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Tissue diagnosis of invasive fungal infections: Current limitations and the emerging use of molecular techniques

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Abstract

Invasive fungal diseases (IFD) due to opportunistic fungi are commonly treated using empirical antifungal therapy. Therefore, a comprehensive study of organisms associated with IFD is essential to define successful empiric therapies in each setting. Current diagnostic tests, such as culture, histology and serology are suboptimal, leading to delays in the initiation of antifungal therapies and resulting in high mortality rates despite the availability of several new antifungal agents. Using molecular methods to identify fungal pathogens directly from formalin-fixed, paraffin-embedded tissues is emerging as a diagnostic approach. The goal of this molecular approach is to complement conventional diagnostic tests through the reliable detection and identification of fungal nucleic acids or antigens in tissues so as to direct antiinfective therapies and improve patient outcomes. Here we review challenges and recent advances in the identification of fungal pathogens from tissue samples by conventional and molecular methods.

Introduction

Invasive fungal diseases (IFD) continue to be a serious threat to a growing number of patient groups, such as cancer patients, organ transplant recipients, the critically ill and other immunocompromised hosts. Despite considerably improved antifungal treatment options, these infections continue to be associated with mortality rates of up to 50% in some patient groups; this is in part due to the nonspecificity of clinical signs. Consequently, IFD are among the most often missed clinical diagnosis in cancer-, AIDS-, and critically ill patients [1-3]. In addition, limitations in diagnostic tests hinder the timely identification of causative agents, impeding the optimal selection of antifungal treatments.

Fungi are widespread in the environment and a diverse array of fungi can be found on mucous membranes of humans. Using pyrosequencing, between 9 and 23 species, including culturable and uncultured fungi, have been detected from the oral mucosa of healthy people, making them potential agents of endogenous infections in immunocompromised states [4●]. This poses a significant problem to the interpretation of diagnostic tests based on the detection of fungi from non-sterile specimens. A distinction between colonization and IFD is often not possible. Cultures from involved tissues on the other hand, are only positive in 50% of cases with proven invasive infection [5,6]. Given these limitations in diagnostics, postmortem examinations classifying the morphology of fungal elements in tissues played a critical role in documenting changes in the epidemiology of IFD, such as a decrease in Candida-infections and an increase in mould infections during the 80´s and the increasing importance of mucormycosis in cancer patients in the 90´s [5,7]. However, it is becoming increasingly recognized, that the classification of IFD into these broad categories is not sufficient, as fungi with different *in vitro* susceptibilities can display identical

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morphologies. Therefore, treatment decisions based on tissue morphology are not possible in many cases, reducing the chances for optimal patient treatment.

In this review, we summarize limitations of culture and histopathology from tissue specimens in determining fungal infection etiology. We argue that tissue samples stored in pathology archives provide an important resource for applying molecular methods to advance our understanding of the etiology of IFD. The ability to accurately identify fungal etiology in tissue may also be useful as a gold standard to evaluate diagnostic tests from other surrogate samples, such as respiratory secretions or blood.

Invasive fungal diseases can be proven by histopathology but species identification is limited

The demonstration of fungal elements in tissue is of major importance for establishing the diagnosis of invasive fungal infections [8]. Several morphologic characteristics allow for the differentiation of several groups of fungi such as yeasts, hyaline moulds, dematiaceous fungi and the mucorales [9●●]. However, as several fungi share similar morphology in tissue samples, a species diagnosis is only rarely possible by histopathology. This limits the usefulness of histopathology in guiding antifungal treatment decisions. In addition, fungal classification by histopathology can be difficult. This is highlighted by a recent study comparing histopathologic classification of fungal elements in tissue with culture results from biopsy samples as a gold standard for species identification. Discrepant diagnoses were observed in 21% of the samples. Errors in the histopathologic classification resulted from the misidentification of septate, nonseptate and yeast forms, and a false sense of the ability to categorize fungal organisms by genus on the basis of morphologic features. Among the 8 discrepancies with clinical follow-up available, two potential adverse clinical consequences were reported. As a consequence, the authors generated templates to provide standardized reporting and to deemphasize attempts at definitive species identification by fungal tissue morphology [10●●]. Additional factors that may impair the correct classification of fungal elements include antifungal pretreatment that can influence fungal morphology. Further complicating this picture, different fungal elements in tissue have been reported in 3-5% of proven invasive fungal infections in a review of autopsy cases performed in Japan. While this suggests infections by different groups of fungi, such as hyalohyphomycetes with mucorales or yeasts with moulds, the validity on these classifications is difficult to assess in the absence of alternative tests, such as culture, which may prove the presence of mixed fungal pathogens in a tissue sample [11].

