Deciphering the aetiology of a mixed fungal infection by broad-range PCR with sequencing and fluorescence in situ hybridisation

V. Rickerts,1 I. McCormick Smith,1 S. Mousset,2 O. Kommedal3 and D. N. Fredricks4

1Robert Koch Institut, FG 16, Berlin, Germany, 2Haematology, University Hospital Frankfurt, Frankfurt, Germany, 3Isentio AS, Bergen, Norway and 4Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, USA

Summary

Simultaneous infections with multiple fungi may be misinterpreted as monomicrobial infections by current diagnostics with ramifications for the choice of antimicrobial agents that may impact patient outcomes. The application of molecular methods on tissue samples may be useful to decipher the aetiology of mixed fungal infections. We present a leukaemic patient who died from sepsis due to candidaemia. The post-mortem examination documented fungal elements in lung tissue. Fungal DNA was amplified from the lung sample by broad-range PCR assays targeting the 28S ribosomal RNA gene or the internal transcribed spacer 2 (ITS-2). Fluorescence in situ hybridisation (FISH) using differentially labelled fungal probes was applied on the tissue. Sequencing identified the PCR amplicons as Aspergillus fumigatus (28S assay) and Candida tropicalis (ITS-2 assay). As a chromatogram suggested mixed amplicons, the Isentio ripseq® tool for in silico analysis was applied and confirmed the presence of both amplicons in the PCR products of both assays. FISH confirmed the presence of Aspergillus and Candida within the infectious process, a prerequisite for inferring a causal relationship with the infection. The combination of broad-range PCR with sequence analysis and FISH applied on tissue samples is a powerful approach to identify the aetiology of invasive fungal infections, including mixed infections.

Key words: Candidiasis, aspergillosis, PCR, FISH, mixed infection, coinfection.

Introduction

Invasive fungal diseases (IFD) in various patient groups are commonly treated using empirical antifungal therapy. Therefore, a comprehensive study of organisms associated with IFD is essential to define successful antifungal therapies in each setting. Current diagnostic tests such as culture, histology and serology are limited in this respect, especially in difficult to assess cases such as infections caused by mixed species. Adverse outcomes, such as the considerable mortality associated with IFD, may be in part due to the gap in our knowledge of the exact aetiology of IFD, which may lead to a suboptimal use of antifungal agents. Using molecular methods to identify fungal pathogens directly from formalin-fixed, paraffin-embedded tissues is emerging as a diagnostic approach. The goal is to complement conventional diagnostic tests through a reliable detection and identification of fungal nucleic acids in tissues from patients with proven IFD, to direct antiinfective therapies and ultimately improve patient outcomes.1

Here, we describe a neutropenic patient presenting with candidaemia in whom IFD was confirmed at the postmortem examination by the demonstration of fungal elements in lung tissue. To decipher the fungal aetiology, we applied two broad-range ribosomal RNA (rRNA) gene PCR assays with sequencing of the amplicons to identify the fungal DNA from the tissue. In addition, we use fluorescence in situ hybridisation...
(FISH) with specific fungal probes labelled with different fluorophores, to confirm the presence of the amplified fungi in the infectious process.

**Patient and methods**

**Clinical presentation**

A 71-year-old woman was diagnosed with acute myelogenous leukaemia secondary to chemotherapy for breast cancer. Her medical history was positive for diabetes mellitus, liver cirrhosis (CHILD A) and coronary artery disease. The initial blood count showed pancytopenia with neutrophils of 0.08/nl and she remained neutropenic during follow-up. She was transferred to our department and induction chemotherapy with cytarabine 100 mg m⁻² i.v. as continuous infusion was started (day 1). Antibacterial prophylaxis with levofloxacin (500 mg day⁻¹ i.v.) was initiated (day 1). Antibacterial prophylaxis with levofloxacin was started to Piperacillin/Tazobactam (3.45 g day⁻¹ i.v.). Five days later, the patient complained of dyspnoea. A chest X-ray showed pneumonia and Piperacillin/tazobactam was changed to Imipenem/Cilastatin (40.5 g day⁻¹ i.v.). She did not receive antifungal prophylaxis. A day later, mechanical ventilation and high-dose catecholamine’s were required due to septic shock with respiratory failure. A blood culture drawn on day 10 was positive for *Candida tropicalis* the following day, only after the patient succumbed to cardiac arrest. The postmortem examination documented a disseminated fungal infection of the lung, liver, spleen and the kidneys. Histopathology of the lung tissue showed fungal elements consisting of budding yeast and hyphal elements. Tissue samples were not cultivated for pathogens.

