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Enhanced fungal DNA-Extraction from Formalin fixed, paraffin embedded tissue specimens by application of thermal energy

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Summary

Determining the etiology of invasive fungal infections (IFI) is critical for patient management as fungi vary in their susceptibility to antifungals. However, the etiology remains obscure in many cases due to negative culture results. The identification of fungal DNA from pathology blocks by PCR and sequencing is an alternative approach to determine the etiology of IFI. Previous studies identified fungal DNA in only 50% of samples with positive histopathology, probably due to DNA damage by the tissue fixation.

We used realtime PCR to quantify human and fungal DNA from Formalin-fixed, paraffin embedded tissue specimens in order to study the effect of thermal energy during extraction on the yield of amplifiable DNA and subsequent identification of fungal DNA.

Tissue sections from eight patients with proven IFI were subjected to DNA extraction with varying exposure to thermal energy. Amplifiable DNA increased up to 76-fold by increasing incubation temperature from 65°C to 90°C. An additional increase was documented by incubation for up to 6 hours at 90°C. The augmented amplification of fungal DNA was associated with improved species identification by sequencing of the PCR amplicons. This may help illuminate the etiology of IFI and thereby improve patient management by guiding antifungal therapy.

Introduction

The identification of fungal pathogens is of major importance for the management of patients with invasive fungal infections (IFI), as different fungi have different *in vitro* susceptibilities to antifungal agents. Standard techniques, such as culture and histopathology fail to identify causative fungi in many patients, limiting our knowledge of the etiology of IFI´s [1,2]. The failure to identify fungi to the species level leads to suboptimal empiric antifungal therapies, and management of patients can be particularly challenging with progressive disease under standard therapy.

Molecular methods are an attractive approach to enhance the diagnosis of fungal infections, such as through PCR and sequencing of fungal rRNA genes to identify fungi in formalin fixed, paraffin embedded (FFPE) tissue specimens. These samples are stored in pathology archives and involved tissue can be assayed to reveal the epidemiology of fungal infections over many years, if the DNA can be retrieved. However, DNA amplification from FFPE specimens can be challenging as evidenced by lower rates of fungal identification compared to fresh tissue samples [3,4]. Modification of DNA during fixation is thought to explain the poor performance of PCR. Several techniques have been studied in order to reverse DNA modification by the fixation procedure. Applying thermal energy to samples during DNA extraction increased the amount of amplifiable human DNA from FFPE tissue specimens [5]. Fungi with their thick cell walls may impede the penetration of fixatives or water and change this dynamic.

We studied the impact of the application of thermal energy during DNA extraction on the amplification of fungal DNA from FFPE tissue samples obtained from patients with proven IFI. Subsequent fungal identification was performed by sequencing the amplicons produced by broad-range fungal PCR assays.

Materials and Methods

The study was approved by the institutional review board of the Fred Hutchinson Cancer Research Center (track number 5841, substudy 18) and the University Hospital Frankfurt (Main), Germany (track number 375/10).

Patient samples: Tissue samples from patients with proven IFI, e.g. with documentation of fungal elements in tissue samples, were identified in the tissue bank of the University Hospital Frankfurt. Culture results of the samples were not available. FFPE tissue blocks from eight patients were studied (**table 1**). Aliquots of four sections, 5 µm each, were cut from tissue blocks and placed in Eppendorf Biopur® tubes (Eppendorf AG, Hamburg, Germany) for DNA extraction. The first and the last section from each block were placed on slides and stained by Grocott´s methenamine silver stain to document fungal structures.

DNA Extraction: After paraffin removal using octane, DNA was extracted using the MasterPure™ Yeast DNA Purification Kit (Epicentre® Biotechnologies, Madison, WI) with an additional bead beating step to optimise fungal cell lysis as described previously [6]. This kit utilizes a non enzymatic approach to lyse fungal cell walls at 65°C. Contaminating macromolecules were then removed by a precipitation step, followed by DNA precipitation with isopropanol for purification of DNA. Extracted DNA was dissolved in 75 µl of 0,5% Triton X and stored at -20°C until PCR testing.

In order to evaluate the effect of thermal energy on the amount of amplifiable DNA, two modifications of the previously described protocol were studied. First, the initial incubation step in lysis buffer was carried out at either 65°C, as proposed by the manufacturer, or at 90°C for one hour with aliquots of all samples. Second, the duration of incubation at 90°C was increased from one hour to three, six and twelve hours using the samples 1,4,6 and 7 in duplicate extractions.

Quantification of DNA: Real-time PCR was performed with an Applied Biosystems 7500™ instrument. Human DNA was quantified using a PCR assay targeting a 155 base pair (bp) segment of the 18S rRNA gene as previously described [7]. A standard curve was generated using human genomic DNA (Roche, Indianapolis,IN) ranging from 1 to 10000 pg. An internal amplification control was carried out in a separate reaction to rule out inhibition of the PCR reaction as previously described [7].

