SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Development of high resolution melting analysis based PCR assays for the species level identification of clinically relevant *Aspergillus* and *Candida* fungi

by Gábor Fidler

Supervisor:
Dr. Melinda Paholcsek

University of Debrecen
Doctoral School of Molecular Cell and Immune Biology
2021

Development of high resolution melting analysis based PCR assays for the species level identification of clinically relevant *Aspergillus* and *Candida* fungi

by Gábor Fidler MSc

Supervisor: Melinda Paholcsek, PhD

Doctoral School of Molecular Cell and Immune Biology, University of Debrecen

Head of the **Examination Committee**:	Gábor Szabó, PhD, DSc
Members of the Examination Committee:	Margit Balázs, PhD, DSc
	Katalin Gombos, PhD

The Examination took place at the Lecture Hall of In Vitro Diagnostics Building, Faculty of Medicine, University of Debrecen
July 15, 2021, 12:00

Head of the **Defense Committee**:	Gábor Szabó, PhD, DSc
Reviewers:	László Kredics, PhD
	Renátó Kovács, PhD
Members of the Defense Committee:	Margit Balázs, PhD, DSc
	Katalin Gombos, PhD

The PhD Defense takes place at the Lecture Hall of In vitro Diagnostics Building, Faculty of Medicine, University of Debrecen
July 15, 2021, 13:00

Introduction

Both the invasive diseases caused by opportunistic human pathogenic fungal species and the growing number of resistant fungal strains are a worldwide concern, killing more than 1.6 million people each year. High-risk groups include those with hematopoietic malignancies, stem cell transplants and HIV infected patients. These systemic fungal infections occurring as a complication of already severe underlying diseases, significantly worsen patients' life expectancy without timely initiated effective antifungal therapy and increase the duration and cost of hospital care.

The main causes of invasive mycoses are the fungal species *Aspergillus fumigatus* and *Candida albicans*, however, as a consequence of a more frequent use of immunosuppressive therapeutic drugs, broad-spectrum antibiotics, and other factors such as climate change, the incidence of other members of the two genera is increasing. In addition to this growing trend, it is worrying that fungal strains that have recently appeared in the clinic have been shown to have different virulence and antifungal resistance profiles. For this reason, antimycotic-resistant nosocomial epidemic outbreaks have already been reported in the United States and South American regions, with very high mortality among patients at risk. With all this in mind, species-level diagnosis and resistance screening are the two key factors, which can contribute to the selection of the appropriate antifungal agent. Primary prophylaxis and empirical therapeutic strategies may reduce mortality, however, there is a possibility that patients will be unnecessarily burdened with severely cytotoxic antifungal drugs. Early recognition of invasive fungal infections due to often non-specific symptoms (cough, chest pain, fever) is a challenge. The most appropriate clinical indicators of invasive mycoses include neutropenic fever that does not persist with antibiotic treatment. By definition, this means that body temperature stays above 38°C consistently and the neutrophil count falls permanently below the limit of 500 cells/microliter.

The imaging methods used in routine fungal diagnostics are not specific, so it cannot be stated with high certainty that the visual lesion is due to a fungal infection. Cultivation of fungi is time consuming and distinguishing between closely related species due to overlapping morphological features is also a challenge. Although molecular antigen-based methods allow for rapid and sensitive diagnosis, neither the molecular "gold standard" galactomannan ELISA developed to detect *Aspergillus* infections nor the panfungal 1-3-β-D-glucan tests provide species-level results. Ribosomal RNA coding genes (*18S, 28S*) and the internal transcribed spacer (*ITS*) regions between

them are most commonly used for genetic identification of fungal species. The *ITS* genes are suitable for discriminating the *Aspergillus* subgenus, but may allow much more accurate species identification within the different *Aspergillus* species sections if protein encoding, partially conserved genes such as *beta-tubulin*, *actin*, or *calmodulin* are used. In addition to next-generation sequencing, polymerase chain reaction (PCR) – based methods can also be used for diagnostic purposes, but due to the high degree of heterogeneity of assays and protocols developed by different laboratories, standardization remains unsolved. As a result, the routine use of these technologies in fungal diagnostics is still a challenge, moreover commercially available PCR test mostly focus only on the detection of major fungal pathogens (*Aspergillus fumigatus*, *Candida albicans*) at the species level.

Taking into account the factors discussed above, there is a need in clinical mycology to develop nucleic acid-based diagnostic methods that can determine a wide range of pathogens belonging to the genera *Aspergillus* and *Candida* at the species level. The high resolution melting (HRM) PCR method was originally developed for the detection of genetic variants, but nowadays it is increasingly used for the genetic identification of various bacteria, viruses and fungi. Based on literature data, the vast majority of fungal diagnostic HRM PCR tests are also designed to detect *ITS* genes. The main aim of our research was to develop and optimize HRM PCR assay that allow simple, and cost-effective discrimination of several clinically relevant *Aspergillus* and *Candida* fungal species based on sequence differences in fungal *β-tubulin* genes.

Aims

Our primary aim was to design and optimize nucleic acid PCR assay based on high-resolution melting curve analysis to identify a number of clinically important fungal species belonging to the genera *Aspergillus* and *Candida*.

