Current Approach to the Laboratory Diagnosis of Invasive Fungal Infections in Haematology Units

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SUMMARY

Recipients of haematopoietic stem cell transplant, patients with haematological malignancies and patients with neutropaenia are among the most vulnerable patient groups for invasive fungal infections (IFI). This may be due to the immunosuppressive nature of the underlying diseases, their treatment or both. Recent advances in the fields of stem cell transplant and haemato-oncology heighten further the likelihood of IFI, particularly invasive candidiasis and aspergillosis, in these patients. IFIs are associated with high morbidity, mortality and increased patient/hospital costs. The diagnosis of these infections is universally challenging because the clinical symptoms are often non-specific. A high index of suspicion and effective use of the laboratory is necessary to arrive at diagnosis and aid management. Laboratory strategies can be viewed as conventional, contemporary and cutting-edge. Conventional methods including culture and histopathology are not sufficiently sensitive and they often require invasive methods of specimen collection which may be precluded in haematology patients. Contemporary methods include the detection of fungal biomarkers in various specimens. They provide quick diagnosis and may be especially useful for excluding serious fungal infections but many exhibit varying sensitivity and specificity according to patient group. Combination of these biomarkers, particularly in the diagnosis of invasive aspergillosis, has been found to be superior in performance to the use of a single assay. Molecular methods such as polymerase chain reaction (PCR) represent the cutting-edge of diagnosis but most are not standardized and so are largely unavailable in routine clinical laboratories. The diversity and level of complexity encountered in these laboratory methods calls for continuous liaison between the haematologist and clinical microbiologist to expedite management and improve patient outcomes.

Key words: Laboratory; haematology; fungi; transplant

INTRODUCTION

Invasive fungal infections (IFI) have gained prominence in the last few decades as a result of an ever increasing population of immunosupressed individuals.[1] Among the most vulnerable patient groups are recipients of haematopoietic stem cell transplant, patients with haematological malignancies (particularly acute leukaemias) and patients with neutropaenia resulting from immunosuppressive therapy. The increase in IFIs in these patients stems from a number of changes in practice which are

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themselves reflections of the advances in medicine.[2] These include:

1. The use of more intensive chemotherapy;
2. Shifts in the source of haematopoietic stem cell from related to unrelated donor and from bone marrow to HLA-matched or mismatched, peripheral and umbilical cord stem cells;
3. Multiple transplantations;
4. Transplantation with T-cell depletion.[2]

The diagnosis of invasive fungal infections is universally challenging, more so in resource poor settings. This is because the clinical symptoms are often non-specific. Therefore the haematologist must have a high index of suspicion especially in at-risk groups and make effective use of laboratory and other diagnostic modalities such as imaging. Delay in diagnosis has been found to impact negatively on patient outcomes.

The field of medical mycology is arguably still in its infancy and almost non-existent in developing countries such as Nigeria; thus while practitioners in developed countries are familiar with a wide array of diagnostic platforms, the average Nigerian doctor remains oblivious of the spectrum of such investigations beyond the mundane culture using Saboraud dextrose agar. The purpose of this review is to provide an update on the diversity in laboratory methods for diagnosing IFIs as it applies to the specialties of stem cell transplant and haemato-oncology.

DEFINITION OF INVASIVE FUNGAL INFECTIONS

All fungal infections display some level of tissue invasiveness which differentiates them from mere colonization, however, the term invasive fungal infections is generally reserved for systemic, generalized, deep-seated, visceral and/or severe life threatening fungal infections.[3]

For the conduct of clinical trials and evaluation of drug efficacy, the European Organisation for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) consensus group classified IFIs into 3 categories according to the level of probability.[4] The classes of IFI, according to the definitions of this consensus group are “proven”, “probable” and “possible”. A proven IFI is one in which the causative fungus is detected by histopathological examination or culture of a specimen taken from the site of disease. A probable IFI requires the presence of a host factor, clinical criterion and a mycological criterion; a possible IFI while having a host factor and clinical criterion lacks mycological evidence. The mycological criteria in question include non-culture based tests that detect antigen or cell-wall constituents.