Fungal DNA was quantified using two broad-range fungal PCR assays using EvaGreen® (Biotium Inc, Hayward, CA), a fluorescent double stranded DNA binding dye. Dilutions of extracted genomic DNA from *Candida albicans* (ATCC 90028) and *Aspergillus fumigatus* (ATCC MYA-1163) ranging from 10 fg to 1000 pg were used for quantification of fungal DNA as described previously [6]. The ITS-2 (primers 5.8 forward and 1 reverse) assay amplifies a 210-290 bp segment of rRNA operon, the 28S (primers 10 forward and 12 reverse) assay a 332-346 bp amplicon, depending on the fungal organism [8]. Fungal PCR assays were performed in duplicate. The median of duplicate PCR reactions was used for calculation. A positive fungal PCR was defined as an amplicon with the same peak melting temperature (±1°C) of the dissociation curve in both duplicates before a cycle threshold of 40.

No template controls were used to monitor contamination during PCR setup and in the master mix. Extraction-negative controls, consisting of water were processed in parallel with tissue samples to monitor for contamination during the extraction procedure.

Sequencing of amplicons: The amplicons of fungal PCR positive samples were sequenced using the primer 5.8S forward and 28S 10 forward. Etiologic agents were determined by comparison of the obtained sequences with an internal database as described previously [6]. For the identification of fungi at the species level, a similarity of more than 97% was required.

Statistical analysis: The amount of human and fungal DNA amplified at extraction temperatures of 65°C and 90°C was compared using the Wilcoxon matched pairs signed rank test in GraphPad Prism (Graphpad Software Inc., LaJolla, CA, USA). A p-value below 0.05 in a two sided test was considered significant. The mean of the relative changes in the amount of amplified DNA between 65° and 90° incubation temperature with 95% confidence intervals (CI) were calculated in GraphPad Prism. The median amount of fungal DNA of duplicate qPCR measurements at different durations of incubation at 90°C for both fungal PCR assays from two separate extractions is plotted versus the duration of incubation. Standard errors of the mean were calculated and plotted using GraphPad Prism.

Results

The sampled tissues are described in **table 1**. Histopathology documented fungal elements in the first and last slide of all included samples. Three samples showed yeast cells and septated mould hyphae were documented in five samples.

The median amount of amplifiable human DNA, as detected by the 18S rRNA gene assay, increased from 722 pg at 65°C to 55428 pg/sample at 90°C incubation for one hour (p=0,008) or 76-fold (13-190; 95% CI) (**figure 1**).

The broad-range fungal PCR assays detected fungal DNA in six (ITS-2-assay) and five (28S-assay) of the eight samples after incubation at 65°C for one hour (**table 1**). With the increased incubation temperature of 90°C, seven (ITS-2) and eight (28S) samples were positive for fungal DNA. The mean amount of fungal DNA in samples positive at both extraction temperatures increased from 0.53 to 32.74 pg/sample (p=0.02) as determined by the ITS-2 assay and from 0.13 to 18.07 (p=0.03) by the 28S assay (**figure 1**).

Sequencing of the amplicons identified *Aspergillus fumigatus* in four samples and *Aspergillus flavus, Candida albicans, Candida tropicals* and *Cryptococcus neoformans* in one sample each (**table 1**).

Increasing the duration of incubation at 90°C to three hours further increased the amount of amplifiable fungal DNA in all four samples tested (**figure 2**). While incubation for six hours was not detrimental to the amount recovered, further increase reduced the amount of amplifiable fungal DNA.

All extraction controls were negative for fungal DNA. No inhibition was detected by the internal amplification control, suggesting that the improvements in the yield of amplifiable human and fungal DNA is not explained by differences in inhibition of the PCR reaction.

Discussion

We used quantitative PCR to evaluate the effect of thermal energy on the extraction of DNA from FFPE tissue samples from patients with proven IFI. The amount of amplifiable human and fungal DNA significantly increased, leading to a superior identification of fungi by sequencing of the amplicons of broad-range fungal PCR assays.

Improvements in the amount of amplifiable human DNA from FFPE tissue specimens due to the application of thermal energy have been reported previously by researchers using different extraction protocols. This suggests an underlying mechanism that is not dependent on the extraction protocol [5]. The absence of detectable PCR inhibition in our samples and a dose response effect with increasing incubation duration until three hours at 90°C argue that thermal energy may enhance the liberation of amplifiable DNA from FFPE tissue samples. A reversal of formalin induced crosslinking between DNA and proteins is suggested to be the underlying mechanism [5]. The different magnitude of increase seen with different samples may be explained by variations in the sample processing, such as the time a specimen is incubated in formalin or by different tissue to formalin ratios.