**Workup of the formalin-fixed, paraffin-embedded tissue, PCR and sequence analysis**

Sections of 5 µm were prepared from the formalin-fixed, paraffin-embedded lung tissue, and they were placed on slides for conventional histology and FISH. In addition, three sections were placed in Eppendorf tubes for DNA extraction. The presence of fungal elements in the sections was confirmed by Grocott’s Methenamine-Silver (GMS) staining of the first and last section of the tissue block.

DNA extraction, real-time PCR assays targeting a region containing the internal transcribed spacer-2 (ITS-2) and a part of the 28S gene, sequencing of the amplicons and identification of the amplicons have been described previously. In short, both broad-range PCR assays were positive for fungal DNA. Negative master mix- and extraction controls, consisting of water processed in parallel with the samples remained negative.

**Fluorescence in situ hybridisation**

Fluorescence in situ hybridisation was performed using two previously described probes targeting the 28S rRNA of agents of aspergillosis or candidiasis. The probe Asp F (labelled with Cy3, coded as orange) hybridises with the 28S rRNA of *Aspergillus* spp., but not with *Candida* spp. The probe Cand 317 binds to the 28S rRNA of yeast related to *Candida albicans* (labelled with Cy5, coded as red), but not *Aspergillus* spp. All probes were synthesised at Eurofins MWG Operon (Ebersberg, Germany).

Hybridisation buffer consisted of 5x SET [0.75 mol l⁻¹ NaCl, 5 mmol l⁻¹ EDTA, 0.1 mol l⁻¹ Tris (ph 7.8)], 10% Dextran, 0.2% bovine serum albumin, 0.1 mg ml⁻¹ polyadenosine, 20 µg ml⁻¹ salmon testes DNA and 0.02% SDS (all from Sigma Aldrich®; Munich, Germany).

To assess for the appropriate performance of the hybridisation procedure, target- and non-target fungi of the two fungal probes were used as controls concomitant with the tissue sample under study. *Aspergillus fumigatus* (ATCC 9197), *Candida albicans* (ATCC 10231), *Scedosporium apiospermum* (RKI 11_346) and *Candida krusei* (ATCC 6258) were grown in Saboraud-dextrose broth medium, washed twice in PBS, than fixed in Formalin (35%; 1:1 with PBS) for 24 h, washed with PBS once and stored at 4°C in 50% ethanol until use. The formalin-fixed fungi were placed on polylysine-coated slides (Roth, Karlsruhe, Germany) and then air dried. Hybridisation of the fungi was performed with 10 µl of hybridisation buffer containing 10 ng of the positive- or the negative control probes (labelled with Cy3 and Cy5) or the specific fungal probes Asp F (labelled with Cy3) and Cand 317 (labelled with Cy5) and 10 ng of 4',6-diamino-2-phenylindole (DAPI) for counterstaining of double-stranded DNA. Coverslips were placed and the slides were incubated in a humid chamber at 50°C for 12 h. The tissue sample was first deparaffinised by dipping into octane (Sigma Aldrich®, Munich, Germany). After air drying, 60 µl of hybridisation buffer containing 60 ng of the Asp F- and Cand 317 probes and 60 ng of DAPI were applied to the tissue. A coverslip was placed and the slide was incubated together with the controls.

After incubation of the tissue sample and the control slides, the coverslips were removed by dipping the
slides in 4 °C 0.2 SET. Next, the slides were washed in 5x SET buffer three times for 10 min. After air drying in the dark, Vectashield® mounting fluid (Vector laboratories, Burlingame, CA, USA) was applied and coverslips were placed for fluorescence microscopy.

Fluorescence microscopy

Fluorescence microscopy was done using an Axio Imager 2 fluorescence microscope. Pictures were generated using AxioVision software (Release 4.8.2) (Zeiss, Jena, Germany). Identical exposure times were used for the tissue sample and the controls at each channel (Cy 3: 50 ms; Cy 5: 250 ms; Dapi: 10 ms).

Results

Sequencing of the amplicons of the PCR assays were identified as Aspergillus fumigatus (28S PCR assay) and Candida tropicalis (ITS-2 assay) by comparison with an internal database, containing ribosomal RNA gene sequences from publicly available genomes and selected sequences from a previous publication with a similarity threshold of >97% used to designate a species.3

As the electropherogram of the sequenced amplicon from the ITS-2 assay appeared mixed, suggesting that more than one amplicon was amplified from the specimen (Fig. 1), the Isentio ripseq® online tool was used for further sequence analysis.4 Only through this in silico analysis, sequences from both fungal pathogens, A. fumigatus and C. tropicalis, were identified from the amplicons of both broad-range fungal PCR assays.

