FUNGAL BIOLOGY 120 (2016) 279–287

ELSEVIER

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journal homepage: www.elsevier.com/locate/funbio

Identification of fungal pathogens in Formalin-fixed, Paraffin-embedded tissue samples by molecular methods

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ARTICLE INFO

Article history:
Received 26 January 2015
Received in revised form
28 June 2015
Accepted 6 July 2015
Available online 17 July 2015
Corresponding Editor:
Sybren De Hoog

Keywords:
FFPE
FISH
Invasive fungal infection
PCR

ABSTRACT

The etiology of invasive fungal infections (IFI) is incompletely understood due to diagnostic limitations including insensitivity of cultures and failure of histopathology to discriminate between different species. This diagnostic gap precludes the optimal use of antifungals, leading to adverse patient outcomes. The identification of fungal pathogens from Formalin-fixed, Paraffin-embedded tissue (FFPE) blocks by molecular methods is emerging as an alternative approach to study the etiology of IFI.

PCR assays, including species specific- and broadrange fungal tests are used with FFPE samples from patients with proven IFI. Fungal species identification is achieved in 15–90% of the samples. This heterogeneity may be explained by the samples studied. However, comparison of different studies is impaired, as controls ruling out false positive-, false negative test results or PCR inhibition are frequently not reported.

Studies using *in situ* hybridization also vary in the clinical samples included and the targeted fungi. In addition, target sequences, the probe chemistry and the detection of hybridization signals also account for the differences in diagnostic sensitivity.

Using both approaches in parallel yields additive insights, potentially leading to a superior identification of fungal etiology and awareness of the limitations of both molecular diagnostic approaches.

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Introduction

Invasive fungal infections (IFIs) continue to be a serious threat in a growing number of patient groups, including cancer patients, organ transplant recipients, the critically ill, and other immunocompromised hosts (Brown *et al.* 2012).

The clinical diagnosis of IFIs continues to be a challenging task, especially in mould infections. For example, aspergillosis is among the most frequently missed diagnosis in critically ill patients (Tejerina *et al.* 2012). Furthermore, the identification of a causative mould is not achieved in about half of the affected individuals (Chamilos *et al.* 2006; Neofytos *et al.* 2009). This impairs the optimal use of prophylactic measures, optimal treatment strategies, and the development of diagnostic tests. This may in turn lead to adverse outcomes in patients with IFI.

The identification of fungal pathogens by histopathology is an important component in the understanding of the epidemiology of IFI. Cohort studies of patients with IFI proven by histopathology documented etiologic shifts such as an

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http://dx.doi.org/10.1016/j.funbio.2015.07.002
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increase in hyalohyphomycosis, primarily invasive aspergillosis starting in the 1980's, and an increase of mucormycosis during the 1990's (Groll *et al.* 1996; Chamilos *et al.* 2006; Kume *et al.* 2011). The distinction between mucormycosis and aspergillosis, that is often achieved directly by hyphal morphology from histopathology samples, is of great clinical importance. The agents of mucormycosis are resistant to antifungals such as voriconazole and the echinocandins and surgery is suggested to play an important role in patient management (Cornely *et al.* 2014). Given the emergence of fungal pathogens with different therapeutic implications but similar histomorphology, the identification of causative agents to the species level is needed for improving patient care, especially in mould infections refractory to standard therapies (Nucci & Perfect 2008). However, cultures identify fungi only in 30–60 % of histopathology positive samples (Tarrand *et al.* 2003; Rickerts *et al.* 2007). In addition, discrepancies between histopathology and cultivated fungi in 20 % of cases highlight the necessity for the development of alternative fungal identification strategies in order to understand the aetiology of IFI (Sangoi *et al.* 2009; Lee *et al.* 2010).

The identification of fungal pathogens from Formalin-fixed, Paraffin-embedded (FFPE) tissue from patients with proven IFI by molecular tools is an attractive way to investigate the aetiology of IFI. Different techniques, based on the amplification of fungal DNA via PCR or the hybridization of probes to fungal RNA have been successfully applied on FFPE tissues samples. In this manuscript, selected aspects of the identification of fungi from FFPE tissue by molecular techniques reported previously are reviewed.

