Clinical and laboratory diagnosis

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Clinical diagnosis of invasive fungal infections demands a high clinical acumen, but both under- and over-diagnosis are common. Laboratory mycological tests have traditionally been limited by a range of complications. Development of methodologies to detect fungal-specific immunoglobulins, cell wall antigens and nucleic acid have improved dramatically in recent years.

In immunocompromised patients, the commonest fungal pathogens are *Candida* and *Aspergillus*. Diagnosis of these invasive fungal infections is neither sensitive nor specific. Recommendations have been created providing guidance and incorporate currently available diagnostic techniques (Denning et al, 1997). New diagnostic approaches are being developed, which are based on an understanding of the pathogenesis of systemic fungal infection and virulence determinants.

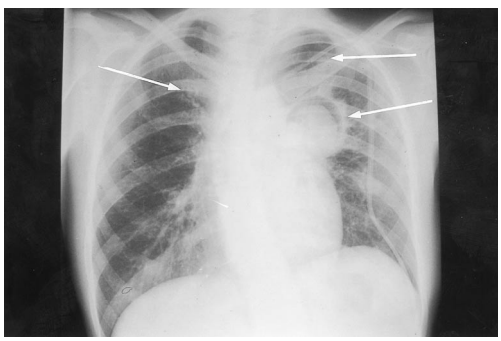

Figure 1. Plain chest radiograph with multiple primary aspergillomata in a bone marrow transplant patient.

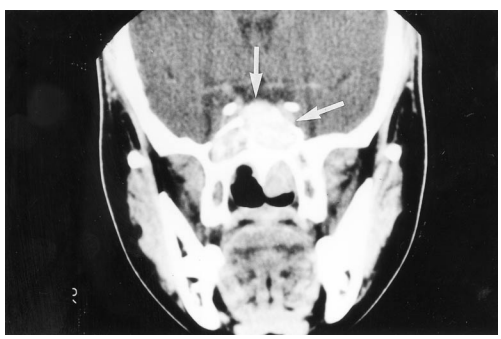

Figure 2. Sinus computed tomography scan showing large Aspergillus *mass eroding skull bone.*

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INVASIVE ASPERGILLOSIS

The clinical spectrum of aspergillosis ranges from simple bronchitis to frankly invasive aspergillosis (IA), e.g. invasive pulmonary aspergillosis (IPA). In the advanced immunocompromised host lung and sinus are the commonest target organs; cerebral, musculoskeletal, gastrointestinal and heart involvement is less common (*Figures 1–4*).

The earliest sign is fever without focal signs or positive microbiology, unresponsive to broad spectrum antibiotic therapy (known as antibiotic-resistant neutropenic fever; ARNF). The three cardinal features of established IPA are fever, unproductive or blood-stained sputum and pleuritic chest pain. Respiratory failure may follow. Plain chest radiog-

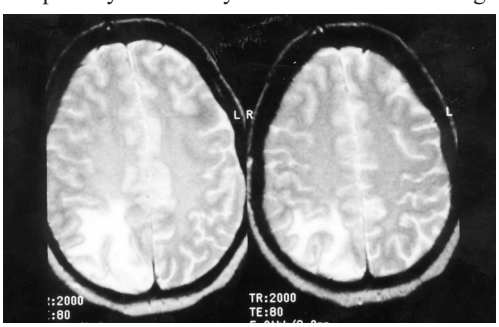

Figure 3. Magnetic resonance image of brain, showing Aspergillus *in occipital lobe.*

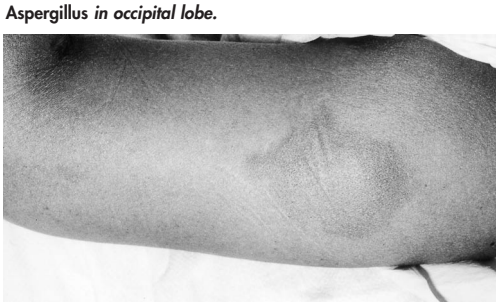

Figure 4. Haematogenous aspergillosis with pre-necrotic skin lesion.

raphy may be initially negative. More sensitive computed tomography (CT) scanning, performed early in the febrile illness, shows the highly suggestive halo sign (*Figure 5*) as a result of the angio-rich peripheral pathology. Advanced disease is characterized by cavitary pleural-based lesions or nodules, which may progress to primary aspergilloma (*Figures 1, 6*). Diffuse pulmonary infiltrates are found in one-third of patients. Early, serial CT scanning at the time of ARNF results in improved outcome (Caillot et al, 1997) (*Figure 7*).