FISH using the fixed control strains demonstrated the correct performance of the hybridisation procedure by hybridisation with the unspecific probe EUK 516 binding to eukaryotic ribosomal RNA. Unspecific binding was excluded by the negative hybridisation result with the nonsense-probe non-EUB. Specific binding of the fungal probes to target organisms but not with non-target organisms was demonstrated (Fig. 2). FISH of the tissue specimen showed hybridisation of round fungal elements and hyphae with the Asp F probe, representing hyphae and probably cross sections through hyphae of A. fumigatus. Fungal elements consisting of budding yeast as well as hyphal elements not hybridised with the Asp F probe but hybridised with the Cand 317 probe, suggesting yeast and hyphal elements of yeast related to C. albicans as the aetiologi cal agents of this mixed fungal infection (Fig. 3). The overlap between the morphology of both fungi in the tissue morphology, deciphered by differential labelling of specific probes, highlights the challenges in inferring fungal aetiology from the morphology of fungal elements in formalin-fixed, paraffin-embedded tissue sections. In addition, differential labelling of the specific probes proves that both fungi were present in the infected tissue, a prerequisite for inferring aetiology due to fungi widespread on mucous membranes and in the environment.

Discussion

The case presented here demonstrates the ability of molecular methods to decipher the aetiology of invasive fungal diseases from formalin-fixed, paraffin-embedded tissue samples, even in cases of mixed infections. Broad-range fungal PCR supplemented with sequencing and in silico analysis of the amplicons identified the amplified fungal DNA to the species level. In addition, we report for the first time that FISH using differentially labelled probes can localise mixed fungal pathogens in infectious processes in tissue samples from patients with proven IFD.

It has been hypothesised that polymicrobial infections are an underappreciated entity in cancer- and probably other immunocompromised patients.5 Mixed infections represent diagnostic challenges and may
predispose to adverse clinical outcomes due to challenges in antiinfective therapy. The true incidence of mixed fungal infections is unknown. Whereas mixed infections are not being regularly reported in the clinical literature, some recent reports include information on mixed infections. A recent cohort study documented concomitant fungal infections in 20% of solid organ transplant recipients with IFD, but it is unknown if these are mixed infections or subsequent diseases due to different fungi. Simultaneous infections due mixed Candida species are documented from 3% of candidaemia cases by blood cultures. More than one mould can be cultivated from respiratory secretions of 7% of patients at risk for mould infections as suggested by a recent report. Although the number of distinguishable fungal tissue morphologies is limited, a postmortem study reported infections due to fungi representing different tissue morphology in 5% of patients with IFD. However, given the limitations in diagnostic tests, these numbers of mixed infections as documented by conventional diagnostic tests likely represent a conservative estimate of the true incidence of polymicrobial infections.

Molecular methods applied to formalin-fixed tissue samples may be useful to evaluate the occurrence of mixed infections among patients with proven IFD. The combination of different molecular methods applied in the reported case highlights important issues concerning the identification of fungal pathogens from formalin-fixed, paraffin-embedded tissue samples by molecular methods. First, although PCR-based techniques successfully amplified fungal DNA from up to...
mucous membranes. Of note, colonisation with 9–23 different fungal species can be detected on mucous membranes of healthy subjects when sensitive molecular tests are used. Finally, differential labelling of FISH probes can be used to directly visualise mixed infections. While a mixed infection in the reported case was already suggested by conventional histopathology, a differentiation between fungi with similar morphology in tissue specimens, i.e. various yeast such as Candida albicans, and Candida krusei or moulds such as Aspergillus fumigatus and Scedosporium prolificans which require different antifungal therapies may be achieved using FISH. Moreover, by a combination of a specific probe and an unspecific probe, samples may be systematically screened for mixed infections.

In conclusion, the diagnostic approach taken in this case report, the combination of amplification and hybridisation-based molecular methods to identify pathogens associated with IFD from formalin-fixed, paraffin-embedded tissues is useful to define the aetiology of IFD, even in mixed infections. This diagnostic approach may help to delineate potentially successful antimicrobial therapies that will improve the outcomes of patients with IFD.

**Competing interests**

DF has intellectual property related to the use of PCR for the detection of fungal pathogens. OK has intellectual property related to the algorithm used to identify fungi from mixed amplicons. He is the founder of the company isentio. The other authors declare that they have no competing interests.

**References**


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