The British Society for Medical Mycology proposed standards of care for patients with invasive fungal infections. Included therein are recommendations on how tissues from immunocompromised patients with suspected IFD should be stained, how fungal elements may be classified and how

positive results should be reported [12]. A subsequent audit demonstrated that less than 50% of pathology departments in the UK provided sufficient detailed descriptions of fungal morphology, as suggested by the guidelines [13]. Comparable data for other countries are not available.

Cultivation of fungi from infected tissue is insensitive

As the morphology of fungal elements in tissue is limited, while the diversity of fungal pathogens is great, cultivation from tissue specimens is useful for identifying fungal species. Growth of fungi from tissue specimens of patients with invasive fungal infections has been reported in 50% of cases with proven invasive fungal infection [6,14]. First, culture conditions such as temperature and duration of cultivation affect the recovery of moulds from various specimens [15]. Second, antifungal treatment is known to impair the recovery of fungi in cultures [16]. Third, several studies suggest that the mucorales are less likely to be recovered by culture from clinical specimens than other moulds. This may be partly due to the tissue processing prior to cultivation and also due to subsequent culture conditions [6,17,18]. Finally, fungi may be inhibited by co-cultured bacterial or fungal organisms, necessitating the use of selective media for sensitive cultivation of the pathogen. For example, *Scedosporium* species are slow growing moulds that can be cultured from the respiratory tract of patients with cystic fibrosis. These patients are often colonized with bacteria, yeasts, and moulds of the genus *Aspergillus*. In these patients, the cultivation of *Scedosporium* from respiratory secretions is much more likely when selective media are used that impair the growth of yeasts and Aspergillus species [19].

Beside the lack of sensitivity of cultures in growing fungi from tissues of patients with proven IFD, it is possible that current culture and identification methods underestimate the frequency of mixed fungal infections. Up to 30% of patients at risk for developing candidemia, such as bone marrow transplant recipients and patients at surgical intensive care units, are colonized with more than one yeast species as assessed by conventional culture [20,21]. As these colonizing strains form the reservoir for endogenous infections in these patients, blood cultures may underestimate the frequency of mixed infections due to overgrowth with the dominant fungus; indeed, documented mixed infections are reported in 4% of patients with candidemia, and this should be considered a lower bounds for a more common problem. Interestingly, a recent report described the cultivation of more than one mould from respiratory specimens of patients with suspected invasive mould infections in 7% [22]. However, as histopathology may not be able to differentiate between most of these fungi and the cultivation of moulds may represent colonisation rather than infection, the frequency of mixed mold infections remains unknown and conventional diagnostic tests may not be able to establish its importance.

