REVIEW

Laboratory Diagnosis of Fungal Infection – A Review.

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Abstract

Introduction: Though decades back the fungi were considered as inconsequential causes of infection but in recent years there has been a surge. This could be due to AIDS epidemic or because of advances in the medical care and treatment. A number of diagnostic procedures are being used but each has its own limitations. Accurate diagnosis relies precisely on a combination of microbiological, histopathological and serological evidence.

Aims: This article focuses on specific conventional and advanced techniques concerning a practical approach to the laboratory investigations of fungal infections.

Methods: Data was obtained and analyzed from previously published literature and electronic database searches from PubMed and Google Scholar.

Results: Traditional methods of diagnosis include direct microscopic examination of samples, culture, serology and histopathology. Nowadays with advances in diagnostic methods, molecular diagnostics and antigen detection in clinical samples is being highly recommended.

Conclusion: Although, the number of species that are routinely seen causing infection is quite low, yet new species are continually being implicated. This has forced the investigators to develop molecular methods for fungal identification. These methods have and will continue to have a major impact on the diagnosis and appropriate treatment of fungal infection. Analytical parameters of these methods need to be standardized to optimize sensitivity and specificity and comparative studies need to be performed to determine which are best to use in the laboratory.

Keywords: Fungal infection, PCR, PCR-ELISA, Loop Mediated Isothermal Amplification, Multilocus enzyme electrophoresis.

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Introduction

It has been found that frequency and intensity of fungal infection has increased in recent years.[1] Decades back the fungi were considered as inconsequential causes of infection. It was not until the mid-twentieth century that fungi came to be considered as significant causes of infection.[2] The cause for this might be the AIDS epidemic which enhanced the life-threatening infections by the opportunistic fungi *Cryptococcus neoformans* and *Pneumocystis jiroveci* and by *Histoplasmacapsulatum*. Secondly, advances in medical care and treatment have led to increases in the number of opportunistic infections in immunocompromised patients.[1] The most common fungi that cause disease in transplant recipients and other immunocompromised patients are *Candida* and *Aspergillus* species.[3,4] The diagnosis of fungal infections depends entirely on the selection and collection of an appropriate clinical specimen for microscopic analysis and culture. Many fungal infections are similar clinically to microbial infections, and often the same specimen is cultured for both fungi and mycobacteria.[2]

Diagnosis of fungal infection by traditional methods such as direct microscopic examination of clinical samples, histopathology and culturehave proved to be conventional and insensitive. The growth in the frequency of fungal infections has forced the scientists to develop and apply new methods. As a consequence, there is an increased emphasis on the use of molecular methods and antigen detection tests as surrogates for culture in diagnosis of fungal diseases.[1] Also, antigen assays such as the galactomannan and glucan detection systems are frequently used, yet these tests vary in sensitivity and specificity, depending on the patient population involved. Molecular-based assays are not yet clinically validated.[5]

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Specimen collection and transport

The key role in the recovery of fungi is the rapid transport of the sample to the clinical laboratory.[2] Penicillin (20 U/mL), streptomycin (1,00,000µg/mL) or chloramphenicol (0.2 mg/mL) may be added to the specimen to prevent the overgrowth of other commensals. In cases of delay, the specimen should be stored under refrigeration at 4°C for no longer than 24 hrs as it preserves the viability of pathogens and also reduces the growth of contaminants. The latter factor is crucial when semiquantitative cultures or quantitative cultures (e.g., cultures of sputum or urine) are necessary for the interpretation of results. Specimens that should not be refrigerated include blood, which should be kept at room temperature or in an incubator at 35°C and CSF, which should be transported at room temperature.

The specimens can be transported in media like brain heart infusion broth or in anaerobic media as long as it can be easily and completely retrieved from the medium.[6]

Methods for fungal Isolation

1. Direct microscopic examination methods are -
   - Wet mounts – KOH, calcofluor white, India ink
   - Fluorescent antibody staining
   - Histopathology[7]
2. Fungal culture
3. Nonculture methods
   - Serology
   - Antibody detection
   - Antigen detection
   - Immunohistochemistry.
4. Molecular methods
   - Polymerase chain reaction (PCR)
   - Microarrays.

