients at high risk of developing invasive aspergillosis.¹³³ A positive result may enable the start of early targeted antifungal chemotherapy, while expensive and potentially toxic antifungal drugs can be withheld with persistently negative results. Testing for (1,3)- β -D-glucan could be also be useful in this regard. When the assays are used in this manner, a positive result should also serve as a trigger for additional diagnostic evaluation—eg, a high-resolution computed tomography scan of the thorax—to investigate the possibility of a subclinical focus of infection. The success of galactomannan (and validated PCR) as a screening tool is largely dependent on the underlying prevalence of invasive aspergillosis, which varies according to the specific host group and institution; thus, the requirement and extent of galactomannan screening may vary accordingly.

An alternate diagnostic strategy is to reserve galactomannan and validated PCR for situations in which clinical and radiological data are suggestive of invasive aspergillosis; in this scenario, galactomannan and validated PCR applied to serum, and other tissues and fluids, may enable a definitive diagnosis of invasive aspergillosis to be secured. Although this approach does not facilitate early antifungal therapy, it may minimise the use of invasive diagnostic modalities. Furthermore, a more definitive diagnosis enables the administration of specific anti-aspergillus therapy and would be of

considerable benefit for future diagnostic and therapeutic research.

Future challenges

Invasive aspergillosis continues to pose many challenges. From a diagnostic point of view, improving the test accuracy remains a priority for patient care, therapeutic research, and future diagnostic research. The question, of course, is the manner in which these improvements can be achieved. The progressive refinement of existing techniques and development of new diagnostic technologies is clearly a priority. Substantial work remains in areas related to cost-effectiveness and whether patients who undergo intensive diagnostic testing have improved outcome. Just as importantly, however, is the generation of a clinical environment and culture that is amenable to high quality diagnostic research, the provision of adequate funding, multicentre participation, international collaboration, and rigorous study design.

Conflicts of interest

WWH is supported by an unrestricted educational grant from Merck & Co and the Fungal Research Trust. TJW and DWD have no conflicts of interest to declare.

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