In connection with this, we have set the following goals:

- *In silico* validation of primers specific for *β-tubulin* genes designed to detect *Aspergillus* and *Candida* fungal species, examining possible cross-reactions based on sequences from public reference databases.
- Setting HRM PCR reaction parameters: determination of optimal $MgCl_2$ concentration, primer concentration and annealing temperature to maximize PCR reaction efficiency.
- Validation of the PCR amplicons by sequencing and agarose gel electrophoresis.
- Determination of melting temperature ranges specific for *Aspergillus* (*A. fumigatus*, *A. lentulus*, *A. terreus*, *A. flavus*, *A. niger*, *A. welwitschiae*, *A. tubingensis*) and *Candida* (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. dubliniensis*) fungi.
- Measurement of PCR efficiency: determination of the range of analytical sensitivity (Limit of detection: LOD) and reliable species-level identification (Limit of identification: LOI).
- Investigation of assay reproducibility, determination of iner- and intra-assay variance.
- Experimental study of assay cross-reactivity with other clinically relevant microorganisms.
- Clinical validation of the assays is one of our future plans. Related to this is the development of a user-friendly software that allows quick and easy evaluation of the PCR results for clinical laboratories.

Materials and Methods

Reference strains and clinical isolates

During the method optimization, a total of 104 fungal and 16 bacterial strains were examined. The clinical isolates were gathered from the microbiological collections of Szeged and Debrecen, and the reference strains were ordered from different international microbiological collections: Fungal Genetics Stock Center (FGS); Centraalbureau voor Schimmelcultures, Fungal and Yeast Collection (CBS); Northern Regional Research Laboratories (NRRL); American Type Culture Collection (ATCC). Of the molds, a total of 43 clinical isolates and 6 reference strains of *Aspergillus* were tested, and for yeasts we examined 38 clinical isolates and 5 reference strains of *Candida* fungi. Possible assay cross reactions were studied with *Fusarium, Scedosporium, Lichtemia, Rhizopus* fungal species and various Gram-positive (*Staphylococcus, Enterococcus*) and Gram-negative (*Enterobacter*) bacteria found in clinics. The cultivation of microorganisms was carried out in cooperation with the Department of Microbiology of the University of Szeged and the Department of Microbial Biotechnology and Cell Biology of the University of Debrecen.

Cultivation methods

Aspergillus fungal strains were first plated on standard minimal nitrate medium and then, after spore formation, the spores were inoculated into liquid minimal medium. Cultivation temperatures of 37°C for *Aspergillus fumigatus* and *Aspergillus niger*, 25°C for *Aspergillus lentulus, Aspergillus terreus, Aspergillus flavus, Aspergillus tubingensis* and 30°C for *Aspergillus welwitschiae* were used. Cultures were grown for 18 h with 220 rpm shaking. *Candida* fungal strains were cultured for two days in yeast peptone D-glucose medium. Bacterial species were grown on Müller-Hinton agar medium under aerobic conditions.

Preparation of spore suspension solutions

Concentrated spore suspensions stock solutions were prepared from liquid cultures of *Aspergillus* and *Candida* fungal strains. Spore counts were determined with a Bürker chamber cell counter. From this solution, a 7-fold 10x serial dilution (10^6-1 spores/ml) was prepared with sterile nuclease free water (Ambion™, Thermo Fisher Scientific, Maryland, USA).

Cell lysis

Green Beads ceramic beads (Roche Diagnostics, Risch-Rotkreuz, Switzerland), and MagNA Lyser instrument (Roche Diagnostics, Risch-Rotkreuz, Switzerland) were used for mechanical lysis of fungal cells. The lysis was performed at 5000 rpm, 50 second parameters. For bacterial cell lysis a milliliter log phase bacterial culture containing 10^8 cells was used after the cells was harvested by centrifugation. For Gram-positive bacteria, the cell wall was digested with a lysis cocktail containing lysozyme enzyme (20 mg/ml enzyme in 20 mM TrisHCL, pH 8.0, 2mM EDTA, 1.2% TritonX100).

Genomic DNA extraction

For pilot experiment and cross reactivity studies, genomic DNA was isolated with the Masterpure™ Yeast DNA purification kit (Epicentre Biotechnologies, Madison, USA) for fungi and for bacteria, DNA extraction was performed with the E.Z.N.A.® Bacterial DNA Kit (Omega Biotechnologies, Norcross, Georgia, USA) according to the manufacturer's instructions.

For HRM PCR experiment, the High Pure Viral Nucleic Acid Large Volume (Roche Diagnostics, Risch-Rotkreuz, Switzerland) silica membrane kit was used, that allows working with large starting sample volume (3-4ml), which is an important factor for pathogen detection in blood samples. DNA extraction was performed according to the manufacturer's instructions, with the addition of incorporating a ceramic bead mechanical cell digestion. For the mechanical lysis Green Beads ceramic beads (Roche Diagnostics, Risch-Rotkreuz, Switzerland) MagNA Lyser instrument (Roche Diagnostics, Risch-Rotkreuz, Switzerland) were used at 5000 rpm for 30 seconds. The quantity and quality of the extracted DNA was checked in each case with a NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc., North Carolina, USA). The DNA elute was considered adequate if the 260/280 absorbance ratio of the isolated DNA was >1.6 and the 260/230 ratio was at least 1.8.