Amplification methods are superior to culture in fungal species detection

Due to the lack of sensitivity and specificity in the cultivation of fungi from infected tissue and challenges in the classification of fungal elements in tissue by histopathology, there is a growing interest in using molecular tests to detect and identify the etiologic agents of invasive fungal diseases from clinical samples. Studies using PCR to detect invasive mould infections from blood samples are limited by the low fungal burden in blood [23]. This and technical issues such as a suboptimal DNA extraction efficacy, contaminations by fungal DNA and the lack of a consensus on the optimal detection strategy limit the widespread use of this molecular approach [24]. While higher mould DNA concentrations are expected in bronchial secretions and tissue specimens, the differentiation between colonization and infection is becoming a critical issue when bronchial secretions are targeted for diagnostic purposes [25,26]. In contrast, recent studies suggest examples where PCR assays may already be successfully used to supplement conventional diagnostic tests in order to better characterize the etiology of IFD. A recent study in patients with deep-seated candidiasis suggests that PCR from blood is more sensitive in detecting yeasts as compared to blood-cultures, detecting yeasts in 89 versus 53% of patients. In addition, while blood cultures detected more than one yeast in 4%, in accordance with previous studies, PCR did so in 36%, suggesting that blood cultures underestimate the number mixed infections [●27]. Similarly, studies using PCR assays to detect moulds from tissue biopsies detect fungal DNA more often than culture grew moulds [6,18]. However, the interpretation of such findings is difficult to assess in the absence of a reliable gold standard for species level identification of invasive fungal infections.

A reliable approach to identify the etiology of fungal pathogens from tissue using molecular methods: the value of combined amplification and hybridization methods

A successful strategy to evaluate the etiology of invasive fungal infections needs both sensitive detection of pathogens and wide breadth of detection. To be useful as a basis for treatment decisions, single and multiple species should be correctly identified and a distinction between colonization and infection is helpful. Using molecular tools on formalin-fixed, paraffin-embedded tissue samples appears to be a promising path to generate diagnostic tools fulfilling these attributes. Among the most crucial steps in this approach is the extraction of fungal DNA from formalin-fixed, paraffin-embedded (ffpe) tissue specimens, the amplification of fungal DNA using PCR assays, the identification of amplicons using sequencing, and hybridization techniques able to localize fungal nucleic acids in pathologic tissue processes.

The DNA extraction procedure is critical for optimal DNA recovery from fungal pathogens. Using different commercial extraction kits, up to a million-fold differences in the amount of fungal DNA can be documented using quantitative PCR [28]. The extraction of fungal DNA from ffpe tissues of patients with proven IFD poses additional challenges, as documented by a lower amount of positive samples when compared to fresh tissue specimens [29,30]. By increasing the amount of tissue used for extraction, the detection of DNA of the mucorales from experimentally infected tissue could be improved, demonstrating that amplifiable fungal DNA may be retained below a detection limit in FFPE tissue samples [31]. However, increasing the amount of tissue is not always possible with clinical samples that may only have a limited amount of fungal material. In addition, this strategy may also inhibit the detection of fungal DNA in some cases by increasing the amount of non-target DNA or of PCR inhibitors. Several modifications of extraction procedures have been described in order to reverse damage of DNA inflicted by the fixation procedure in order to increase the yield of DNA extraction from FFPE tissue specimens. Among them, the application of heat prior to DNA-extraction has been shown to increase the amount of amplifiable human DNA from FFPE tissue samples, probably by reversing cross linking of DNA with other biomolecules [32]. Recently, we have shown that this is also relevant for the extraction of fungal DNA from FFPE tissue specimens. Improvements in the amount of amplifiable human DNA up to 76-fold were documented, and the number of samples from which fungal DNA was amplified by broad range PCR and successfully identified by sequencing was improved [33]. Recent studies using PCR to identify fungal DNA from FFPE tissue specimens successfully identified fungal DNA in 70-80% of FFPE tissue samples in accordance with culture results, suggesting that this is a feasible approach [34,35].