Amplification of fungal DNA from tissue samples by PCR

Tissue samples provide unique opportunities to establish the aetiology of IFI by molecular methods. Fungi identified in deep tissue samples mostly represent true invasive pathogens. This is in contrast to samples such as bronchoalveolar lavage, where colonization by fungi may be a frequent finding in some patient groups, reducing the specificity of diagnostic tests (Bretagne *et al.* 1995; Rickerts *et al.* 2002). In addition, tissue samples often contain a higher fungal load as compared to blood and bronchoalveolar lavage, which improves fungal identification (Kasai *et al.* 2008). Using PCR assays with adequate molecular targets such as the ITS-region or combinations of different targets typically allows for the identification of fungal pathogens to the genus or even the species level, mostly in line with culture results in culture positive cases (Lau *et al.* 2007; Rickerts *et al.* 2007). Some clinical studies suggest that fungal identification from fresh tissue is even more sensitive using molecular methods than by culture. This appears to be most prominent in moulds difficult to cultivate, such as the mucorales (Willinger *et al.* 2003; Rickerts *et al.* 2007).

Reasons for negative PCR results from fresh tissue showing fungal elements include the pretreatment with antifungals that lowers the amount of fungal DNA in tissue (Vallor *et al.* 2008). In addition, some fungal organisms may not be detected by PCR due to mismatches in primer binding regions. This is possible even when so called panfungal PCR assays are used (Lau *et al.* 2007; Rickerts *et al.* 2007; Khot *et al.* 2009; Dannaoui *et al.* 2010).

Tissue samples also display challenges in the identification of fungal pathogens. First, taking of samples may not be possible in patients with suspected IFI due to contraindications including thrombocytopenia. In addition, invasive sampling is only indicated in patients with progressive disease and additional treatment options. Therefore, available tissue samples can be biased in favour of difficult to treat infections such as mucormycosis. This impairs the generalizability of data from tissue studies on the aetiology of IFI in general. Second, tissue samples can contain high amounts of non-fungal, i.e. human DNA. This can impair the amplification of fungal DNA, especially when broadrange primers targeting conserved region are used that also bind to human DNA (Khot & Fredricks 2009). Third, fresh tissue samples are often not available for diagnostic testing, especially when IFI are not considered as a differential diagnosis. Therefore, FFPE tissue samples are frequently the only samples available to perform molecular tests. In contrast to fresh samples, the amplification of fungal DNA from FFPE tissue samples from patients with proven IFI can be challenging. Studies comparing the amplification of fungal DNA from fresh and FFPE tissue consistently describe a reduced sensitivity from FFPE tissue (Willinger *et al.* 2003; Lau *et al.* 2007). In addition, damaged DNA templates in FFPE tissue may create sequence artifacts interfering with identification of fungi (Do & Dobrovic 2015).

Studies evaluating PCR to detect fungi from FFPE tissue vary in methods used and results

Fungal DNA was amplified by PCR in 15–90 % of FFPE tissue samples from patients with proven IFI. This heterogeneity may in part be explained by the samples studied. First, the presence of fungal elements in tissue was confirmed in some studies using fungal stains (Rickerts *et al.* 2011; Bernhardt *et al.* 2014). This may select for samples with higher fungal load, especially when the performance of molecular tests is restricted to samples with a threshold of fungal elements seen by microscopy. The knowledge of the hyphal morphology may also bias the interpretation of molecular tests when exact criteria for positive or negative molecular tests are not applied. Ideally, a comparison between histopathology and molecular test results should be performed after a molecular diagnosis has been established in a blinded fashion, which was done in a minority of studies only (Bialek *et al.* 2005; Hammond *et al.* 2011). Second, studies that included mould and yeast infections demonstrate superior amplification of fungal DNA from samples of yeast infections (Munoz-Cadavid *et al.* 2010; Rickerts *et al.* 2011). Among samples from mould infections, samples from patients with mucormycosis appear to be the least positive (Lau *et al.* 2007; Rickerts *et al.* 2011). Reasons may include DNA extraction, that has not been optimized for such samples or the use of primers not optimized for mucorales (see below). Third, studies did not report on the antifungal treatment history of patients before tissue sampling. It has been documented that effective antifungal therapy reduces the fungal load in tissue samples

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