EPIDEMIOLOGY OF INVASIVE FUNGAL INFECTIONS

Aetiology

The major IFIs to afflict haemato-oncology and stem cell transplant patients are invasive candidiasis and invasive aspergillosisalthough disease caused by other yeasts and moulds have recently emerged.[5]

Candida albicans is the commonest yeast involved in invasive disease. However there has been an emergence of non-*albicans Candida* with species such *as C.tropicalis*, *C.glabrata*, *C.parapsilosis,* and more recently *C.auris*, being increasingly responsible for blood stream infections.[5,6] *Cryptococcus neoformans*, *Geotrichum spp*, *Trichosporon biegelli* and *Rhodotorula* spp have also been reported as rare causes of systemic infection.[5]

Aspergillus species represent the commonest moulds involved in invasive disease. They cause invasive aspergillosis in recipients of haematopoietic stem cell transplant, patients with haematological malignancies and patients who develop neutropaenia following anti-cancer therapy. The commonest species is *Aspergillus fumigatus* but non-*fumigatus* species such as *A.flavus*, *A.niger* and *A.terreus* may also be involved.[5] There have been an increasing number of cases of invasive disease caused by non-

Aspergillus moulds such as *Fusarium* species and *Scedosporium* species which ordinarily do not cause serious infections in immunocompetent persons.[5] Fungi in the order *Mucorales* such as *Mucor* spp., *Absidia* and *Rhizomucor* occasionally cause invasive disease.

Risk Factorsfor IFI

Major risk factors for IFI include neutropenia (<500 neutrophils/ml) for more than 10 days, haematological malignancies, bone marrow transplantation, prolonged (>4 wk) treatment with corticosteroids; prolonged (>7 days) stay in intensive care unit, chemotherapy, HIV infection, invasive medical procedures, and the use of immunosuppressive agents. Malnutrition, solid organ transplantation, severe burns and major surgery are also frequently implicated.[7]

In patients with haematological malignancies and recipients of allogeneic stem cell transplant, neutropaenia, use of steroids, graft-versus-host disease and mucosal colonization are risk factors for both invasive candidiasis and aspergillosis. Patients whose fungal prophylaxis regimens are inadequate are also at risk.

The use of indwelling central intravenous cathethers and prolonged use of broad spectrum antibacterials have been identified as specific risk factors for invasive candidiasis in patients with haematological malignancies while cytomegalovirus infection and proximity to building works are particularly associated with invasive aspergillosis.

Burden of IFI

Although there is a general consensus that the incidence of invasive fungal infections is on the rise, the real frequency is often underestimated because of diagnostic difficulties.

Invasive candidiasis and candidaemia: *Candida* species cause 9-12% of all blood stream infections. They are the fourth leading cause of nosocomially acquired BSI in the USA and the sixth or seventh most common cause according to European surveys.[8,9] There is a paucity of data in Africa but Oladele *et al* reported a hospital-wide frequency of 5.2% in Ibadan, Nigeria.[10] The incidence in a multicentre prospective study conducted in Italy was 2.7%[11]. Invasive candidiasis, especially candidaemia, is most common in ICU settings. A high proportion of patients become colonised with *Candida* but only 5%-30% develop invasive disease.

Invasive aspergillosis occurs in 8%-15% of patients undergoing allogeneic stem cell transplantation and 5%-15% of those undergoing solid-organ transplantation.[12] Ten percent of AML patients and 5% of ALL patients have also been reported to acquire invasive aspergillosis.[2] Morbidity and mortality range from 30%-70% in such patients.[13]

LABORATORY APPROACH TO THE DIAGNOSIS OF INVASIVE FUNGAL INFECTIONS

In a bid to meet the need for prompt and reliable diagnosis of IFIs in haematology patients, there has been an expansion in the laboratory strategies for detecting these infections. These strategies can be organized into conventional diagnostic methods, contemporary tests and cutting-edge methods which show promise but have not been fully adopted in routine laboratory practice.