Several factors impacting the extraction of fungal DNA from FFPE tissue specimens from patients with IFI have been described. First, the amplification of fungal DNA appears to differ between fungal pathogens. Whereas the amplification appears to be most successful from samples containing yeasts, samples containing moulds, especially the mucorales appear to do worse [6,10]. Our study includes samples from patients with the most common fungal pathogens, yeasts and moulds. The application of thermal energy appears to increase the amount of amplifiable fungal DNA from both. As we did not study samples from patients with mucormycosis, we do not know if DNA from these or other emerging fungal pathogens behaves differently. In a subsequent study, we used a protocol with 3 hours of incubation at 90°C on a larger sample set. This allowed for the identification of fungal DNA from patients with invasive candidiasis in 10 of 11 cases, with septate mould infections in 8 of 10 cases, and with mucormycosis in 10 of 19 cases [6]. This compares favourably with previous studies using nested PCR assays or larger amounts of tissue to identify fungi from FFPE tissue samples [12,13]. Second, the amplicon length of the PCR assay may have an impact on the yield of amplifiable fungal DNA, as evidenced by a higher number of fungal DNA positive samples at 65°C using the ITS-2 assay with a shorter amplicon, and an increasing positivity of the 28S assay at 90°C, which produces longer amplicons that may be more sensitive to the effects of formalin fixation. This is in accordance with recent studies demonstrating superior amplification of shorter amplicons by fungal PCR assays from FFPE tissue specimens [9,10]. Third, different extraction protocols have been shown to vary in the efficiency of fungal DNA extraction. We used a non enzymatic extraction kit supplemented with additional steps to remove the paraffin and for mechanical lysis of fungal cells. While some commercial extraction kits designed for FFPE tissue samples may provide more convenient handling, adaptations of these kits for fungal DNA extraction require careful optimisation in terms of possible contaminations with fungal DNA, or inhibition of the PCR reaction which may adversely affect outcome [10]. The introduction of a heating step into such assays needs careful optimisation to avoid unintended consequences such as the attenuation of enzymatic lysis steps. In addition, increasing the amount of amplifiable human DNA in samples from patients with proven IFI may reduce the amplification of fungal DNA through dilution of relevant targets relative to non-relevant DNA, and through inhibition of PCR at the highest DNA concentrations [11]. By using broad-range primers designed to amplify fungal DNA in the presence of high levels of human DNA and screening for inhibition of the PCR reaction we

were able to increase the number of samples from which fungal DNA could be amplified and identified by sequencing [8].