Aspergillus and *Candida* HRM primers

We searched for potential melting domains in annotated *Aspergillus* and *Candida β-tubulin* gene sequences (*benA*, *TUB2*) available in public databases (EMBL/GeneBank). Multiple sequence alignment was performed with Clustal Omega software. Conserved gene regions of 30-40 bases in

length covering the variable regions were chosen as the primer adhesion site, allowing universal detection of several *Aspergillus* and *Candida* fungal species during HRM analysis.

Optimization of PCR reaction parameters

PCR reactions were performed using LightCycler® 480 High Resolution Melting Master mix (Roche Diagnostics, Risch-Rotkreuz, Switzerland) containing saturating double-stranded DNA intercalating LightCycler® 480 ResoLight dye. For HRM PCR reaction conditions were optimized, with reference fungal strains of *Aspergillus fumigatus* (AF293) and *Candida albicans* (ATCC 10231) on LightCycler® 96 (Roche Diagnostics, Risch-Rotkreuz, Switzerland) real-time quantitative PCR instrument. The *Candida* HRM PCR assay was also optimized for LightCycler® 2.0 and LightCycler® Nano (Roche Diagnostics, Risch-Rotkreuz, Switzerland) PCR instruments. The following parameters were set to achieve adequate PCR reaction efficiency:

- Primer concentration: HRM primers were tested in a final concentration range of 0.2-1.5 μM.
- Annealing temperature: Gradient PCR reactions were performed in the temperature range of 55-72°C in order to determine optimal primer adhesion.
- Determination of optimal $MgCl_2$ concentration: Calibration was performed in the concentration range of 1-3.5 mM according to the manufacturer's instructions.

In the extension phase of the amplification, endpoint measurements are performed to quantify the amount of amplicons and fungal DNA in the sample. The melting curve analysis is performed after amplification, and the release of the fluorescent dye and the denaturation of the amplicons are monitored by continuous heating of the reaction medium. The results of the *Aspergillus* experiments were evaluated exclusively with LightCycler® 96 software (Roche Diagnostics, Risch-Rotkreuz, Switzerland) and for the *Candida* experiments with the dedicated evaluation program of the current PCR instrument (LightCycler® 96, LightCycler® 2.0, LightCycler® Nano).

Monitoring PCR inhibition (SPUD assay)

For controlling PCR inhibition we used a SPUD external inhibition assay designed by Nolan *et al.* (2006). This assay was originally developed to detect the *Solanum tuberosum* (potato) *phyB* gene (GenBank ID: Y14572). The SPUD assay was optimized for the PCR temperatures used in

Aspergillus and *Candida* HRM detection, but in a separate reaction from species identification to avoid interference. For the PCR reactions we used 0.4 μM primer concentration and the determined optimal SPUD template concentration was 200,000 copies, for which the average number of PCR threshold cycles was 21.68 ± 0.29. The melting temperature of the SPUD amplicons was 80.8°C.

Experimental validation of the HRM amplicons

Conventional PCR reactions were performed in the presence of 20 ng of DNA isolated from *Aspergillus* reference strains. Amplification was performed using Phusion High-Fidelity PCR Master mix (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. The size of the resulting amplicons was determined by gel electrophoresis (1% agarose gel in Tris-acetate-EDTA (TAE) buffer) and ethidium bromide dye was used for DNA staining. A voltage of 90V and a current of 55 mA was set on the rectifier during the reaction. The DNA fragment distribution on the agarose gel was examined with UV transilluminator. The resulting DNA bands were excised from the gel using a sterile scalpel, and DNA was recovered from the gel pieces using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After that the DNA molecules were purified with SigmaSpin™ (Sigma-Aldrich, Missouri, USA) according to the manufacturer's instructions. Capillary sequencing was performed with the BigDye® Terminator v3.1 Cycler Sequencing Kit (Thermo Fisher Scientific, Maryland, USA) according to the manufacturer's instruction on an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, USA) in both directions.

In the case of *Candida* strains conventional PCR reactions were performed on LightCycler® Nano (Roche Diagnostics, Risch-Rotkreuz, Switzerland) in the presence of 20 ng genomic DNA. Amplification was performed using Phusion High-Fidelity PCR Master mix (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. On the PCR instrument our previously determined HRM temperature protocol was set. The size of the resulting amplicons was determined by gel electrophoresis (2% agarose gel in Tris-borate-EDTA (TBE) buffer) and ethidium bromide dye was used for DNA staining. A voltage of 90V and a current of 55 mA was set on the rectifier during the reaction. The DNA fragment distribution on the agarose gel was examined with UV transilluminator.

Applied statistical methods

Characterization of PCR reaction kinetics, determination of dynamic ranges

PCR measurements and linear regression analysis were performed on *Aspergillus* and *Candida* genomic DNA panels (10^6-1 GE/PCR reaction). The Cq values obtained were plotted in relation of DNA template concentration and line was fitted on the points. From the equation of this line, the slope and R^2 value was determined, which is used to investigate whether the amount of amplicons increases exponentially per cycle. If true, this variable assumes a value of 1, otherwise the amount of amplicons does not increase exponentially per cycle, which may be due to the presence of inhibitors in the sample. From the slope of the line, the PCR efficiency can be calculated with the following equation:

$$\text{Efficiency}\ (\%) = (10^{\frac{-1}{\text{slope}}}\text{-1}) \text{ x } 100$$

During the experiments, we determined the limit of detection (LoD), ie the smallest amount of fungal genomic DNA that our method can detect. It may happen that, although amplification occurs, the amount of DNA in the sample is not sufficient to identify the fungal species with HRM analysis, so we have determined the smallest amount of DNA at which the species can be clearly identified by the melting curve and peak (LoI).