Conventional methods

Conventional methods of diagnosis include direct microscopy, culture and histopathological examination.

Direct microscopy

Conventionally, direct microscopy is the first step in the laboratory diagnosis of infectious agents. Fungal diagnosis employs stains different from bacteriological stains; the commonest of these is lactophenol cotton blue. Calcofluor white is a fungal specific fluorescent stain which requires the use of a fluorescent microscope. In most instances, direct microscopy is followed by culture and biochemical identification. This method is inherently lacking in specificity especially for invasive aspergillosis as *Aspergillus* sp. rarely

sporulate in vivo and hyphae seen may represent any number of filamentous fungi.[14]

Culture

Culture is undertaken to isolate fungi from tissue biopsy specimens or biological fluids. In certain cases, it may also serve to identify or differentiate between species in a given genus. Fungi-specific culture media include, but are not restricted to, Saboraud Dextrose agar, Potato dextrose agar, and brain heart infusion agar. The isolation of yeasts is typically followed by identification to species level using biochemical phenotypic tests. Due to the emergence of non-*albicans Candida* and their varying susceptibilities to antifungal agents, it is unacceptable to assume all cases of invasive candidiasis are due to *Candida albicans*. Chromogenic agar media hasten the identification of common species of *Candida* by incorporating chromogens which give different colours based on the species. Biochemical identification panels incorporating assimilation and fermentation tests such as API Candida and Vitek are also commercially available for speciation of yeasts. Moulds are typically identified based on the colonial morphology and microscopic appearance which are characteristic for the particular genus and sometimes species.

The advantage of culture is that an isolate is available for sensitivity testing. Furthermore, in an era when obscure fungi are being implicated in the causation of IFI, culture is more beneficial than methods which target expected aetiologies.

While culture remains the gold standard for the diagnosis of most invasive fungal infections, the method is often insufficiently sensitive to detect a large number of infections. Blood culture is positive only in 50% of candidaemia and deep-seated infections are often not detected on culture; <10% of *Aspergillus* species are detected.[12,15] Automated blood culture systems take 1 to 3 days to grow in candidaemia and an additional 1 to 2 days may be needed for identification of the organism; for some other fungi, culture takes days to weeks. Delays in diagnosis of candidaemia and invasive aspergillosis are tantamount to delays in antifungal therapy which results in increased mortality rates and hospitalization costs.

The invasive process required to collect appropriate specimens is also a drawback of culture based methods because the patients who need them are often debilitated. Patients with haematological malignancies and immunosuppression from stem cell transplant are prone to thrombocytopaenia which may preclude biopsy for the diagnosis of invasive aspergillosis.

Histopathology

Histopathological examination can offer rapid, presumptive diagnosis of infectious fungal organisms. It can be used in a complementary fashion with culture to provide insight into the diagnostic significance of some culture isolates. Demonstration of tissue invasion or an inflammatory reaction can help to determine whether an organism represents contamination, colonization, or true infection. Moreover, it is often the only diagnostic platform when material is not submitted for culture.[16]

Examination of tissue sections by routine haematoxylin and eosin staining could reveal the presence of *Aspergillus* hyphae but special stains such as period acid Schiff and Grocott's silver may add sensitivity and should be carried out whenever a fungal infection is suspected. *Aspergillus* hyphae seen in tissue sections tend to be narrow (1–3 um in diameter) and septate; and are usually distinguishable from the wider and pauciseptate hyphae of the mucoraceous moulds such as *Rhizopus*; however they may appear similar to other filamentous fungi including *Fusarium* and *Scedosporium.*[17] This, together with the fact that specimen collection requires invasive procedures which may be precluded in the patient, is the bane of diagnosis by histopathological examination.