The target of PCR-assays used to amplify fungal DNA is critical for the identification of fungal pathogens. So far, multicopy genes such as the ribosomal rRNA genes have been used in order to provide sensitive species identification. Among these genes, conserved regions can be targeted to detect a broad coverage of fungi, while variable regions can be targeted to amplify DNA from distinct species or groups of fungi. Broad range fungal assays have been successfully used to determine the etiology of invasive fungal infections from FFPE tissue samples. Taxon-specific assays may be at least as sensitive in the detection of target organisms, but negative results have to be interpreted carefully as infections due to non-target fungi might be missed. This limitation might be diminished when assays targeting different groups of fungi are used [35]. The more inclusive target of broad-range assays may come at the price of an increased likelihood to detect contaminating DNA and a potential for cross-amplification of human DNA. However, assays can be designed to detect fungal DNA in an excess of human DNA [36]. This might be critical in tissue biopsies where lots of human DNA can be expected. Strategies to overcome contamination and adequate controls to address this issue have been proposed previously [24].

Most studies using PCR to detect fungal DNA in FFPE tissue specimens used Sanger-sequencing for the identification of PCR amplicons. A reliable differentiation up to the species level is possible depending on the target sequence. Difficulties in species identification may arise when mixed sequences are recovered either due to a mixed fungal infection or due to the presence of contamination with fungal DNA. An *in-silico* tool has been introduced to identify up to three bacteria from mixed chromatograms of bacterial DNA amplified by broad range bacterial PCR from mixed infections such as abscesses [37]. This approach might be useful for unraveling mixed chromatograms from fungal PCR assays as well. Further options to decipher mixed amplicons include the generation of clone libraries. Using this approach on clinical samples from mixed bacterial infections allows one to identify more agents than by PCR or culture [38,39●●]. Further species resolution is possible by next generation sequencing [39●●].

Beside the identification of fungi by sequencing ribosomal RNA-genes, further applications for PCR and sequencing from tissue specimens may emerge. Vallor et al. successfully amplified the single copy FKS gene of *A.fumigatus* from tissue of an animal model of invasive aspergillosis [40]. With improvements in DNA extraction, such genes may successfully be targeted in FFPE tissues. Targeting single-copy genes may allow for the identification of molecular siblings, fungi that cannot reliably be differentiated by morphology or sequencing of ribosomal RNA-genes. The distinction between such sister species may have clinical implications as they may display different in vitro susceptibilities to antifungal agents [41,42●]. In addition, genetic elements related to in vitro resistance have been successfully detected directly from FFPE tissue of a patient with invasive Aspergillosis [43]. Such assays would be critical to use on FFPE tissue specimens to study reasons for antifungal treatment failure.

Since its introduction in the late 1980´s fluorescence in situ hybridization (FISH) has become a widely used method for the identification and quantification of phylogenetically defined microbes in populations [44]. The basic steps of FISH include the fixation of samples to allow for the uptake of labeled synthetic DNA probes into target cells. Identification of microorganisms is achieved by binding of probes, designed to specifically hybridize with unique sequences of the ribosomal RNA of target organisms. After washing away unbound probe, hybridized cells can be identified by epi-fluorescence microscopy. Due to the high rRNA content of cells, a sensitive detection and identification of microbes can be achieved [45].

In the field of fungal diagnostics, FISH so far has been studied for the identification of agents of candidemia. DNA-probes have been described to differentiate between frequent isolates from patients with candidemia such as *C. albicans*, *C. krusei*, *C. glabrata* and *C. parapsilosis* cultivated from blood culture bottles [46]. In addition, DNA probes targeting *Cryptococcus neoformans* and

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Cryptococcus gattii have been developed that differentiate these agents of cryptococcosis from other ascomycetes and basidiomycetous yeasts. Using these probes allowed for the identification of cryptococci directly from cerebrospinal fluid of patients with cryptococcosis [47]. More recently, peptide nucleic acid (PNA) probes, chemically modified DNA probes with increased affinity for complementary nucleic acids, have been used to discriminate between cultivated yeasts. Close relatives such as *C. albicans* and *C. dubliniensis* were differentiated *[48]*. Also, the distinction between frequent agents of candidemia, requiring different antifungal therapies such as *C. albicans* and *C .glabrata* was shown to be possible directly from positive blood culture bottles with sensitivities of 98.7 and 100% specificity in a multicenter trial. Of note, some species not regularly described as causes of candidemia, such as *Candida nivariensis* or *Candida bacarensis* were also stained with the probe aimed at the detection of *C .glabrata*, highlighting potential limitations in the species resolution with short probes [49]. Additional PNA- probes have been evaluated to further broaden the spectrum of cultivated yeasts (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*) identifiable by PNA-probes. These yeast-species accounted for 99% of all yeasts cultivated in a diagnostic laboratory [50].