1. Direct microscopic examination

This has been used for many years; however its usefulness should be reemphasized.[8] This important procedure can often provide the first microbiologic proof of the cause of disease in patients with fungal infection and guide the selection of appropriate media to support growth.[2] Preparations for direct examination of clinical specimen include KOH, India ink, and calcofluor white; in addition, a few staining techniques such as Giemsa and periodic acid Schiff (PAS) are effective.

KOH

To distinguish the patterns of fungal presentation, a potassium hydroxide mount is very useful. If yeasts and hyphae are in the same microscopic field, it is likely to be *Candida albicans*. *Aspergillus* fungus are usually present as thin septated hyphae that branch at acute angles. In contrast, *Mucorales* fungus have nonseptated, broad, ribbonlike hyphal structures that have 90° branching. Cryptococcus shows a capsule. Blasatomyces is a yeast with broad budding pattern in figure of eight. Histoplasma capsulatum is a very small fungus that is intracellular and the spherule with endospores is suggestive of *Coccidioidesimmites*.[9]

Calcoflour with KOH

Calcoflour white dye with KOH is useful for showing the presence of fungal cells in clinical specimens because it binds to β 1–3, β 1–4 polysaccharides present in fungal cell walls. The dye fluoresces on exposure to shorter wavelengths of UV light. A fluorescence microscope is needed for detecting fungal cells prepared with Calcofluor White. A chalk-white or brilliant apple-green fluorescence is shown in presence of yeast cells, pseudohyphae, and hyphae. This is usually dependent on a filter used which separates them from background material. The need of fluorescence microscopemight be the disadvantage of using this method.[2]

Gram's stain

Fungi like Cryptococcus sp. show only stippling and stain weakly in some instances. Some isolates of Nocardia sp fail to stain or stain weakly.[2]

India ink

India ink is useful for indicating the presence or absence of extracellular polysaccharide capsules of fungal cells especially *Cryptococcus neoformansin* CSF. Through this stain, the encapsulated yeast cells can be readily detected against the dark background.[10]

Lactophenol cotton or aniline blue wet mount

It is the most widely used method. Lactic acid preserves fungal cell wall structures and thus the slides can be made permanent.[10]

PAS

One of the most widely used stains for fungal histopathology is PAS . The principle of mechanism is that it detects glycogen in tissues and since fungal cell walls contain it in sufficient amounts thus PAS can be used for screening for fungal organisms.[11]

Gomori's methenamine silver stain (Grocott's modification)

In this method, aldehyde groups are released and identified by the reduced silver method that projects the polysaccharides present in the fungal cell walls. The aldehydes cause reduction of methenamine silver nitrate complex, that results in brown-black staining of fungal cell wall. The Gomori'smethenamine silver stain is better than other fungal stains as it stains both live and dead fungi in contrast to PAS, which stains only live fungi.[12]

Fluorescent antibody staining

This technique may be used to detect fungal antigen in clinical material such as pus, blood, CSF, tissue impression smears, and in paraffin sections of formalin fixed tissues. The main advantage of this technique is the detection of *fungi* even if only a few organisms are present as seen in pus from sporotrichosis.[7]

Histopathology

Histologic examination of tissues to detect fungal cell wall remain an important tool to define the diagnostic significance of positive culture isolates, including fungal invasion of tissue and vessels as well as the host reaction to the fungus.[11]