Contemporary methods

Contemporary techniques are largely based on immunological methods that detect either antigen or antibody in patient specimens. Their

main attractive feature is the considerable reduction in turn-around time. Presumptive diagnosis can be made in a matter of minutes to hours while definitive culture results are being awaited. In some cases, they may be the only evidence of infection. Some of these tests such as the β-D-glucan and galactomannan assays exhibit variable performance depending on study design, type of patient population, method of screening (whether serial or punctual testing) and the criteria used to define cut-off values for positivity. Due to varying specificities and sensitivities, these require careful interpretation. Targeting the right situation and the right patient population is necessary for optimal benefit.[18]

Beta-D-glucan assay

(1-3)-β-D-glucan is an abundant cell wall polysaccharide found in most fungi except cryptococci, the zygomycetes and *Blastomyces dermatitidis*. This pan-fungal marker has become very useful in the diagnosis of a broad range of fungal infections including those caused by *Candida*, *Aspergillus* and *Pneumocystis jirovecii.*[19]At least four assays for the detection of β-D-glucan are commercially available including chromogenic and turbidimetric quantitative enzyme immunoassays. The clinical utility of these assays improves with serial testing. They are also useful for monitoring antifungal treatment and serve as prognostic markers for patient response. The preferred specimen for testing is serum but other specimens such as BAL fluid and CSF may be used often with decreased sensitivity. The sensitivity in serum is 60%-80% while specificity is 80%-95%.[20]False positive results may occur with prior antibiotic use (beta lactam antibiotics such as amoxicillin-clavulanic acid, ampicillin-sulbactam and cefazoline), gauze packing, cellulose depth filters from haemodialysis machines e.t.c.[21]

Galactomannan assay

Galactomannan (GM), a cell wall component, is a biomarker specifically detected in infections with *Aspergillus* species but the test may cross react with other fungi including *Paecilomyces* spp., *Penicillium* spp., *Fusarium* spp. and *Histoplasma capsulatum.*[18] Currently available tests for detection of GM include the Platelia Aspergillus enzyme immunoassay which is validated for use in serum and bronchoalveolar lavage (BAL) fluid samples. In some cases, it can be used to test CSF for diagnosis of cerebral aspergillosis. The sensitivity and specificity vary according to the specimen used- for serum, sensitivity is 60-80% while specificity is 80-95%; for BAL, 85-90% and 90-95% respectively; for CSF, 85-90% and 95-100% respectively.[18]False positive results may be due to concomitant administration of piperacillin-tazobactam, although newer formulations of the drug pose lower risk. GM measurement in serum can be used to monitor response to therapy. The combined use of a β-D-glucan assay and a GM enzyme immunoassay (Platelia *Aspergillus*) improves the specificity of diagnosis for invasive aspergillosis.[22] In a multicentre prospective study, the combination of β-D-glucan assay and galactomannan showed sensitivity, specificity, positive predictive value and negative predictive value of 92%, 93%, 94% and 90% respectively.[23] Combination of GM testing with molecular methods such as PCR improves performance even further with sensitivity and specificity of 97% each.[24]

Candida albicans germ tube antibody (CAGTA) assay

This is an indirect immunofluorescence assay which detects antibodies to the surfaces of *C. albicans* germ tubes. Sensitivity of 91-100% and specificity of 77% to 89% have been demonstrated in limited studies.[25] It is particularly useful in ICU settings since it is not affected by Candida colonization or intake of antifungal agents. Its utility in the diagnosis of candidaemia associated with deep seated infections has also been documented.

Candida mannan/ Anti-mannan antibody test

These tests are often of value when carried out concurrently. The combination of positive results

in both tests has a sensitivity of 73% and specificity of 80% for diagnosis of invasive candidiasis in neutropaenic fever.[7] Mannan is a major component of the cell wall of *Candida*.