These studies document that FISH using DNA- or PNA probes targeting rRNA can be used to differentiate between cultivated yeasts and suggest that their use may also be possible directly in uncultured samples. To our knowledge, these probes have not been used on fresh or formalin-fixed tissue samples from patients with deep-seated fungal infections.

Most studies that sought to identify fungal pathogens in formalin-fixed, paraffin-embedded tissue samples used conventional DNA probes targeting the 5S, 18S or 28S rRNA for in situ hybridization. Using this approach, hybridization was achieved in 60-80 % of samples from yeast-infections, hyalohyphomycosis, phaeohyphomycosis and infections due to dimorphic fungi with documentation of hyphae by histology [51-54]. More recently, Montone used locked nucleic acid (LNA) probes, chemically modified DNA probes with increased affinity for the RNA-target with sequences identical to the DNA probes previously reported by Hayden. Using ffpe tissue samples from patients with culture confirmed coccidioidomycosis, aspergillosis and fusariosis, they reproduced the high specificity of the probes and suggested that the signal obtained by LNA-probes is superior to that obtained with DNA probes [55-57].

These studies using ffpe tissue specimens were performed with tissue obtained from culture proven clinical samples and tissue from proven non-target fungi as negative controls. While this approach of testing the sensitivity and specificity of probes is highly accurate for diseases caused by a limited number of causative agents, such as *Histoplasma capsulatum*, the definition of target species and

outside hits of these probes are not as clearly defined as for the probes reported for candidiasis and cryptococcosis.

Recently, reports applying FISH-probes to study the etiology of IFD from tissue specimens used cultivated fungi to experimentally evaluate target organisms and outside hits of the probes. Shinozaki developed a probe targeting the 28S rRNA of *Fusarium* spp.. They used a target sequence conserved among frequent agents of Fusariosis that displayed several mismatches in the target region of other frequent fungal pathogens such as *Aspergillus*, *Candida* and the mucorales making hybridization with these fungi unlikely. The specificity of the probe was assessed using formalin fixed, paraffin embedded hyphae from a panel of type strains and tissue from mice infected with *Candida*, *Aspergillus*, mucorales, *Fusarium* and *Pseudallescheria*. The designed Fusarium-probe was found to specifically stain hyphae from cultivated type strains and hyphal elements in tissues from animals with fusariosis [58]. In a comparable approach, we used a panel of type strains to generate DNA probes to identify *Candida* spp. and *Aspergillus* spp. in tissue samples by FISH and compared the results to those obtained by broad-range fungal PCR targeting the 28S ribosomal RNA-Gene with sequencing of the amplicons [34]. In this study, PCR was more successful in identifying the etiology of IFD. As in previous studies using ISH, the distinction of the probe signal was impaired in necrotic tissue background in samples from mould infections [34,51,52]. This limitation may be overcome by superior probe chemistries such as PNA-, or LNA probes [45]. In addition, the hybridization signals can be restricted to parts of fungal elements in tissue [34,58,59]. This is in accordance with data demonstrating that rRNA signals obtained by PNA probes is most intense in the apical compartment of hyphae [60].

Ultimately, a panel of probes with well-defined target species detecting frequent agents of IFD could be designed. Recently, online tools allowing the prediction of probes generating strong signals have been introduced. These tools will help characterize probes to reduce time needed for experiments [61]. Using such fungal probes with non-specific probes labeled with different fluorophores may help visualize uncommon agents and localize them to the pathologic process in tissue. Improvements in microscopy may facilitate the application of different numbers of fluorescently labeled probes allowing for the detection of multiple species in a single sample [62].