Aspergillus sinusitis may have absent, subtle or florid signs such as facial pain, swelling and bloody nasal discharge.

LABORATORY DIAGNOSIS OF FUNGAL INFECTION IN THE IMMUNOCOMPROMISED

The presence of fungi in normally colonized body sites of immunocompromised patients does not prove infection. They are rarely isolated from infected deep organs, blood or tissues. There are four approaches:

1. A thorough examination of respiratory secretions by direct microscopy
2. Isolation of the organism
3. Detection of antibody or antigen
4. Histopathological evidence of invasion (*Figure 8*) (LaRocco and Burgert, 1997).

Isolation of fungi from otherwise sterile sites provides vital information to the clinician. Sputum cultures that yield *Aspergillus* spp. should be considered significant unless proved otherwise. In diffuse disease bronchoalveolar lavage (BAL) or bronchial biopsy provide more reliable and accurate diagnosis (60% positive), by direct microscopy and Calcofluor white® staining for fungal elements. In focal lung lesions radiographically-guided fine needle biopsy is helpful. Until recently, serological tests have not been very successful in the early diagnosis of invasive disease.

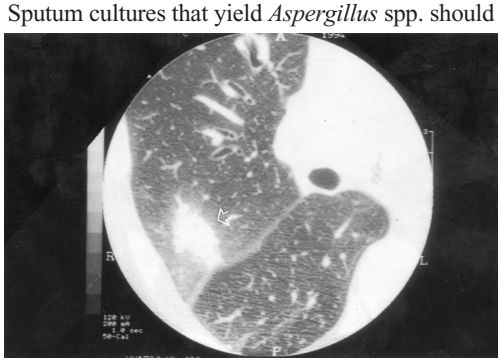

Figure 5. Halo sign ('blush' around dense central area), a result of the angio-invasiveness of Aspergillus.

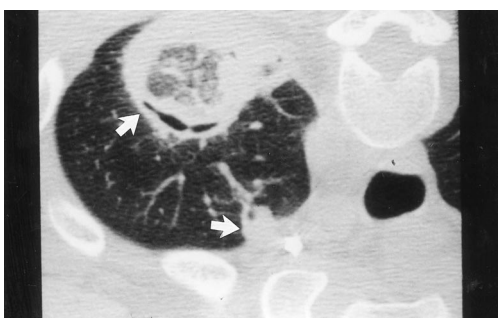

Figure 6. Advanced cavitary, pleural-based aspergillosis lesions on computed tomograph of the chest.

Antibody and antigen detection

Mycological confirmation of *Aspergillus* infection requires tissue culture. Appropriate invasive procedures are difficult and hazardous in neutropenic, thrombocytopenic patients. Histology may only suggest *Aspergillus* — culture is definitive.

New, commercially-available kits are currently being evaluated for usefulness in detecting antibodies of individual immunoglobulin classes in IA. Antibody tests may be negative as a result of the immunocompromised status. However, highly sensitive methods detect very low antibody levels. Using analytical isoelectrofocusing in conjunction with immunoblotting, 11 of 13 patients with proven/highly probable IA had anti-*Aspergillus*

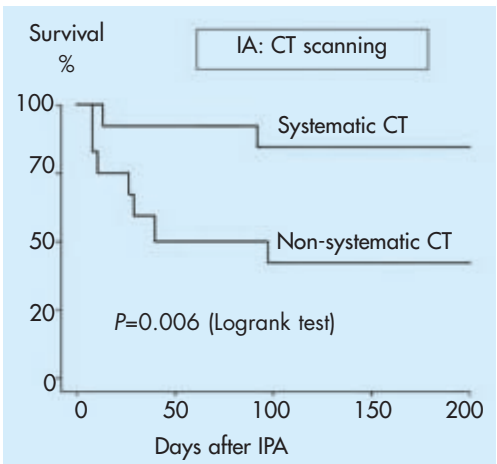

Figure 7. Kaplan–Meier plots of survival of patients undergoing systematic computed tomography (CT) scanning vs those not undergoing such intensive monitoring. IA = invasive aspergillosis; IPA = invasive pulmonary aspergillosis. From Caillot et al (1997).