2. Fungal culture

Culture from a tissue has always been the key diagnosis for detection of fungal infections. Moreover, culture allows for susceptibility testing.[1] Some fungal cultures include Sabouraud dextrose agar (SDA), SDA with antimicrobial agents, potato dextrose agar or the slightly modified potato flakes agar and BHI agar enriched with blood and antimicrobial agents. Gentamycin or chloramphenicol and cycloheximide are the anti microbials usually included with fungal media. The first two inhibit bacterial growth whereas cycloheximide inhibits bacteria and many of the environmental fungi typically considered as contaminants. Culture can be done in Petri dishes or large test tubes. Former have the advantage of a larger

surface area but are more prone to dehydration. Latter are safer to handle and less susceptible to drying.[13]

Cultures are incubated at 25°C–30°C and 37°C as many fungal pathogens grow at temperature below 37°C. After noting the colony characteristics like color and texture of growth, slide mounts should be made in lactophenol cotton blue stain to study the morphological details. For molds that grow in 7–14 days or that have a cobweb aerial mycelium, one of the dimorphic species should be considered.[8]

The most common procedure for microscopic examination of fungal cultures is a direct mount of the fungal isolate. This is achieved by preparing tease mount or cellophane tape mount. Many fungi can be identified by these two methods but when fungi are atypical a slide culture should be prepared.[13]

3. Nonculture methods

Serology

Serological diagnosis is the method that is based on detection of either antibodies or antigen in the patient serum and has several advantages. First, it is more sensitive than culture methods and gives the results even when the samples are very less. Second, if positive, serological resultsmay reduce the need for culture of potentially hazardous fungi, for example, Coccidioides spp. Also, it is minimally invasive. Low levels of sensitivity and specificity are the disadvantages of serology. A negative serologic testshould not exclude the presence of fungal infection.[14]

Limitation of the serology is to distinguish between presentand past infection. Finally, sensitivity is dependent on the type of disease and the timing of testing relative to the disease process, for example, early versus late.[1]

Latex particle agglutination, immunodiffusion, countercurrent immunoelectrophoresis, immunofluorescent antibody, ELISA, and western blotting/immunoblotting are the various serological techniques used. Particularly useful tests include the measurement of organismspecific antigens like Galactomannans, which are considered specific for the diagnosis of aspergillosis. Serum β glucan detection is used as a diagnostic tool forthe detection of a broad spectrum of fungal pathogens, with the exception of *Mucoralesand Cr. neoformans.*[15-16]

4. Molecular Diagnostics

In the field of clinical mycology, molecular methods for fungal diagnosis and identification directly address the declining numbers of clinical mycologists because they are not dependent on classical phenotyping methods. Similarly,molecular methods have the power to identify the increasing numbers of fungi found to produced is ease in humans and animals.[1]

Some of the latest techniques employed in the detection of Fungi, including fluorescence in situ hybridization, DNA array technology, multiplex tandem PCR, realtime PCR, PCRELISA, RAPD, and loopmediated isothermal amplification (LAMP).[15]

Fluorescence *in situ* hybridization

Fluorescent in situ Hybridization (FISH) is a powerful method for the in situ detection of active growing organisms in environmental samples. This method can locate the exact position of particular DNA or RNA sequences in the biological materials. FISH probes are used to target sequences of ribosomal RNA or mitochondrial genes due to presence of sequence databases and result in multiple copies in each cell. Limitations of the FISH method can include fungal and substrate inherent autofluorescence, insufficient permeability of cell walls, non-specific binding of probes, and low ribosome contents.[17]

DNA array Hybridization

DNA array hybridization or Reverse Dot Blot Hybridization (RDBH) or macroarray, is a hybridization method which is considered a practical technique to detect and identify fungi and other microbes in culture[18].

PCR

PCR is the prime method for diagnosis of many diseases. This generally work best when pure cultures are available. Molecular identification can work in the absence of live cells if template nucleic acid is available in patient specimens, including fixed tissue[1].