Investigation of intra- and inter assay variance

In the experiments, *Aspergillus* and *Candida* genomic DNA PCR panels were assembled, which represented the different fungal strains in three technical replicates. Measurements were made on a LightCycler® 96 (Roche Diagnostics, Risch-Rotkreuz, Switzerland) instrument and the coefficient of variation (CV) was determined from the Cq values obtained. In the case of PCR assays, two types of coefficients can be distinguished: a) intra- and b) inter assay variance. The first shows the technical variance between sample replicates, i.e. within a PCR panel, which should optimally not exceed 10%. The latter, on the other hand, expresses the variance between the different PCR runs, i.e. the reproducibility of the measurement, which is optimally below 15%. The coefficient of variation (CV%) can be determined by averaging the standard deviations between the measurements.

Results

Determined optimal HRM PCR reaction parameters

The HRM PCR reaction parameters were optimized on *A. fumigatus* (Af293) and *C. albicans* (ATCC 10231) reference strains, respectively.

At the optimal $MgCl_2$ concentration, the amplification curves sowed a lower number of cycles with a higher plateau, while the fluorescence intensity of the melting peaks was as high as possible. For the *Aspergillus* HRM assay, 3 mM $MgCl_2$ proved to be the most appropriate. In the case of the *Candida* HRM assay, we also obtained the highest peak in the presence of 3.5 mM $MgCl_2$, however, amplification was the most efficient at 2 mM $MgCl_2$ because we obtained the lowest number of cycles, so it was ultimately optimal.

Regarding the annealing temperature, the amplification curves were also authoritative. In case of the experiments with *Aspergillus* fungal species, the annealing temperature of 58°C seemed to be the optimal, but cross-reaction was observed with other fungal species, so it was raised to 62°C.There was less than 1 cycle difference between the two annealing temperatures, so this change had no significant effect on amplification efficiency. In addition, the pattern of the melting curves was almost identical in both cases, so we assume that no significant difference can be measured in the melting peaks either. The annealing temperatures tested with the *Candida* HRM assay showed close amplification curves in the 55-68°C, however, cross-reaction was observed at 55-58°C, so a 60°C annealing temperature proved to be appropriate. No differences were found in the melting curves obtained during the HRM analysis as observed in the *Aspergillus* HRM assay.

The efficiency of the *Aspergillus* assay was best when primers with a concentration of 0.2-0.2 μM were used in a 1:1 ratio. For the *Candida* assay, a primer concentration of 0.4 μM was optimal.

Validation of HRM PCR amplicons with defined reaction parameters

Specific binding of *Candida* HRM PCR primers to the target region was confirmed by agarose gel electrophoresis. The gel image yielded a well-defined DNA band for all seven *Candida* species tested and ranged in size from to 311 bp fragment length, similar to the amplicon lengths determined *in silico.* Non-specific products could not be seen.

In the *Aspergillus* HRM PCR reaction, two *β-tubulin* gene segments were amplified simultaneously with two primer pairs (ASP1, ASP2), therefore we considered it reasonable to determine the sequence of PCR products belonging to the *Aspergillus* species detected by the method. This was done by capillary sequencing. The mean length of DNA segments amplified with ASP1 primers was 139.5 ± 1.5 base pairs, depending on the *Aspergillus* species detected. The estimated melting temperature of the amplicons produced during the PCR reactions ranged from 83.8 to 85.3°C. The average length of the PCR products observed for the ASP2 primers was 132± 5.97 base pairs and their estimated melting temperatures ranged from 79 to 82.2°C. The determined sequences were identical to the reference sequences found in the NCBI database, thus confirming that the desired *β-tubulin* gene regions were amplified specifically for both primer pairs during PCR reactions. Based on our results, we concluded that the target sequences belonging to the studied *Aspergillus* species are sufficiently diverse and their melting temperature differs sufficiently to distinguish the *Aspergillus* fungi at the species level by HRM PCR.

Characterization of *Aspergillus* HRM PCR melting curves

During HRM analysis, non-overlapping species-specific heteroduplex melting peaks characteristic of ASP1-ASP2 amplicons were identified. Based on melting temperatures and peak distances, we are able to distinguish the fungal species *A. fumigatus*, *A. lentulus*, *A. terreus*, *A. flavus*, *A. niger*, *A. tubingensis* and *A. welwitschiae*. The ASP2 melting domain is characterized by a lower fluorescence intensity and lower melting temperature (81-85.60°C) compared to the ASP1 melting domain (85.61-89°C). The complete melting spectrum (81-89°C) characteristic of *Aspergillus* species can be divided into six melting clusters, three of which include the melting peaks of the ASP1 amplicons (Tm: 85.61 – 89°C) and the other three clusters include the melting peaks of the ASP2 amplicons (Tm:81.00 - 85.60°C). The characteristic temperature ranges for each cluster are as follows: cluster_1 (81.00-82.72°C); cluster_2 (82.73-83.61°C); cluster _3 (83.62-85.60°C); cluster _4 (85.61-87.10°C); cluster _5 (87.11-87.64°C); cluster _6 (87.65-89.00°C). Based on the combination of ASP1-ASP2 clusters, seven unique two.digit codes can be assigned to each *Aspergillus* species to distinguish them: *A. fumigatus* (1,4), *A. lentulus* (3,4), *A. terreus* (3,6), *A. flavus* (3,5), *A. niger* (2,5), *A. wewitschiae* (1,5) és *A. tubingensis* (2,7).