Emerging applications of molecular tissue diagnosis

Tissues samples from patients with invasive fungal infections are crucial for understanding the etiology on IFD. Molecular tests are emerging as tools to characterize the etiology of IFD from FFPE tissue specimens, circumventing shortcomings in culture and histopathology. While amplification based methods offer sensitive detection and identification of organisms, hybridization tests localize

and identify fungi within tissue pathology, proving causality. Several attributes may allow a comprehensive study of organisms associated with IFD. First, culture negative mould infections constitute up to 60 % of all mould infections found at autopsies in cancer patients. Molecular methods may provide a clear picture of the etiology of mould infections in these patients. Second, polymicrobial infections in cancer patients are increasingly being recognized and can be a therapeutic challenge. They may originate from abdominal infections such as neutropenic colitis [63]. Mixed infections are diagnosed in up to 23% of patients in Candidemia [64]. In polymicrobial communities, microbes interact, either directly, by secreted molecules or via competition for nutrients [65]. These interactions may modify antimicrobial susceptibility [66] and therefore have therapeutic or prognostic implications. Deciphering the etiology may stimulate research on interactions between microorganisms [67]. Third, the etiology of IFD is difficult to assess in patients colonized with multiple fungi, such as patients with cystic fibrosis. While IFD may be rare in these patients, they do occur after lung transplantation, that is an increasingly used therapeutic option to treat end stage pulmonary failure and that is associated with mortality rates of 29% despite antifungal therapy [68].

Conclusion

The tissue diagnosis of IFD is important for understanding changes in the epidemiology of opportunistic fungal infections. The proper histopathologic description of fungal elements in tissues is critically needed but current technology falls short and cultivation suffers from similar challenges. The use of ffpe tissue samples to study the etiology of IFD by molecular tests is a promising strategy. A combined approach using amplification techniques with sequencing and hybridization based assays has the potential to overcome limitations of culture and histopathology. A better understanding of the etiology of invasive fungal infections will improve empiric antifungal therapies, may guide treatment decisions in patients with break-through infections, and can help improve the development of non-invasive tests.
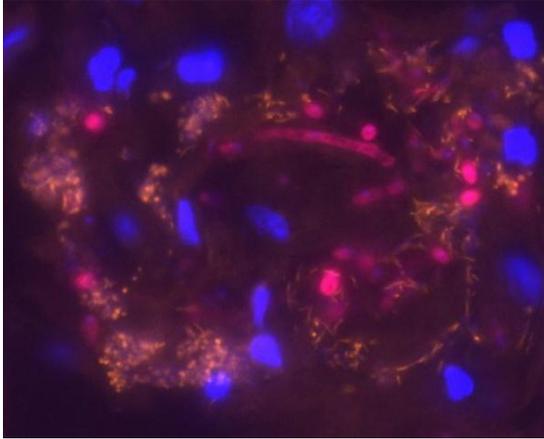
Figure 1: Gut biopsy of a patient with relapsed acute myelogenous leukemia who died from sepsis.
While blood cultures were sterile under antimicrobial therapy, Fluorescence in situ hybridization (FISH) targeting different ribosomal RNA sequences of microbes documents a mixed infection. Cocci and rods (stained with the bacterial probe EUB 338 [45], labeled with the fluorescent dye Cy3 (coded as organge) and yeasts (stained with the fungal probe Cand 317 [34] labeled with the dye Cy5 (coded as red) between human cells. Nuclei of human-, and fungal cells stained with 4´,6-diamidino-2-phenylindole (DAPI) (blue). The yeast was identified as *C.albicans* by sequencing of the amplicons of broad-range fungal PCR assays. Bacteria where not further identified.

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