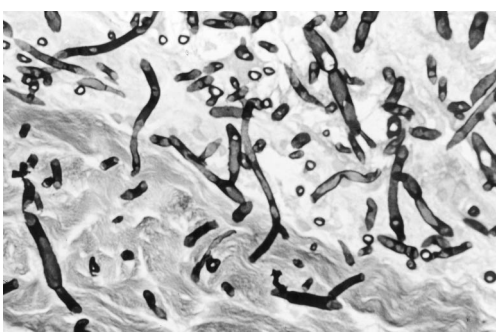

Figure 8. Silver stain of Aspergillus *spp in tissue biopsy.*

IgG to multiple antigenic preparations of *A. fumigatus* (Hearn et al, 1995). Use of a spectrum of antigenic fractions is advisable, given the variability in the immune response of individual patients.

The *Aspergillus* antigen galactomannan can be detected in plasma or serum samples from patients with IA by the commercially-available sandwich enzyme-linked immunosorbent assay (ELISA) (Platelia Aspergillus, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France). This assay has the highest sensitivity (67–100%) and specificity (81–98%) when performed with sera from patients receiving treatment for haematological malignancies (Verweij et al, 1996), and has good inter/intralaboratory reproducibility. It appears in serum first, in higher concentrations and more consistently compared to urine. Galactomannan can also be detected early in BAL samples.

A positive ELISA galactomannan test occurs early in the infection, sometimes 6–13 days before clinical features. The highest concentration of galactomannan is released in terminal disease. Antigenaemia lasts from 1 week to 2 months. ELISA also allows monitoring of antigen titres during treatment — a decreased concentration is indicative of a response to treatment (Rohrlich et al, 1996).

Polymerase chain reaction

Polymerase chain reaction (PCR) methodology detects *Aspergillus* DNA fragments in urine, BAL, serum and plasma (Verweij et al, 1996; Bretagne et al, 1998). However, positive amplification reactions have been observed in 6–23% of BAL fluid samples from patients without invasive infection, limiting the diagnostic value of this test (Yamakami et al, 1996). Another approach for the molecular diagnosis of IA involves the use of universal primers common to all fungi combined with restriction fragment length polymorphism, hybridization of the amplified DNA fragment with a specific probe, or single stranded confirmational polymorphism (Sandhu et al, 1995), thus detecting to species level a wide range of important opportunistic fungi. The diagnostic value of PCR remains to be established in prospective studies.

PCR analysis of serum/whole blood is preferred to BAL since false positivity (environmental contamination) is low and blood sampling is easy and repeatable. PCR positivity occurs later than galactomannan detection (Bretagne et al, 1998). However, the combined use of PCR and ELISA should result in a definitive diagnosis of IA, even in the absence of obvious clinical signs. PCR data raise an interesting question as to the origin of the *A. fumigatus* DNA, since the organism is not usually cultured from blood, even in late disease.

The G-test

The detection of circulating 1,3-β-D-glucan (a component of *Aspergillus* and other pathogenic fungi cell walls) is another investigative strategy for diagnosis of IA. Plasma 1,3-β-D-glucan has been measured in febrile episodes (Obayashi et al, 1995). With a plasma cut-off value of 20 pg/ml, 37 of 41 episodes of proven fungal infections were detected. All episodes of non-fungal infections, tumour fever or collagen-vascular diseases had concentrations below the cut-off value (specificity 100%). The positive predictive value of the test is 59%, the negative predictive value 97% and the efficacy 85%. The test is now available commercially (Fungitec G-test, AMS Biotechnology (Europe) Ltd), but is expensive and requires validation in selected patients.

CLINICAL AND LABORATORY DIAGNOSIS OF IA IN PRACTICE

One approach to early detection and management of IA combines antigen testing (ELISA galactomannan) with thoracic CT and radionuclide imaging (Severens et al, 1997). High-risk haematology patients undergo twice-weekly galactomannan testing. If two consecutive tests are positive a CT scan is performed. Treatment is indicated on the basis of a combination of positive radiography and antigenaemia, thus reducing the number of patients who would receive empiric antifungal treatment. Serial galactomannan concentrations provide assessment of therapeutic response.