We also examined the ASP1-ASP2 melting domains in relation of the heteroduplex melting peak distances. Two large groups could be distinguished. The first group included the species *Aspergillus fumigatus*, *A. niger*, *A. welwitschiae*, whose points describing the melting peak temperatures were visibly well separated from each other. The second group consisted of the species *Aspergillus terreus*, *A. flavus* és *A. lentulus*, with a smaller distance between them. For the *A. lentulus* some salient values overlapped with *A. flavus*. This may have been due to measurement error because the center of the *A. lentulus* group was clearly separated from the *A. flavus* values.

Characterization of *Candida* HRM PCR melting curves

Based on our results it can be concluded that the *Candida* HRM PCR assay is suitable for the genetic identification of seven *Candida* species based on the melting temperature differences between amplicons. The lowest melting points was observed for *Candida dubliniensis*, followed by *C. tropicalis, C. albicans, C. krusei, C. parapsilosis, C. guilliermondii és C. glabrata* in ascending order. The kinetics of the normalized melting curves determined during HRM analysis and the melting peaks proved to be species-specific, this result was also supported by the difference graph. The *Candida* HRM assay was optimized for three different PCR instruments (Roche LightCycler® 96, Roche LightCycler® Nano, Roche LightCycler® 2.0). Species-specific *Candida* melting patterns were reproduced on all three PCR platforms. Due to the technological differences between the devices, a negative (LightCycler® 2.0) or positive (LightCycler® Nano) temperature shift was observed, so it was necessary to recalibrate the reference values determined during HRM analysis.

Assay cross-reaction with non-target microorganisms

In the presence of human genomic DNA, no cross-reaction was observed in any of the HRM assays, however, when examining the 11 non-*Aspergillus* and *Candida* fungal strains and 16 bacteria, we made the following findings:

1. *Aspergillus* HRM PCR assay: *Candida* type strains (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. dubliniensis*) showed poor false positivity, however, despite the high DNA template concentrations amplification efficiency was also low (Cq>38). During HRM analysis, the kinetics of the melting curves for the *Candida* species were different than usual and the melting temperature of the amplicons was lower than the

temperatures measured for *Aspergillus* species (Tm <81°C). For *Aspergillus udagawe* and *A. viridinutans*, only one melting peak was obtained (Tm: 85.38-85.41°C; 86.12-86.17°C). In the case of more *Fusarium* strains HRM analysis resulted one melting peak as well: *F. napiforme* (Tm: 87.61-87.68°C), *F. delphinoides* (Tm: 87.60°C), *F. verticillioides* (Tm: 87.81-87.90°C), *F. oxysporum* (Tm: 87.30-87.56°C), *F. solani* (Tm: 87.44-87.57°C), *F. incarnatum* (Tm: 86.31-86.44°C). For the two *Scedosporium auranticum* isolates, an amorphous melting peak was also seen (Tm: 84.11-84.22°C).

2. *Candida* HRM assay: When examining assay cross-reactions with the genus *Aspergillus* positive reactions were seen in the case of *A. fumigatus*, *A. lentulus*, *A. viridinutans* and *A. udagawe*. Their melting temperatures were above the range typical for *Candida* species (Tm: 86.35±0.16°C), moreover, the obtained melting peaks were not species-specific, and overlapped with each other. Amorphous melting were obtained for *Rhyzopus oryzae* (Tm: 77.38°C) and *Scedosporium auranticum* (75.73°C), and very low amplification efficiencies were observed even in the presence of large amounts of DNA.

We did not find any cross-reaction with any of the bacterial strains we tested (*Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Enterococcus hirae, Enterobacter aerogenes, E. gergoviae, E. cloacea*).

Analytical sensitivity and reliability of the HRM assays

Dynamic ranges, limit of detection (LoD)

For *Aspergillus* reference strains, the lower limit of detection was 3pg DNA (LoD: 10^2 GE) overall. An expection to this was the *Aspergillus tubingensis* gDNA panel (LoD: 10 GE), however, during HRM analysis, only one melting peak was obtained in this case, so at such a low concentration, species identification is unreliable because of the absence of heteroduplex melting peaks. The efficiency of the PCR reactions was determined by linear regression analysis. Tje slope of the obtained regression lines ranged from -3.1714 to -3.6281, depending on the species. The R^2 value was greater than 0.99 for each *Aspergillus* species. The efficiency of the reactions (E) was highest for the *Aspergillus fumigatus* and *Aspegillus lentulus* (E: 2.07; 2.05) and lowest for *A. flavus* (E: 1.89).

The sensitivity of the *Candida* HRM PCR assay was determined on three PCR instruments. The sensitivity of Roche LightCycler® 96 and Roche LightCycler® 2.0 was found to be similar in the case of the *Candida* reference fungal strains we tested, but the Roche LightCycler® Nano showed reduced sensitivity for several *Candida* species. In case of *Candida albicans*, *C. glabrata*, *C. tropicalis* és *C. krusei* species, the lower limit of detection was 10 CFU/ml (0.2 GE/PCR reaction) regardless of the PCR instrument used. Overall, lower sensitivity was observed for *Candida parapsilosis* (100 CFU/ml). In the case of *Candida guilliermondii* and *C. dubliniensis*, different sensitivities were observed on the PCR platforms. The efficiency of the PCR reactions exceeded 1.9 in almost all cases, except for *Candida guilliermondii* on the LightCycler® 96 and LightCycler® 2.0 platforms.

