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Original article

Diagnostic value of a semi-nested PCR for the diagnosis of mucormycosis and aspergillosis from paraffin-embedded tissue: A single center experience

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ABSTRACT

Objective: The main aim of our study was to investigate the diagnostic value of a molecular method for the diagnosis of mucormycosis and aspergillosis from formalin-fixed and paraffin-embedded (FFPE) tissues.
Methods: A retrospective chart review identified all cases with histology reports mentioning the presence of fungi with morphological characteristics of either *Aspergillus* or mucormycetes, for the period 2005–2012. Paraffin blocks were retrieved from the archives of the Department of Pathology. A semi-nested PCR specific for the detection of mucormycetes and *Aspergillus* species was applied in FFPE tissue from the above blocks. Results were compared with those of histological (gold standard) and microbiological methods.
Results: Twenty cases with fungal hyphae in tissue were revealed. Mucormycetes were detected in 9 cases (45%) by PCR, in only 4 of which culture was available. Species of *Aspergillus* were detected in 8 cases (40%) by PCR, two of which were co-infection with mucormycetes. Five patients had other fungi, non-detectable with this specific PCR. At least one sample per patient was positive by PCR. Seven out of 30 samples tested overall were false negative. The calculated sensitivity of this method in our setting was 79.3% (95% CI: 60.3–91.9%); specificity was 100%.
Conclusions: The specific PCR used appears to be an easy and useful tool for the prompt and accurate diagnosis of mucormycosis and aspergillosis, in combination with histology and direct examination. Mucormycosis was more frequent than aspergillosis during the study period, highlighting the importance of continuous epidemiological surveillance of these serious infections.

Introduction

An increase of invasive mycoses due to filamentous fungi has been observed during the last 20 years. These mycoses have high morbidity and mortality and affect mainly immunocompromised patients, including those with hematologic malignancies, hemopoietic stem-cell or solid organ transplantation, diabetes mellitus or long term treatment with corticosteroids. Prompt initiation of specific treatment is crucial for a better outcome [1–7]. Species of *Aspergillus* are susceptible to voriconazole and the echinocandins, but zygomycetes of the order of Mucorales (mucormycetes) are usually susceptible only to amphotericin B, and less frequently to posaconazole [8]. Therefore, knowledge of the local epidemiology is of utmost importance both for prophylaxis and the initiation of diagnostics-driven or empirical treatment. Aspergillosis is considered the most frequent hyphomycosis; yet, in recent years, fungal infections with a rapid development and even higher fatality rate, such as those caused by mucormycetes, are increasing [9] and more data characterizing the epidemiology of the aforementioned mycoses in Greece are necessary.

Diagnosis of these infections is challenging, due to the lack of specific clinical signs. Radiology, only contributes to the assessment of lesion extension, and serology is very limited, concerning only aspergillosis (e.g. detection of galactomannan). Microbiological investigation, consisting of direct microscopy of fresh tissue

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and culture, is very important. Direct microscopical examination with KOH (10–20%) and preferably with a fluorescent brightener, has a high diagnostic value as it allows rapid presumptive diagnosis distinguishing mucormycosis from aspergillosis or other hyalohyphomycosis [3]. Culture, apart from its time-consuming nature and low sensitivity e.g. approximately 50% in the case of mucormycosis [10], is an ideal method in terms of providing the potential for exact identification of the fungus and its antifungal susceptibility profile. However, fresh bioptic tissue is not always available to the microbiology laboratory. Diagnosis is usually achieved by means of histological examination, which although considered the 'gold standard', exhibits limited specificity [11]. In view of the above, the development of molecular methods has greatly contributed to the accurate identification of fungi. These methods can be applied in any biological material including formalin fixed and paraffin embedded (FFPE) tissues, despite the detrimental effect of formalin upon DNA during the fixation procedure [12]; it has nevertheless not been extensively tested in the detection of hyphomycetes.

The goal of the present study was to investigate the diagnostic value of a molecular method specific for the detection of species of *Aspergillus* and mucormycetes, applied to FFPE tissues. Secondarily we examined the prevalence of mucormycosis in cases with histological presence of fungal hyphae, in a tertiary academic hospital in Greece, between 2005 and 2012.