SYSTEMIC CANDIDOSIS

Candidosis is a spectrum of infections that may be classified as cutaneous, mucosal or deep invasive. Deep invasive infection is further classified: fungaemia, tissue-proven disseminated acute or chronic candidosis, and single organ candidosis.

Diagnosis of systemic candidosis

The difficulties for clinical diagnosis of systemic candidosis lie in the absence of specific clinical signs (Anttila et al, 1997a). In hepatosplenic candidosis (*Figure 9*) fever persists for weeks or months following neutrophil recovery, with weight loss, upper abdominal pain, hepatosplenomegaly and alkaline phosphatase elevation. Imaging may show typical foci. Blood, tissue culture and histology are frequently negative (Anttila et al, 1997a). Laparoscopy-guided liver biopsy enhances the diagnosis (Anttila et al, 1997b), if performed after neutropenia recovery.

Disseminated candidiasis similarly presents with ARNF, or relapse of fever following a treated bacterial infection. Subtle liver and renal biochemical abnormalities and pulmonary infiltrates occur.

Rarely a septic shock presentation predominates. Skin lesions (*Figure 10*) and ocular target focal lesions (cotton wool/haemorrhagic retinal exudates, uveitis, iritis, hypopyon) suggest the diagnosis.

G-test: High levels of 1,3-β-D-glucan are found in patients with candidaemia and other forms of systemic candidosis (Obayashi et al, 1995) — this warrants further evaluation.

Arabinitol detection: Arabinitol of fungal and non-fungal origin have been distinguished and many publications have indicated the clinical usefulness of arabinitol determination by gas liquid chromatography (Walsh and Chanock, 1998). Diagnosis of disseminated infection in patients with acute leukaemia occurs 12–21 days after the first elevated D-arabinitol (fungal origin) and L-arabinitol (part of normal human metabolism) ratio. Positive urinary D-arabinitol:L-arabinitol ratios are found in children with confirmed invasive candidosis (Christensson et al, 1997). Regular monitoring of D:L-arabinitol ratios in urine is promising in the diagnosis of systemic candidosis in immunocompromised children.

Antigen detection: Immunoassay detection of *C. albicans* protein antigens of 47 and 48 kDa (enolase) is promising (Walsh and Chanock, 1998). Use of some of the commercially-available kits has been restricted by their prohibitive cost, and by the unknown nature and function of the target antigen (Örmälä et al, 1995).

Detection of anti-*Candida* antibodies: These assays were developed as precipitin tests or passive haemagglutination techniques using crude cell wall or cytoplasmic antigens, but lacked specificity and sensitivity. Recently detection of antibodies to germ tubes of *C. albicans* (Garcia-Ruiz et al, 1997) and enolase has been possible (Van Deventer et al, 1994). Commercial kits are undergoing evaluation.

Combinations of tests: Joint consideration of both antigenaemia and antibody response may provide an insight into the progression and nature of infection: patients with systemic candidosis have been shown to be positive for enolase and anti-enolase (M Richardson, unpublished data, 2000). Commercial ELISA kits for detection of *Candida* mannoprotein and anti-*Candida* immunoglobulin (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) are available. The antigen test (Platelia Candida Ag) detects mannan with a sensitivity of 0.1 ng/ml. The anti-*Candida* test (Platelia Candida Ab) detects antimannan antibodies.

In candidaemia 84% of patients have at least one serum sample positive by one test. In 75% positivity by one test is associated with negative results by the other test for any of the tested sera. Sensitivity and specificity were 80% and 93% respectively when the results of both tests were combined (Sendid et al, 1999), showing a disparity between mannanaemia and detectable antimannan antibodies. Use of both ELISAs may be useful for routine diagnosis of systemic candidosis. Other commercial tests are available for detection of individual immunoglobulins to *Candida* but await further evaluation.

Molecular diagnosis of candidosis

Three broad approaches are being explored: early, rapid detection of *Candida* in clinical specimens, rapid genus/species identification either directly or from a positive culture, and monitoring of drug susceptibility and response to therapy.

