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Development of a novel qPCR, using mitochondrial genes for the detection of mucormycosis

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INTRODUCTION

Mucormycosis is a life threatening infectious disease. In 2021, mucormycetes caused epidemics in COVID-19 survivors in India and Brazil. Globally, incidences of mucormycosis are on the rise due to an increase of at-risk patient cohorts. Still, there is a diagnostic gap to detect and differentiate mucormycosis from other invasive fungal infections. An early diagnosis and early-targeted therapy is essential to improve patient outcome.

Mucormycetes are particularly challenging to diagnose due to their proneness for degradation in clinical samples.

AIM OF THIS WORK

To overcome this problem, we aim to establish robust mitochondrial markers known from forensic sciences. A first dye-based pan-Mucorales qPCR assay on these markers was published by Lackner et al. (2019), based on the *rnl* gene [1].

The goal of this work is to transform the current dye-based assay into a probe-based pan-Mucorales qPCR.

EXPERIMENTAL PROCEDURE

Determination of key characteristics for the pan-Mucorales qPCR assay

SENSITIVITY: limit of detection (LOD) + PCR-efficiency

SPECIFICITY: cross reactivity → fungal and animal DNA

RESULTS - SENSITIVITY

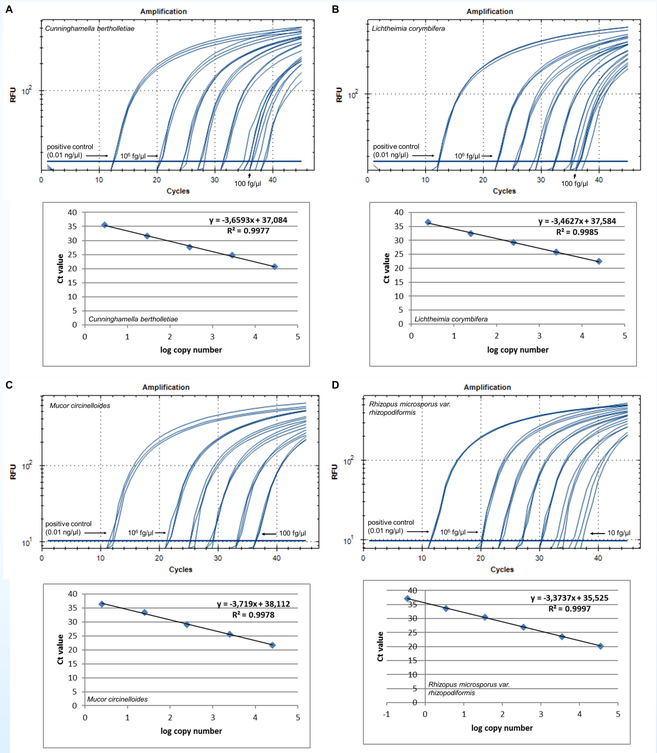

Figure 1: Determination of analytical sensitivity (LOD and genome copies) and PCR-efficiency of the *rnl* qPCR assay for **A)** *Cunninghamella bertholletiae*, **B)** *Lichtheimia corymbifera*, **C)** *Mucor circinelloides* and **D)** *Rhizopus microsporus var. rhizopodiformis*. DNA concentrations of 10^6 fg/µl, 10^5 fg/µl, 10^4 fg/µl, 1000 fg/µl, 100 fg/µl, 10 fg/µl, 1 fg/µl, 0.1 fg/µl, 0.01 fg/µl, 10^{-3} fg/µl, 10^{-4} fg/µl and 10^{-5} fg/µl were used to determine LOD. Ct values were plotted against the log of DNA copies and PCR-efficiency was calculated (Efficiency = 10(-1/slope)).

Table 1: Analytical sensitivity (LOD and genome copies) and PCR-efficiency of the pan-Mucorales qPCR assay for a selection of eight mucoralean strains.

species	LoD	copy number	PCR-efficiency [%]
Cunninghamella bertholletiae	100 fg	2.93	87.62
Lichtheimia corymbifera	100 fg	2.49	94.44
Lichtheimia hyalospora	100 fg	2.74	94.00
Lichtheimia ramosa	100 fg	2.86	95.90
Mucor circinelloides	100 fg	2.49	85.73
Mucor janssenii	1000 fg	25.01	89.25
Rhizopus delemar	100 fg	1.98	89.14
Rhizopus microsporus var. rhizopodiformis	10 fg	0.35	97.88

RESULTS - SPECIFICITY

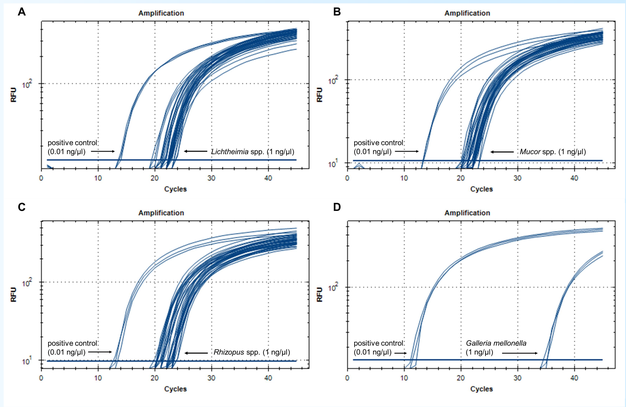

Figure 2: Determination of analytical specificity of the *rnl* qPCR assay by testing cross reactivity with fungal (non-Mucorales and Mucorales) and animal DNA (*Galleria mellonella*, mouse, human). DNA concentration of 1 ng/µl was used. Results for **A)** *Lichtheimia* spp. **B)** *Mucor* spp. **C)** *Rhizopus* spp. and **D)** *Galleria mellonella* are shown.

NEGATIVE AMPLIFICATION

- non-Mucorales → *Candida*, *Aspergillus*, *Cryptococcus*, *Penicillium*, *Fusarium*
- mouse
- human

→ no cross reactivity with non-mucoralean, murine and human DNA

POSITIVE AMPLIFICATION

- Mucorales → *Apophysomyces*, *Cunninghamella*, *Mucor*, *Rhizomucor*, *Lichtheimia*, *Rhizopus*
- *Galleria mellonella*

→ detection of clinically relevant mucormycetes

→ slight cross reactivity with *Galleria mellonella*

CONCLUSION AND OUTLOOK

Concluding from our first set of evaluations, our probe-based pan-Mucorales qPCR was found to successfully detect a wide diversity of clinically relevant mucormycetes. Detected cross reactivity was ruled out *in silico* and confirmed by qPCR. Specificity, amplification efficiency (85.73 % to 97.88 %) and sensitivity (100 fg/µl or 2-3 genome copies) were promising.

Next steps are the evaluation of a novel internal control, which will complete our duplex probe-based pan-Mucorales qPCR assay. The final assay will be evaluated on a comprehensive set of formalin-fixed-paraffin-embedded (FFPE) tissue specimens head to head with qPCRs based on classical-nucleic-markers.

[1] Caramalho, R., Madl, L., Rosam, K., Rambach, G., Speth, C., Pallua, J., … Lackner, M. (2019). Evaluation of a novel mitochondrial Pan-Mucorales marker for the detection, identification, quantification, and growth stage determination of mucormycetes. *Journal of Fungi*, *5*(4). https://doi.org/10.3390/jof5040098