Cutting-edge methods

Molecular methods represent the cutting edge of diagnosis in every aspect of clinical microbiology. The adoption of molecular methods has the potential to identify the increasing numbers of fungi that cause invasive infections in man.[2]These molecular tests may be nucleic acid-based or non-nucleic acid based. Another method of classification is into culture-based or non-culture based tests. Culture based tests require growth of the organism in pure culture followed by the use of molecular techniques for identification.

Polymerase Chain Reaction (PCR)

In its own right, PCR is not a novel testing method and can conveniently be described as contemporary or even traditional in most advanced settings. It represents the bedrock of most molecular techniques. Excellent details of PCR may be found in a review by Arvantis *et al.*[26] Traditional PCR was unable to quantify the amount of amplified DNA and thus could not be used for differentiating colonization from infection. This problem was solved by development of real-time PCR which has replaced conventional PCR methods in clinical laboratories. The drawbacks of fungal diagnosis using PCR include the difficulty in lyses of fungal cell walls which reduces sensitivity of some assays and the potential for contamination of the assays since fungi are ubiquitous. For these and other reasons, PCR is not widely used in diagnosis of IFI. PCR assays exist for *Aspergillus* spp and Invasive candidiasis.

Multiplex PCR refers to a platform that can detect a wide variety of fungi by using primers that amplify a region that is conserved among different fungal genera.

Nucleic acid sequence based amplification (NASBA)

This is very similar to PCR but it involves amplification of mRNA instead of DNA and it is isothermal. It can detect active disease instead of latent or past infections. It is also less susceptible to contamination.

Fluorescent in situ Hybridisation (FISH)

FISH uses fluorescent probes to identify target areas on the genome of fungal pathogens in human samples which can then be detected using a fluorescent microscope. Peptide nucleic acid probes have also been developed which have a peptide backbone rather than deoxy ribose sugar. Their advantage is that they bind more tightly and can enter microbial cell more effectively. PNA-FISH is used for direct detection of *Candida* from blood culture bottles. A multiplex PNA probe is also available for differentiating *C. albicans* from other *Candida* species.

Matrix assisted laser desorption/ ionization- Time of Flight (MALDI-TOF)

This technique is a fast growing non-nucleic acid sequence based molecular diagnostic assay that generates species-specific spectra based on mass-to-charge ratio (mass spectrometry) representing a unique signature characteristic of the species. This is referred to as proteomic profiling/fingerprinting. The technology has been commercialized and instruments are available from a number of biotechnology companies.

The technique is in three basic steps- the sample is mixed with a matrix (an energy-absorbent organic compound) which crystallizes it and facilitates ionization by a laser beam; the positively charged ions are accelerated through high voltage; the time of flight component is a tube through which excited ions travel separating from each other on the basis of mass-to-charge ratio, the transit time of individual ions provides the method of identification.[2] Characteristic spectra called peptide mass fingerprints are generated on a detector for each organism in the

specimen. Generated spectra are screened against a library of reference spectra that corresponds to individual species

MALDI-TOF technology has several benefits- sample preparation is minimal; sample analysis is rapid; there is no need for downstream steps (unlike PCR which requires post-amplification detection). One limiting factor is the need for an existing spectral library to compare the generated spectra with. Other drawbacks include the proprietary nature and instrument specificity of libraries, capitalization costs and non portability of the instrument.

Conclusion

The recent advances in haematology as a specialty have translated to a rise in the population at risk for IFIs. Conventional methods such as microscopy, culture and histopathology while retaining a useful place are no longer sufficient to avert the morbidity and mortality associated with delayed diagnosis of these infections. Newer and more rapid tests are now available but they require understanding of their strengths and weaknesses to be put to good use. This calls for close liaison between the Haematologist and clinical Microbiologist to be able to navigate the diverse and complex testing available for the diagnosis of these infections since delayed diagnosis brings about poor patient outcomes.

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