Materials and methods

Histology reports issued between January 2005 and December 2012 with the finding of fungal hyphae consistent with either *Aspergillus* or Mucorales, as well as the relevant paraffin blocks were retrieved from the archives of the Department of Pathology. DNA was extracted from FFPE tissues obtained from the above blocks. Each sample consisted of three FFPE tissue scrolls 10 μm thick, placed in an Eppendorf tube. A semi-nested PCR was applied, as described by Bialek et al. [13]. In brief, for DNA extraction the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) was used according to manufacturer's instructions. This includes a 60 min heating step at 90 °C after lysis with proteinase K, in order to reverse formalin-induced protein-DNA cross-linking [12], and thus enhance nucleic acid yield. For the detection of mucormycetes the 18S rDNA was amplified. The external primers used were ZM1 (5′-ATT ACC ATG AGC AAA TCA GA-3′) and ZM2 (5′-TCC GTC AAT TCC TTT AAG TTT C-3′) and for the semi-nested PCR the ZM1 and ZM3 (5′-CAA TCC AAG AAT TTC ACC TCT AG-3′) (177 bp product). For the detection of *Aspergillus* the external primers P2 (5′-CTT TGG TTG CGG GTT TAG GGA TT-3′) and Asp2 (5′-GGG AGT TCA AAT CTC CCT TGG G-3′) complementary to mitochondrial transfer RNA (GenBank accession number L37095), were used. For the semi-nested PCR the primers used were P1 (5′-GAA AGG TCA GGT GTT CGA GTC AC-3′) and P2 (135 bp product). Primers G1 (5′-GAA GAG CCA AGG ACA GGT AC-3′) and G2 (5′-CAA CTT CAT CCA CGT TCA CC-3′) detecting human β-globulin (nucleotides 70400–70667; accession number, NG_000007.3) were used to ensure DNA existence and absence of inhibitory factors. In case of negative results for human β-globulin gene, a second DNA extraction was performed using a new sample from the paraffin block. A strain of previously sequenced *Rhizopus arrhizus* and *Aspergillus fumigatus* ATCC 204305 from pure culture were used as positive controls. Reaction mixture with no DNA was used as negative control in each experiment.

The nested-PCR results were compared to the findings of the previously performed histological and microbiological examinations. Histological examination was based upon Hematoxylin–Eosin (H–E) stain, as well as PAS and Grocott methenamine silver stains for the identification of fungi [14]. Mycological investigation had been performed by fresh tissue culture on Sabouraud glucose agar with chloramphenicol but without cycloheximide, and direct examination with Blankophor® (Bayer, Leverkusen, Germany, 0.25 mg/mL in 20% KOH) by means of fluorescence microscopy at 420 nm.

In some cases, non-specific bands were observed on the agarose gel, especially in the *Aspergillus* PCR; therefore, we considered positive for aspergillosis only the cases with products matching the 135 bp fragment of the control strain and sequencing of the PCR products was not considered necessary for confirmation of our results. Sequencing from the paraffin block was performed however in one case (by a collaborating laboratory), and confirmed the identification of *Aspergillus flavus* (case 15, Table 1), which also grew in culture.

Results

Twenty cases with histological evidence of the presence of fungal hyphae in tissues were found during an 8-year period, i.e. between 2005 and 2012 included. Demographic and clinical data of the patients are shown in Table 1. Culture data were available in 9 cases, only four of which had an *Aspergillus* species or Mucorales grown (Table 1). In the remaining 5, other types of fungi were grown: 1 *Bipolaris* spp., 1 *Scedosporium apiospermum*, and 3 *Candida* spp. After the use of the molecular method, mucormycetes were detected in 9 out of 20 (45%) and an *Aspergillus* species in 8 out of 20 (40%) cases. In two cases PCR was positive for both mucormycetes and *Aspergillus*; one had two types of fungal hyphae identified on histological examination (case No. 3, Table 1, no culture data available), whereas in the second case (No. 8, Table 1) only hyphae characteristic for mucormycetes were observed (broad, ribbon-like, aseptate hyphae with right angle branching); however culture was positive for both *R. arrhizus* and *A. fumigatus* (Table 1). The 5 cases with other types of fungi had a negative PCR. Of the 9 patients with mucormycosis, 5 had an underlying hematological condition, 3 had diabetes and one had previously undergone a knee surgery (Table 1). All 6 patients with aspergillosis as the only diagnosis had either an underlying comorbidity or sinus aspergilloma (Table 1).

Overall, 30 FFPE samples from the above mentioned patients, i.e. one to 5 per patient (Table 1), were examined with the semi-nested PCR. Seven samples tested negative despite microbiological or histological evidence of disease. However, at least one sample of each patient tested positive for either of the two fungi. In one representative experiment, concerning patient No. 8, one sample was positive and another one negative (columns 5 and 6, Fig. 1), in spite of the presence of hyphae in the histological examination. In the same experiment, fresh frozen tissue from another patient used for comparison was positive whereas fresh tissue from the same patient after surgical debridement was negative (columns 10 and 11, respectively, Fig. 1). Both direct microscopy with Blankophor® and histology were also negative in the post-surgical debridement sample, indicating successful therapy. Based on the results of our experiments the sensitivity of the method in our setting was 79.3% (95% CI: 60.3–91.9%) with a 100% specificity (95% CI 47.5–100%).

Discussion

Our study demonstrates the successful use of a semi-nested PCR [13] for the diagnosis of Mucorales and *Aspergillus* spp in FFPE tissue. Such tissue, beyond its role in establishing the histological diagnosis, may serve as a valuable, readily available archived material for molecular fungal testing due to the acceptable conservation of DNA. Several specific molecular methods have been developed for the detection of fungi in FFPE tissue, quantitative (real-time PCR) and real-time-PCR-based multiplexed molecular methods among