Multiplex tandem PCR (MT-PCR)

MT-PCR is a technique used for highly multiplexed gene expression profiling and for the rapid identification of pathogens. MT-PCR is suggested to be used for rapid identification of fungal elements directly from specimens.[15]

Real time PCR

Real-Time PCR has revolutionized the whole process of quantification of DNA and RNA fragments with greater reproducibility. It is a sensitive method and provides accurate quantification of the species. Advantages include the ease of quantification, greater sensitivity, reproducibility and precision, rapid analysis, better control of quality in the process and a lower risk of contamination.[19]

PCR-ELISA

PCR-ELISA works immunologically in 3 steps: amplification, immobilization, and detection. PCR-ELISA method (i) is about 100-fold more specific than conventional PCR method, (ii) has faster result output (iii) allows multiple sample testing (iv) is able to do quantitative and qualitative analyses (v) reduces risk of contamination (vi) omits the use of mutagen-staining materials and (vii) is an easy-to-use method as it only requires the use of basic lab equipment.

Loop Mediated Isothermal Amplification (LAMP)

LAMP is a powerful and novel nucleic acid amplification method that amplifies a few copies of target DNA with high specificity, efficiency, and rapidity under isothermal conditions (do not require a thermal cycler). It has a set of four primers and a DNA polymerase with strand displacement activity. The cycling reactions can result in the accumulation of 109 to 1010-fold copies of target in less than an hour. As LAMP has advantages of rapid amplification, simple operation and easy detection, it is used for clinical diagnosis of infectious diseases in developing countries without requiring delicate equipments or skilled personnel.[15]

Electrophoretic karyotyping (EK)

In this technique, intact DNA molecules migrate through an agarose gel matrix under the influence of pulsed fields, which permits easy separation of DNA molecules of several megabases. Chromosome-length polymorphism is evaluated by EK analysis, which uses electric fields of alternating orientation to move intact chromosomes through an agarose gel matrix. Electrophoretic karyotyping helps in analysis of chromosomal binding patterns, known as electrophoretic karyotypes, and in the detection of karyotypic variations within the species. EK has been extensively used to fingerprint *C. albicansand* other Candida species. It has a moderate discriminatory power, however, shows good reproducibility.[21]

Multilocus enzyme electrophoresis (MLEE)

MLEE evaluates the polymorphism of isoenzymes or alloenzymes of the isolates. Proteins from cell extracts are separated by

electrophoresis under native conditions, and the enzymes are visualized by specific enzyme-staining procedures[7]. The main advantages of this method is its high discriminatory power when a sufficient number of enzymes is evaluated, and the very low probability of homoplasy in clonal organisms.[21]

Proteomics Profiling/Fingerprinting

The most commonsequence of non-nucleic acid based molecular diagnostic assay for fungi is MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight). The method shows a species-specific spectrawhich gives a unique signature specific to the species. The sample is prepared by mixing with solution of anorganic compound called matrix which when crystallizes on drying, causes the sample entrapped within the matrix to be crystallised which is then ionized in an automated mode with a laser beam. All these generate singly protonated ions from analytes in the sample. The charged analytesare measured using different types of mass analyzers like quadrupole mass analyzers, ion trap analyzers, time of flight (TOF) analyzers etc. The strength of this method lies in the rapid sample analysis (minutes). Weaknesses is the requirement of an existing spectral library to compare generated spectra to, and potential variability in results of unknown fungi.[1]

Conclusion

Considering the large number of fungi in the environment that are capable of causing human disease, molecular methods will have to be replaced by conventional methods soon.

Overall, molecular methods have and will continue to have a major impact on the diagnosis and appropriate treatment of fungal infection. Analytical parameters of these methods need to be standardized to optimize sensitivity and specificity and comparative studies need to be performed to determine which are best to use in the laboratory. Ideally tests should be as simple as possible to perform so that most clinical laboratories can use them. If these criteria are met, most of the newly developed molecular-based tests will be available to all of the patients with fungal infection . They will be especially useful for non-culturable, slow growing, pleomorphic opportunistic fungi. The time required to achieve the molecular skills is minimal compared to the time required to be trained as a classical mycologist. However, regarding the vast number of fungi exists in the environment which may infect particularly immunocompromized patients, a strong partnership between classical mycology and molecular biology is needed.[22]

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