Improving the yield of fungal DNA extraction from FFPE tissue samples from patients with proven IFI may allow investigators to target lower copy number genes, such as determinants of acquired *in vitro* resistance and genes needed to distinguish between molecular siblings that share identical ribosomal RNA sequences but differ in *in vitro* resistance to antifungals. This information provides the potential to improve fungal diagnostics and improve our knowledge of the etiology and epidemiology of IFI. Data on species level identification of fungal pathogens will help allow targeting of antifungal therapy, leading to better patient outcomes. Furthermore, empiric use of antifungals needs to be driven by knowledge about the most common fungal pathogens in a clinical setting, and improved PCR assays can be useful for collecting these data.
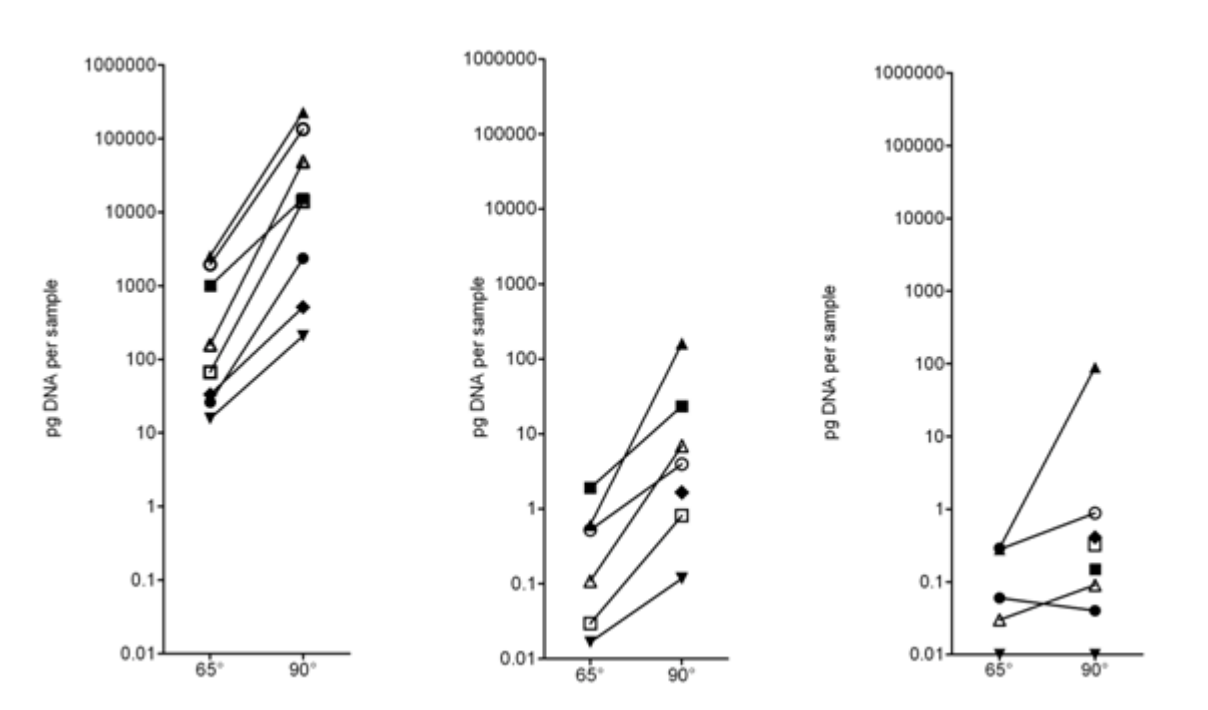
Figure 1. Amount of DNA as detected by three quantitative PCR assays at different incubation temperatures. Open symbols represent samples from yeast infections (sample 4:∆, sample 6: □, sample 2:○), closed symbols from mould infections (sample 1:▲, sample 3:●sample 5:■, sample 7:▼sample 8:♦)
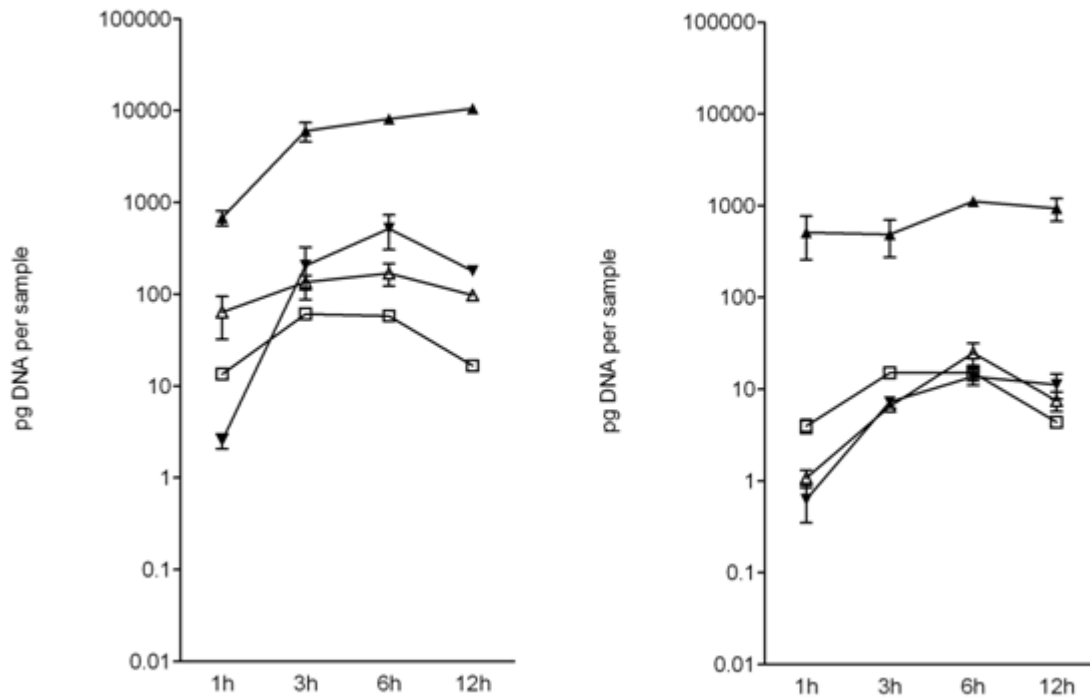
Figure 2. Amount of fungal DNA at different incubation times at 90°C incubation temperature as determined by two broadrange fungal PCR assays (left: ITS-2 assay, right: 28S assay). Open symbols represent samples from yeast infections (sample 4:∆, sample 6: □), closed symbols from mould infections (sample 1:▲, sample 7:▼).

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Disclosure of Conflict of Interests

All authors: No conflict of interest

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