Limit of identification (LoI)

At low template DNA lavels, no significant melting temperature shift was observed for any of the HRM PCR assays. The *Aspergillus* showed a species-specific heteroduplex melting peaks patterns up to 10^2 GE, but in presence of lower amount of template DNA, a decrease in the fluorescence intensity of the ASP1 melting peak was observed. When examining the *Candida* HRM PCR assay, no DNA template concentration dependence was observed in the melting peaks, and no temperature shift was detected. The lower limit of species identification (LoI) was the same as the values determined during the analytical sensitivity test for the assay.

Reliability, intra- and inter assay variance of HRM PCR methods

The reproducibility of melting peaks determined by *Aspergillus* HRM analysis was examined on three clinical *Aspergillus* genomic DNA panels. The intra assay variance calculated from the cumulative mean of the melting temperatures ASP1-ASP2 of the technical replicates was less than 4% for all seven *Aspergillus* species. The largest variance was observed in the melting temperature of *Aspergillus welwitschiae* (3.15-3.19%). The inter assay varaincies were also very low (0.01-0.06%). The overall variancy calculated based on the mean values of the variancies proved to be 2.48%. The reliability of the *Candida* HRM assay was examined with four clinical *Candida* gDNA panels. For melting peaks associated with clinical isolates, the veariance in both technical replicates within a single PCR run and the melting temperatures of the species proved to be low (0.06-0.21%). We investigated the veriance of the melting temperatures of seven *Candida* reference strains in the

presence of different amount of DNA templates (7.5ng-75fg/10µl). The standard deviation was less than 1% in all cases, and no significant temperature shift was measured even in the lower DNA concentration ranges. The calculated inra assay variancy on these panels was between 0.01-0.2% and the overall variancy was also acceptable (0.11%).

The MycoAnalyzer software

An online application (MycoAnalyzer) has been developed that allows user-friendly and fast evaluation of the HRM PCR results. After registering the Tm and Cq values, the program automatically performs the identification of the *Aspergillus* or *Candida* fungal species in the sample based on the pre-installed reference values. For the *Candida* HRM assay, there is an option to select the PCR instrument (LightCycler® 96, LightCycler® 2.0, LightCycler® Nano), in which case the application takes the temperature shifts into account, and automatically corrects the reference values for the type of PCR instrument selected. It is possible to register any number of samples to which unique IDs can be assigned. The analysis will start after pressing the „Submit" button, and the identified species name will be written in the „Result" column. In case of successful species identification the row of the sample will be highlighted with green, otherwise if the Tm values are out of range the row collor will be chamged to red, and „Not identified *Aspergillus*/*Candida* species" text will be appeared in the „Result" column. In case of high cycle values (Cq>40), the sample row warns the user with a yellow color code.

Discussion

Rapid and reliable species-level identification of human pathogenic fungi is still a major challenge for microbiological diagnostic laboratories. Characterization of fungal cultures based on phenotype or biochemical signatures is a time-consuming and complex task, so there is a great need in this field for the development of new, rapid and species-specific molecular diagnostic methods. In fungal diagnostics time is a key factor, as mortality associated with candidemia for example can increase by as much as 20% in the 12 hours after infection if antifungal treatment is not initiated.

A major percentage of invasive mycoses are still associated with the genera *Aspergillus* and *Candida*, but the incidence of *Aspergillus fumigatus* and *Candida albicans*, the two main pathogens, is on a declining trend. The main risk factor of invasive fungal infections is immunosuppression, but as a result of new life-saving medical interventions due to technological advances, the number of fungal infections continues to increase and as a consequence, more and more fungal species need to be detected. *Aspergillus terreus*, known as the third most common fungal pathogen causing invasive aspergillosis, in many cases has primary amphotericin B resistance. An epidemiological study conducted between 2004 and 2008 found that out of 5036 patients diagnosed with candidemia, non-albicans infections was detected in nearly half of the cases (49.6%), with a significant percentage identifying *Candida glabrata*. Within the genus *Candida*, the occurrence of antifungal resistance is already so common that several species are on the WHO (World Health Organization) and CDCA (Centers for Disease Control Antibiotic) risk list. PCR techniques are well suited for fungal diagnostics due to their ease of use, speed, high sensitivity and low cost. PCR methods based on high-resolution melting curve analysis have been successfully used as an alternative to sequencing for rapid, cost-effective species level identification of bacterial cultures.

During my Ph.D. work, I participated in the development of high-resolution melting curve (HRM) based fungal diagnostic PCR assays that proved to be suitable for the genetic discrimination of 14 clinically relevant *Aspergillus* and *Candida* fungal species. One advantage of these methods is that the species is determined on the basis of the unique species-specific melting pattern of the amplicons formed during the PCR reaction. For this reason precise knowledge of the gene sequence is not required for species identification, nor are sequence specific probes used.