The published methods encompass many different genomic amplification protocols, various DNA extraction methods, either narrow or broad target primers, uni- or multicopy primers and amplicon detection formats. Although the sensitivity is one colony-forming unit (cfu) or 10–20 fg of yeast DNA there is very little work on clinical specimens. A PCR method to amplify a 350 base pair segment of the P-450 lanosterol 14alpha-demethylase gene has been developed (Morace et al, 1999). Restriction enzyme analysis (REA) of the resultant amplicons was used to identify *Candida* spp. involved in human infection. In preliminary studies, PCR-REA detected as little as 200 fg of *Candida* DNA, or as few as five cfu of *Candida* spp. per ml of blood. Daily serum samples from patients with haematological malignancy, neutropenia and fever were analysed by conventional blood culture and PCR-REA (Morace et al, 1999).

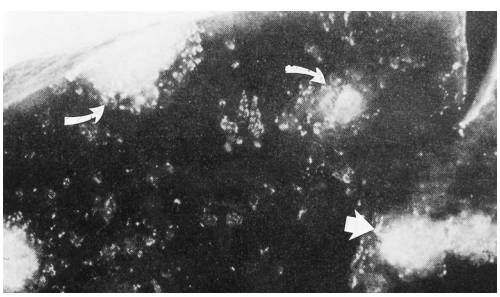

Figure 9. Spleen section in patient who died from hepatosplenic candidosis: multiple abscesses.

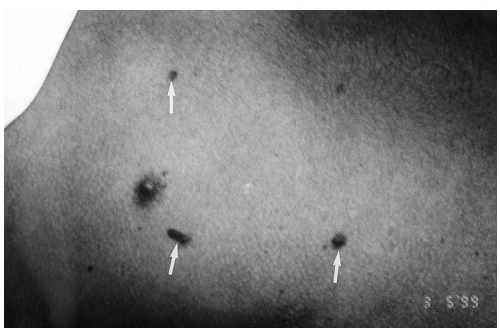

Figure 10. Multiple skin lesions of disseminated candidosis.

The method was more sensitive than conventional blood cultures with a high negative predictive value (97.5%) for the development of disseminated candidosis in neutropenic patients.

Any new immunoassay or DNA detection method for systemic candidosis must distinguish cutaneous/mucocutaneous from systemic disease. There has to be a greater understanding of the expression of antigens in relation to progression of disease.

WHAT IS THE VALUE OF THE NEW APPROACHES TO THE DIAGNOSIS OF SYSTEMIC FUNGAL INFECTION?

In clinical practice the following are frequently asked questions:

- How frequently should sera be examined?
- How sensitive is the assay in different forms of the disease?
- How sensitive is the assay at different stages of infection?
- Is serology likely to make the diagnosis before other diagnostic procedures?
- Is sensitivity affected by the underlying condition, e.g. neutropenia, AIDS?
- Can serology be used to distinguish between systemic infection and colonization?
- Can serology be used prognostically
- Can serology be used to monitor response to treatment and to detect relapse?
- Is the antigen detected resistant to proteinase-treatment, freezing or thawing?
- Is the assay species specific?

In relation to aspergillosis and candidosis, very few answers exist. Only in the diagnosis of cryptococcosis and the systemic fungal infections seen principally in the Americas is serology the definitive diagnostic test.

FUTURE DIRECTIONS

Successful pathogenic fungi invade host tissue and cause an increase in fungal-specific products as a result of normal excretory or secretory mechanisms, natural autolysis and an attempt by the host to damage or destroy the invading pathogen. These excretory, secretory or breakdown products are being explored as they could potentially be used as specific markers of systemic disease. **HM**

KEY POINTS

- Persistent protracted neutropenia with antibiotic unresponsive fever, and a 'halo' computed tomography sign is highly suggestive of invasive aspergillosis.
- Hepatosplenic candidiasis in the post-neutropenic recovery stage is characterized by prolonged persistent fever, hepatosplenomegaly, elevated alkaline phosphatase and negative blood cultures, in contrast to disseminated candidiasis (neutropenic fever, pulmonary infiltrates, sepsis).
- Detection of fungal antigen galactomannan, 1,3-β-D glucan, enolase, antibodies to cell wall components, polymerase chain reaction detection of DNA fragments and metabolites (arabinitol) — alone or in combination — are promising useful alternative/adjunctive diagnostic tools.

Figure 5 *reproduced courtesy of Dr D Denning.*
Conflict of interest: none.

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