With *in silico* examination, we identified regions within the *β-tubulin* gene of the genera *Aspergillus* and *Candida* that have sufficient sequence differences between the species. Based on these, species-specific reference melting temperature ranges were determined with HRM analysis. The *Aspergillus* HRM PCR assay uses two primer pairs to discriminate between *Aspergillus fumigatus, A. lentulus, A. terreus, A. flavus, A. niger, A. welwitschiae és A. tubingensis* fungal species vie heteroduplex melting peaks. The *Candida* HRM PCR assay is able to identify *Candida albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. guilliermondii, C. dubliniensis* fungi at the species level with a single primer pair. The specificity of the primers was experimentally confirmed by capillary sequencing and agarose gel electrophoresis. A total of 49 *Aspergillus* and 42 *Candida* fungal strains were tested during *in vitro* standardization of the assays. Validation of the *Aspergillus* duplex HRM PCR assay was performed on a LightCycler® 96 instrument, and the *Candida* HRM assay was optimized on three different PCR platforms (LightCycler® 96, LightCycler® 2.0, LlightCycler® Nano). It was statistically confirmed that the results of our HRM assay are reliable and reproducible, significant melting temperature shifts were not observed even at low DNA concentrations. The lower limit of detection (LoD) for the *Aspergillus* HRM assay was 100 GE in general, the analytical sensitivity of the *Candida* HRM assay proved to be 10 GE, but a reduced sensitivity was found in the case of *Candida parapsilosis* (LoD: 100 GE). Moreover, the LightCycler® Nano had a lower detection limit of 100 GE for *C. guilliermondii* and *C. dubliniensis* in addition to *Candida parapsilosis*.

Fungal diagnostic PCR tests for invasive fungal infections focus on direct pathogen detection from blood. This is ont he one hand due to the high sensitivity and negative predictive value associated with the method (if the patient was not recieving antifungal prophylaxis) and ont he other hand to several studies supporting fungal nucleic acids appearing earlier in the bloodstream such as glactomannan. This allows invasive aspergillosis to be confirmed before GM positivity and the onset of clinical symptoms. Egger et al.'s 2020 paper provides a comprehensive, objective picture on this subject, but the title following the analogy of the classic wastern film („Blood Aspergillus: The Good, the Bad and the Ugly") aptly illustrates that the above-mentioned beside the strengths, limiting factors must also be taken into account. Of these, it should be noted that antifungal prophylaxis in blood samples significantly reduces the diagnostic efficiency of PCR tests. Most health centers that care hematological cancer paitents use and antufingal prophylactic antifungal therapeutic approach recommended by international guidelines. As a consequence of antifungal

prophylaxis and intensive immunosuppressive therapies, the occurrence of non-Aspergillus molds in becoming more common in the clinic. For this reason, it is important that PCR diagnostic assay have high analytical specificity and be able to differentiate between several *Aspergillus* species, whitout cross-reacting with other molds. This is important because non-Aspergillus infectioins may require different antifungal treatments or some *Aspergillus* species may already have polyene or azole resistance.

Most commercial fungal diagnostic PCR assays (MycAssay *Aspergillus*, AsperGenius, MycoGenie és SeptiFast PCR) target ribosomal loci using universal primers, hovewer, beside the major fungal pathogens (*Aspergillus fumigatus, Candida albicans*), only a few species can be identified with them. In terms of the number of species distinguished, the our assay outperform these commercial PCR tests, however, their sensitivity is lower. For thi reason our assay is more suited for the identification of fungal cultures at the species level. As a preliminary result, we successfully identified *Candida glabrata* and *C. albicans* from hemoculture and cannula samples with our method.

Beside species identification, screening of gene mutations associated with antifungal resistance mechanisms is also of paramount importance. Commercially available tests are only suitable for the identification of azole resistance markers within the *Cyp51A* gene, however, for example polyene (amphotericin B) resistance is not reported. The HRM methods are sufficiently sensitive to detect various polymorphisms and to identify antifungal resistant strains. HRM has been successfully used to screen for azole-resistant *Candida albicans* strains associated with *ERG11* gene mutations. MALDI-TOF mass spectrometry is also very fast and efficient in identifying fungi at the species level, but a limiting factor is that the determination is currently only possible from pure culture, which can delay the diagnosis by days. The advantage if PCR methods is that pathogen detection is possible directly from clinical samples (tissue, blood, BAL), however, special care should be taken to check for false-negative results due to PCR inhibition or false-positive results due to environmental contamination, especially in case of fungal diagnostic assays. False positive results originating from laboratory contamination are common in case of ubiquitous fungi. For this reason, the purity of the reagents used in DNA extraction should be monitored by introducing appropriate isolation controls, and negative controls should also be used in PCR runs.

During the development of the *Aspergillus* and *Candida* HRM methods, cost-effectiveness, ease of use and reproducibility were considered in accordance with international guidelines. In addition, supporting the routine use of our method, we created an online application (MycoAnalyzer) that allows samples to be analyzed in a user-friendly manner. If sample processing or the PCR instrument other than that described in the dissertation is used, recalibration of the system with reference fungal strains due the melting temperature shift is required, in which case the MycoAnalyzer software cannot be used for species identification. The HRM PCR test can be combined well with other diagnostic methods without significantly increasing the cost.

Our future plans include testing HRM assays on fungal cultures, hemocultures, cannula and catheter samples isolated from patients. Moreover, we plan to determine the melting pattern of multidrug-resistant *Candida auris* with the *Candida* HRM assay.

In summary, we believe that the *Aspergillus* and *Candida* HRM PCR assays we developed may provide a good alternative for microbiological laboratories, and allow simple, rapid and cost-effective species-level identification of clinically relevant fungal pathogens.

Summary

Our primary aim was to develop and optimize HRM PCR fungal diagnostic assays that allow genetic distinction of clinically relevant *Aspergillus* and *Candida* species. We identified regions in the *β-tubulin* genes of these two genera, which proved to be diverse enough in sequence to serve as unique species specific genetic fingerprint in HRM analysis. Based on this finding we managed to identify reference melting temperature ranges in case of *Aspergillus fumigatus*, *A. lentulus*, *A. terreus*, *A. flavus*, *A. niger*, *A. welwitschiae*, *A. tubingesis* and by using another *Candida* genus specific assay we can discriminate between *Candida albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. dubliniensis* and *C. guilliermondii* species. We optimized the HRM PCR reaction parameters experimentally. The *Aspergillus* HRM PCR assay was validated on Roche LightCycler® 96 instrument while the *Candida* HRM assay was optimized for three different Roche PCR instrument (LightCycler® 96, LightCycler® 2.0, LightCycler® Nano).

Altogether the analytical sensitivity of the *Aspergillus* HRM assay proved to be 100 GE, however, the *Candida* HRM assay was generally more sensitive (LOD: 10 GE), except in case of the *Candida parapsilosis* (LOD: 100 GE). The assay accuracy and reproducibility above the detection limit was statistically confirmed.

To advance the routine use of our method, we designed a complete fungal diagnostic protocol including cell disruption, DNA isolation, HRM PCR working protocol and interpretation of the results. The MycoAnalyzer online application was developed in order to achieve fast and user friendly species identification from the HRM PCR results. Our HRM PCR tests can be used in combination with other diagnostic methods without increasing the costs significantly.

Based on the number of identified species, simplicity, fast turn-around time and cost-effectiveness we think that our method can be useful for clinical microbiology laboratories in species identification of *Aspergillus* and *Candida* cultures isolated from patients.

Acknowledgement

I would like to take the opportunity to thank all those who contributed to the dissertation. First of all, I would like to thank my supervisor Dr. Melinda Paholcsek and Prf. Dr. Sándor Biró for their trust in me, for allowing me to work in the Department of Human Genetics, for providing me with financial background, professional guidance and human help I have provided over the years. I could learn a lot from them.

Special thanks go to Dr. Éva Leiter, Dr. Sándor Kocsubé, Dr. Anikó Stágel, Dr. Judit Szabó, Dr. László Majoros, and Dr. Varga János for their valuable professional help.

I thank all my past and present colleagues.

Finally, I would like to thank to all my family who have always stood by me, without them I would not have gotten this far.

Appendix – List of publications

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/205/2020.PL
Subject: PhD Publikációs Lista

Candidate: Gábor Fidler
Neptun ID: SU3X95
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology
MTMT ID: 10057432

List of publications related to the dissertation

1. **Fidler, G.**, Leiter, É., Kocsubé, S., Biró, S., Paholcsek, M.: Validation of a simplex PCR assay enabling reliable identification of clinically relevant Candida species.
   BMC Infect. Dis. 18 (393), 1-13, 2018.
   DOI: http://dx.doi.org/10.1186/s12879-018-3283-6
   IF: 2.565
2. **Fidler, G.**, Kocsubé, S., Leiter, É., Biró, S., Paholcsek, M.: DNA Barcoding Coupled with High Resolution Melting Analysis Enables Rapid and Accurate Distinction of Aspergillus species.
   Med. Mycol. 55, 642-659, 2017.
   DOI: http://dx.doi.org/10.1093/mmy/myw127
   IF: 2.799

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

List of other publications

3. Biró, A., Markovics, A., Fazekas, M., **Fidler, G.**, Szalóki, G., Paholcsek, M., Lukács, J., Stündl, L., Gálné Remenyik, J.: Allithiamine Alleviates Hyperglycaemia-Induced Endothelial Dysfunction. *Nutrients. 12* (6), 1-13, 2020.
   DOI: http://dx.doi.org/10.3390/nu12061690
   IF: 4.171 (2018)
4. **Fidler, G.**, Tolnai, E., Stágel, A., Gálné Remenyik, J., Stündl, L., Gál, F., Biró, S., Paholcsek, M.: Tendentious effects of automated and manual metagenomic DNA purification protocols on broiler gut microbiome taxonomic profiling.
   Sci. Rep. 10 (1), 1-16, 2020.
   DOI: http://dx.doi.org/10.1038/s41598-020-60304-y
   IF: 4.011 (2018)
5. Paholcsek, M., **Fidler, G.**, Kónya, J., Rejtő, L., Méhes, G., Bukta, E., Loeffler, J., Biró, S.: Combining standard clinical methods with PCR showed improved diagnosis of invasive pulmonary aspergillosis in patients with hematological malignancies and prolonged neutropenia.
   BMC Infect. Dis. 15 (1), 251, 2015.
   DOI: http://dx.doi.org/10.1186/s12879-015-0995-8
   IF: 2.69

Total IF of journals (all publications): 16,236
Total IF of journals (publications related to the dissertation): 5,364

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

24 June, 2020

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *