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

Evaluation and validation of PCR based diagnostic methods detecting aspergillosis

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detecting aspergillosis

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INTRODUCTION

Aspergillus is a genus that consists the largest number of mold species. There are numerous plant-, animal-, and human pathogens (*Aspergillus fumigatus, Aspergillus terreus, Aspergillus lentulus, Aspergillus flavus, Aspergillus niger, Aspergillus nidulans*) among them.

Recently invasive mold diseases rank among diseases with the highest morbidity and mortality in the developed countries. Their prevalence shows a gradually increasing tendency. Literature data estimate that at least as many, if not more, people die from invasive fungal diseases than malaria or tuberculosis.

The mortality rate can be as high as 100%. Literature data report that in certain patient groups every single hour spent without adequate antimicrobial therapy significantly increases the mortality. According to hospital statistics in the European population, *Aspergillus fumigatus* is responsible for about 7% of all hospital mortalities. Recently the occurrence of invasive mold infections caused by *Aspergillus terreus* species increased significantly as well.

Along with *Candida* species, *Aspergilli* provoke most frequently different nosocomial fungal infections. People with certain immunodeficiency, especially those with severe haematological problems (neutrophil count $<500 \times 10^6$/liter) belong to the risk group. People suffering from acute myeloid leukaemia (AML) have the highest chance to develop disease.

Since fungal diseases notably affect the life span, and the quality of life of people in the haemato-oncology wards, the hospital infection-control has gained more importance.

Settling an adequate diagnosis is hard. Verification of the presence of fungi in the attacked tissues is possible only after time consuming culturing or histopathological techniques, requiring invasive procedures (broncho-alveolar lavage sampling; BAL).

Serology unlike culturing techniques can provide results within three hours. These methods detect unique but general fungal cell wall constituents, and are not suitable to detect the causative agents on species specific level. Combination testing with other methods screening for biomarkers (more often PCR) is crucial.

Recently, the nucleic acid based screening methods in virtue of their rapid and easy workflow, and good reproducibility are more popular and fancy. Validated PCR methods are able to detect *Aspergilli* also on species specific level.

Given by the presence of ubiquitous pathogenic agents however, highly sensitive PCRs can lead to false-positives and very low specificity. The extraordinary high false-positive rates of serological procedures can be observed among patients being treated immediately before diagnosis with *Piperacillin-Tazobactam*, *Ampicillin-Sulbactam* and *Amoxicillin-Clavulanic Acide* beta-lactame type antibiotics and with the combination of beta-lactamase inhibitors and other antibiotics (*PenicillinG*, *Ceftriaxon*, *Imipenem*, *Ciprofloxacin*, *Vancomicin*, *Gentamicin*, e.g.*)*.

The ISHAM (International Society for Human and Animal Mycology) - EAPCRI (European *Aspergilllus* PCR Initiative) is a non-profit organisation whose members are engaged to collaborate and develop a standard for *Aspergillus* PCR methodology and to validate different in-house methodologies in clinical trials so that PCR could be incorporated into future consensus definitions for diagnosing invasive fungal infections (IFIs).

The members of the laboratory working party meet in every 2-3 years, analyse the results of former executed studies, design further international cooperations and identify the most important variables that influence the performance of PCR. The goal of the clinical working party is to analyse and summarize the novel issues of the laboratory and statistical working party in cooperation with the two other academic groups; EBMT (European Society for Blood and Marrow Transplantation), and EORTC (European Organisation for Research and Treatment of Cancer).

The apathogen, saprophytic Gram-positive *Streptomyces* bacteria belong to the phylum *Actinobacteria* and to the order *Actinomycetales* composing about 20% of the normal soil bacterial population and sharing common ecological niche with the former mentioned *Aspergilli*. Their growth comprises complex morphological and biochemical processes representing remarkable differences in liquid and on solid medium. Their complex life cycle is regulated by pleiotropic auto-regulators. One of them is Factor C.

Investigation of the regulatory roles of Factor C - which is up to now the only one known auto-regulator protein - was commenced at the University of Debrecen (UD), Institute of Biology (recently UD, Department of Human Genetics) after its isolation, identification, cloning and sequencing from the 72 hours old liquid fermentation of the producer strain *Streptomyces griseus* 45H (according to the new nomenclature *S. albidoflavus* 45H). The secreted protein constitutes 324 amino acids.

By using the amino acid sequence of the mature Factor C protein PSI-BLAST (Position-Specific Iterative Basic Local Alignment Search Tool) sequence homology search algorithm was used to find similar (orthologous) proteins in other species. In this wise we found similar proteins in four human pathogenic filamentous fungal species, in further two apathogen fungi, in certain other *Streptomyces* species, and in certain Gram-positive, not *Actinomyces* type, low G+C bacteria, and in their phages respectively.

Based on sequence data of the pathogenic filamentous fungal species *facC* gene could be found only in *Aspergillus fumigatus* and *Aspergillus terreus*. Based on our own research data the genome *Aspergillus lentulus* - which is still not sequenced - possesses *facC* genes as well. Moreover, in the genome of *Aspergillus fumigatus facC* genes can be found on two chromosomes Chr#3 and Chr#5 respectively.

The presence of *facC* orthologous genes could only be affirmed in the genomes of eukaryotic, human pathogenic species known as primary causative agents of invasive aspergillosis, furthermore are not present in the genomes of species possible present in the blood stream of immunocompromised hosts colonizing and evoking systemic infections (bacterial and fungal). Based on these facts it was a great opportunity for us to develop nucleic acid based assays detecting these unique *facC* orthologous genes.

OBJECTIVES

- To develop real-time assays to diagnose invasive aspergillosis based on the presence of the *facC* orthologous genes of the major causative agents of *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus lentulus*.
- To determine the reaction efficiency, analytical sensitivity and analytical specificity of our *Aspergillus fumigatus* and *Aspergillus terreus* specific Taqman® assays.
- Identification of *Aspergillus fumigatus* and *Aspergillus lentulus* species based on their specific melting points (melting analysis) and melting curves (HRM analysis).
- To estimate the dynamic ranges of our Taqman® assays in automatic nucleic acid extraction system and on large number serum samples spiked with *Aspergillus fumigatus* and *Aspergillus terreus* conidia.
- To validate of our Taqman®-LNA3 real-time assay in a prospective case-control study at the University of Debrecen in collaboration with the Department of Internal Medicine, Institute of Medical Microbiology and Institute of Pathology. To perform combined biomarker testing and to monitor episodes with neutropenic fever for the presence of *Aspergillus* specific antigenes (galactomannan) and for the presence of *Aspergillus fumigatus facC* orthologous genes on three consecutive days. We compare the results of biomarker analyses with each other and with those of conventional methods and estimate the diagnostic efficiency of every single method.
- To participate in annual cross-control studies in order to test and improve our in-house real-time assays and to estimate our performance as compared to those of the other groups.

MATERIALS AND METHODS

Bioinformatic methods screening for Factor C homologs

We searched databases with the PSI-BLAST for the presence of homologous amino acid sequences of the mature Factor C protein.

Developing of Taqman® MGB and Taqman® LNA assays

We developed eight different Taqman® hydrolysis assays (4 Taqman®-MGB and 4 Taqman®-LNA assays) for the detection and identification of *Aspergillus fumigatus* and *Aspergillus terreus* species. In the case of our different mono-copy systems there is only one target sequence available. Since in the *Aspergillus fumigatus* genome there are two *facC* orthologous genes present (AFUA_3G14910, AFUA_5G00540) we developed dual-copy assays as well. In this case we run two simultaneous PCR amplifications using different target sequences (duplex PCR) involving the usage of four different sequence specific primers and two different probes labeled with the same fluorophore dye in one tube.

Cultivation of *Aspergillus* strains and purification of the gDNAs

As making inoculums we used the spore suspensions of three (*Aspergillus fumigatus* AF293) and seven days old (*Aspergillus terreus* NCCB IH2624 and *Aspergillus lentulus* SZMC3118) cultures and we let them grow on liquid, glucose containing minimal medium. Mechanical lysis was performed with liquid nitrogen and than we purified the gDNA (Thermo Scientific, Maryland, USA) according to manufacturer's instructions.

PCR amplification controls

The term genomic equivalent (GE) is equivalent with the amount of genomic DNA present in one cell. We incorporated appropriate internal amplification controls within every single run protocol during the real-time analyses of the samples with different *Aspergillus fumigatus*, *Aspergillus terreus* and *Aspergillus lentulus* gDNA contents (10^2-10^3 GE).

Determination the reaction efficiency and specificity of the Taqman® MGB and Taqman® LNA assays on gDNA panels

After construction of standard series (panels) from the gDNA isolates of filamentous fungi we estimated the reaction efficiency, limit of detection (LoD) and the linear dynamic range of every single assay. We used 25-member serial dilution panels across 7 orders of magnitude (15 ng - 15 fg) by using the gDNAs of *Aspergillus fumigatus* AF293, *Aspergillus terreus* NCCB IH2624 strains and nuclease free water (Ambion PCR grade water). We run every sample in triplicate. For the graphical representation of the standard curves after the successful PCR amplification reactions mean Cp values of the triplicates were taken and plotted as a function of the $\log_{10}$ input values of genomic contents (GEs). In order fit the data to a straight regression line, we used the slopes of these regression lines ($y = mx + b$) and we calculated the reaction efficiency values.

We investigated the specificity of our Taqman®-MGB assays on human DNA and on numerous pathogenic and apathogenic fungal gDNA samples respectively. The specificity of our Taqman®-LNA4 assay was investigated on the ISHAM-EAPCRI panel performed and released in the year of 2013 (2013/08/20).

Melting point analysis for the detection of *Aspergillus fumigatus* and *Aspergillus lentulus* species

After evaluating the results of the 2013/08/20 ISHAM-EAPCRI cross-control panel we observed that our *Aspergillus fumigatus* specific Taqman®-LNA3 assay cross reacts with the gDNA of *Aspergillus lentulus*. From these facts we concluded that the genome of *Aspergillus lentulus* (whose genome is still not sequenced) may also contain *facC* alike orthologous gene. We designed primers for the amplification of *Aspergillus fumigatus* AFUA_3G14910 and AFUA_5G00540 genes and using *Aspergillus fumigatus* Af293 gDNA as template and we optimized the reaction parameters. Using the same primers under the same reaction conditions we successfully amplified *facC* like fragments on *Aspergillus lentulus* SZMC3118 purified gDNA template as well. After sequencing these *facC* amplicons of *Aspergillus lentulus* origin we designed PCR primers matching to *facC* alike genes present of both species; *Aspergillus fumigatus* Af293 and *Aspergillus lentulus* SZMC3118 to differentiate among them on the basis of the different melting curves (HRM analysis) of their templates.

Measuring the reaction efficiency of *facC*-HRM assays on gDNA panels

We constructed *Aspergillus fumigatus* AF293 and *Aspergillus lentulus* SZMC3118 gDNA panels (Afu-HRM panel-1, Alent-HRM panel-2, Afu-Alent-HRM panel-3) on 7-fold dilution series (10^6-1 GE/PCR reaction).

Measuring the dynamic ranges of our Taqman® assays on conidia panels

The fungal nucleic acid purification of biological samples was done in a Roche MagNA Pure LC 2.0 robotic system. We performed 6-membered whole blood panels across 6 orders of magnitude (10^6-10^1) to determine the dynamic ranges of our Taqman®-MGB (Taqman®-MGB3, Taqman®-MGB4), and Taqman®-LNA (Taqman®-LNA3, Taqman®-LNA4) assays. After these we further investigated our Taqman®-LNA assays on 5-membered serum panels across 4 orders of magnitude (10^1-10^4).

According to our extraction run protocols we also analysed negative control samples in accordance with those containing of fungal conidia in different amounts. 5% positivity of the extraction negative control samples resulted in a disapproval of the extraction run results.

Fungal conidia were disrupted with MagNA Lyser Instrument (Roche Applied Science) and ceramic beads according to our optimized protocol (ceramic bead-beating) in 250 µl and in 1250 µl sample volumes respectively.

We ran the Taqman®-MGB real-time PCR amplification reactions on Applied Biosystems 7500 Real-Time PCR, while the Taqman®-LNA real-time assays ran on a Roche LightCycler® 480 Instrument II in 20 µl reaction volumes by the incorporation of adequate PCR-internal controls and PCR-NTCs.

The 2014 ISHAM-EAPCRI BAL panels

In November of 2014 we investigated the cross-control panels released by the ISHAM-EAPCRI international organisation. For measuring the analytical sensitivity of the different home developed and optimized real-time diagnostic methods there were guinea pig BAL samples made in the Cardiff central microbiology laboratory of the organisation.

We received two identical BAL panels (BAL panel-D1, BAL panel-D2) for investigation. In one case the *Aspergillus fumigatus* fungal nucleic acids were extracted in a robotic system from the BAL samples while in the other case we purified the nucleic acid on a manual platform.

We performed mechanical lysis in every cases in order to extract the fungal nucleic acids from the BAL samples. We investigated the two BAL panels in different real-time systems (Roche LC 2.0, LC 96). We tested all together 4 different methods (method-1, -2, -3, -4).

In the case of method-1 and method-2 the volume of the samples (250 µl), the mechanical lysis protocol (ceramic bead-beating) and the robotic sample extraction platforms (Roche MagNA Pure LC 2.0) were identical. It was only the real-time platform that differed. In the case of the method-1 we ran the samples in Roche LC 2.0 capillary PCR system while in the case of the method-2 samples ran in Roche LC 96 well plate system.

Method-3 was identical with the method-1 but the mechanical lysis procedure. In the case of method-3 we applied the Roche SeptiFast Lysis Kit according to manufacturer's instructions.

Method-4 substantially differed from the above presented. In the case of this latter the template volume was 2,5 times as much compared to the formers and the BAL panel-D2 was only analysed with this latter. Fungal cell wall extraction was according to our optimized bead-beating protocol followed by manual DNA extraction. According to this we settled the elution volume to 45 µl which was completely monitored during post-elution in Roche LC 2.0 capillary system.

Results were scored as follows: three points for detecting an essential sample ($\geqq$10 CFU/GE for fungal DNA containing samples and 0 CFU/GE in the case of negative control samples) and two points for detecting a desirable (<10 CFU/GE) sample. The maximum score achievable was 32 (24 for essential samples and 8 for desirable), for the separate detection of conidia or DNA the maximum score is 16, respectively.

The maximum score achievable for analysing *Aspergillus fumigatus* conidium and *Aspergillus fumigatus* DNA panels was 16-16 for each.

PATIENTS AND METHODS

Study population

Between June 06, 2012 and May 08, 2013, 27 febrile neutropenia episodes (FNEs); 16 males with the median age (yr) ±(SD) of 42.5 ±21.72, (range; 14-75), and 11 females with the median age (yr)±(SD) of 45.5 ±15.31, (range; 19-66) with different haematological malignancies (mainly acute leukemias) receiving stem cell transplantation and intensive chemotherapy were included at the onset of febrile neutropenia (neutrophil count < $0.5x10^9$ cells/L; temperature > 38°C recorded twice or > 38.5°C recorded once) in our local prospective case-control study in cooperation with the Institute of Internal Medicine of the University of Debrecen.

Case group, control group

On the basis of serological, radiological, microbiological, and histological data signs and symptoms of IPA were collected together along with host criteria and episodes were retrospectively stratified according to revised European Organization for the Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) criteria in order to define case-group of "proven" or "probable" IPA (7 patients) and control-group "possible" IPA (20 patients with no EORTC/MSG evidence of IA).

Diagnostic strategy of invasive pulmonary aspergillosis

At the onset of fever broad-spectrum antibiotic treatment (e.g., (pl. *cefepime, carbapenem*) was administered according to the published IDSA (Infectious Diseases Society of America) guidelines. Standard chest CT radiology was invariably performed and BAL analysis was performed as clinically indicated.

Furthermore, for FNE, combined biomarker testing was commenced. Serum samples were prospectively screened for the presence of genus and species specific biomarkers (GM and *facC* genes) with Platelia *Aspergillus* GM-enzyme immunoassay and *facC*-PCR.

Consecutive serum samples (3 x 3 ml) were routinely piped together with clinical test request formulas of the episodes from the onset of febrile neutropenia to the Department of Medical Microbiology, Debrecen, Hungary for GM antigen monitoring.

Screening for GM-antigenemia a sandwich ELISA test (Platelia *Aspergillus* GM-EIA; Bio-Rad Laboratories, Hungary) was used according to the developer's instructions. GM-EIA cutoff values were determined using the $OD_{450/620}$ value of sample / $OD_{450/620}$ value of control. While evaluating single specimens any value above the $OD_{450/620} \geqq 0.5$ cutoff value was considered positive as requested for *in vitro* testing.

In parallel with GM-serology we performed *facC* orthologous genes based nucleic acid diagnosis as well (*facC*-PCR) at the Department of Human Genetics. In this study there were triplicate *facC*-PCR reactions were performed pro specimens. In a triplicate on the basis of a single positive PCR the specimen was considered PCR positive. In optimal cases there were three GM-EIA and three *facC*-PCR results assigned to every single episode.

In the case of fever refractory to broad spectrum antibiotic treatment, and/or positive GM-serology, and/or positive chest CT there were BAL examinations also performed.

When performing postmortem analysis Hematoxylin and Eosin (H&E) staining is able to visualize the fungal hyphaes in lung parenchyma but in the case of tissue necrosis further supplementary staining procedures like as Grocott-Gömöri methenamine silver staining (GMS) and/or the Periodic Acid-Schiff (PAS) staining are suggested to perform. Lung sampling was performed from three independent parts of the potentially infiltrated lung parenchyma and carefully examined for the presence of fungal filaments of *Aspergillus* origin.

Therapeutic strategy of invasive pulmonary aspergillosis

Recurrent or persistent fever refractory to broad spectrum antibiotic treatment, concomitant positive (more than one) GM-serology result along with either positive CT and/or positive BAL was considered sufficient evidence to initiate broad spectrum antifungal treatment. In the case of ceased fever, negative chest CT and/or BAL we suggest to extend the combined biomarker monitoring two times on a weekly basis.

DATA INTERPRETATION

Patients enrolled in statistical analyses were subclassified into two groups (cases and controls) according to the disease investigated (proven, probable aspergillosis) and their actual health profile. Along with estimating the categorical (positive test result/presence of the disease, negative test result/absence of the disease) and continuous (Se, Sp, PV^+, PV^-) variables we calculated mean, median values, standard deviation (SD±) and confidence intervals (95% CI) as well.

ROC analysis

To measure the diagnostic accuracy of the two screening tests results from GM-EIA and *facC*-PCR testing (77 GM-EIA indexes and 249 *facC*-PCR Cq values) were converted to qualitative (positive, negative) indexes considering the presence (cases) or absence (controls) of IPA infection of the episodes providing the biological samples.

In the case of *facC*-PCR every single run was evaluated independently. After calculating the series of sensitivity (Se) and specificity (Sp) reports at every single decision threshold the receiver operating characteristic (ROC) curve was edited by plotting the true positive values (sensitivity values on y- axis) versus the false positive rates (1-specificity values on x-axis). Area under the ROC curve (AUC) was estimated with 95 % confidence intervals (95 % CIs) and with standard errors (±SD). Along with these we also calculated positive and negative likelihood ratios (LR^+, LR^-) and positive and negative predictive values (PV^+, PV^-) as well.

Comparison of the two classifiers with Kappa-test

Cohen's Kappa test was used for assessing sample concordance between the two raters; the gold-standard (Platelia *Aspergillus* GM-EIA) and the new method being tested (*facC*-PCR). Categorical (qualitative) outcomes of the screening methods were compared with no regard to patient statuses estimating the agreement occurring by chance.

RESULTS

We confirmed the presence of *facC* orthologous genes in human pathogen species causing aspergillosis

When searching databases with the amino acid sequence of the mature Factor C protein with PSI-BLAST sequence homology search algorithm we found *facC* alike orthologous genes, presumably with the same evolutionary origin in the genomes of the human pathogen filamentous strains of *Aspergillus fumigatus* Af293, *Aspergillus terreus* NIH2624 among others. In the genome of *Aspergillus fumigatus* Af293 there are two copies of the gene. One is on the Chr#3 (AFUA_3G14910) and the other is on the Chr#5 (AFUA_5G00540). In the genome of *Aspergillus terreus IH2624* there is only one copy of the *facC* gene.

Dynamic ranges of the Taqman®-MGB and Taqman®-LNA assays on gDNA panels

We developed and tested eight different Taqman® assays for the specific detection and identification of the species *Aspergillus fumigatus* (MGB1, MGB2, MGB3, LNA1, LNA2, LNA3) and *Aspergillus terreus* (MGB4, LNA4). Whit this we assessed on *Aspergillus fumigatus* Af293 and *Aspergillus terreus* IH2624 gDNA panels the reaction efficiencies and limit of detection values (LoD) of our real-time assays. Based on our results we concluded that the Taqman®-LNA3 assay proved to have the highest sensitivity to detect *Aspergillus fumigatus* Af293 (LoD = 1 GE), while Taqman®-LNA4 assay was the most sensitive in detection of *Aspergillus terreus* IH2624 (LoD = 1 GE) species.

Analytical specificity of Taqman®-MGB and Taqman®-LNA assays on gDNA panels

We proved experimentally that Taqman®-MGB hydrolysis assays (Taqman®-MGB3, Taqman®-MGB4) hybridizing to certain regions of *facC* orthologous genes present in *Aspergillus fumigatus* Af293 and *Aspergillus terreus* IH2624 genomes do not cross react with each other or with numerous other species possessing *facC* orthologous counterparts. We examined the analytical specificity of our *Aspergillus fumigatus* specific Taqman®-LNA3 and our *Aspergillus terreus* specific Taqman®-LNA4 assay on the ISHAM-EAPCRI 2013 panel. According to this our *Aspergillus fumigatus* and *Aspergillus terreus* specific assays did not cross react with each other nor with the gDNA of several species with one exception only. Interestingly our *Aspergillus fumigatus* specific Taqman®-LNA3 assay cross reacted with the gDNA of *Aspergillus lentulus*.

Diagnostic accuracy of *facC*-HRM assay on *Aspergillus fumigatus* and *Aspergillus lentulus* gDNA panels

We proved the presence of two *Aspergillus fumigatus facC* alike and presumably *Streptomyces facC* orthologous genes in the genome of *Aspergillus lentulus* SZMC3118 species being also similar in size (900-950 bp) to the ancestor gene. The genes were first amplified and than sequenced with sequence specific primers designed to the AFUA_3G14910 and AFUA_5G00540 *facC* orthologous genes present in *Aspergillus fumigatus* genome. Using the sequence data, we successfully engineered primers for the sensitive detection and identification of *Aspergillus fumigatus* Af293 and *Aspergillus lentulus* SZMC3118 strains on the basis of the characteristic melting points of their amplicons. After performing *Aspergillus fumigatus* Af293 and *Aspergillus lentulus* SZMC3118 gDNA panels we estimated the diagnostic accuracy, amplification efficiency, the limit of detection of our *facC*-HRM assay and the characteristic melting points of the different amplicons for the specific identification of the species.

Diagnostic accuracy of Taqman® assays on *Aspergillus* conidia containing blood panels

When performing linear regression analysis, we assessed the dynamic ranges of our *Aspergillus fumigatus* (Taqman®-MGB3, Taqman®-LNA3) and *Aspergillus terreus* (Taqman®-MGB4, Taqman®-LNA4) specific real-time assays on whole blood and serum samples containing different amounts of fungal conidia. On the basis of our data we concluded that the amplification efficiency proved to be better in the case of Taqman®-LNA hydrolysis assays as their amplification efficiency data fall into the optimal range (E = 103-107%) when testing whole blood samples. Moreover, both our Taqman®-LNA3 and Taqman®-LNA4 assays were able to detect single *Aspergillus fumigatus* and *Aspergillus terreus* conidia (LoD = 1 GE) in biological samples. Therefore, we further tested the diagnostic utility and dynamic range of our Taqman®-LNA assays on large number of serum samples. The reaction efficiency of our Taqman®-LNA3 real-time assay on large volume, conidia containing serum samples was 117,54%, while for the Taqman®-LNA4 assay it was 107,93%.

Infection control of invasive pulmonary aspergillosis (IPA)

Case group, control group

During the one-year follow-up period there were 27 episodes enrolled in our case-control study. Episodes were retrospectively stratified according to revised European Organization for the Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) criteria in order to define a case-group of “proven” or “probable” IPA and a control-group possible IPA

Empiric antifungal therapy

There were 15 patients with neutropenic fever receiving antifungal treatment. Later it was proved however that 60% of these patients were loaded unnecessary with the side-effects of strong cito- and hepatotoxic agents.

Diagnosis of IPA with conventional methods

Concordant negative *Aspergillus* GM and negative CT supported by PCR have been shown to have the highest discriminatory power to exclude IPA. Their results proved to be concordant in 94,5% of the cases not supporting IPA. The negative predictive value of the two methods was; PV^-: 0,94. Invasive BAL procedure was performed in 77% of episodes when indicated. Concordant negative chest CT and BAL results showed high discriminatory power to exclude IPA; PV^-: 0,92. Based on our results we determined that the invasive BAL procedure have a strong capacity (PV^-: 0,93) to prove the absence of invasive aspergillosis in 93% of the controls investigated (15 people) which means that there was only one patient that was misdiagnosed.

Diagnostic accuracy and diagnostic utility of the biomarker-screening assays

We performed ROC (receiver operating characteristic curves) analysis and area under the ROC curve (AUC) was estimated for both classifiers; Platelia *Aspergillus*-EIA and *facC*-PCR. The area under the ROC-curve (AUC) for *Aspergillus* GM-EIA was 0.7283 (95% CI 0.6031-0.8535); *P* value of < 0.0012 ±0.0639 representing a „fair” agreement while that for the *facC*-PCR was 0.8004 (95% CI 0.6976-0.9031); *P* value of < 0.0001 ±0.05243 representing a „good” agreement.

Diagnostic performance parameters were calculated (by doing the 2x2 contingency tables of cases and controls); sensitivity (Se) values, specificity (Sp) values, discriminatory properties of post-test probabilities (likelihood ratios; LR^+, LR^-), predictive values (PV^+, PV^-) and accuracy indexes (DORs) with 95% confidence intervals (95% CI) for GM screening (GM-EIA) and for the nucleic acid based diagnostic method (*facC*-PCR) respectively.

Sample concordance was assessed between the two classifiers; Platelia *Aspergillus* GM-EIA and *facC*-PCR showing a fair agreement generating an observed ratio of 47.17% and a Cohen kappa statistic of 0.258.

Mortality data

The overall mortality rate of our case-control study was 48.14%. 13 of 27 patients died during the surveillance period. 43.47% of patients died from the GM-EIA negative and 75% of the patients from the GM-EIA positive group.

Case-studies

Post-mortem histology (PMH) was performed in 53.85% (7/13) of episodes. There were major discrepancies observed between clinical and post-mortem findings in the case of two episodes.

Autopsy proved hyphal tissue invasion in the case of one patient only (patient ID 4). Interestingly this patient with ceased fever in response to antibiotic treatment provided negative CT result along with 7 consecutive negative GM-EIA indexes but successively positive PCRs generating 10 runs of 11 that were positive thus was classified “possible” and remained undiagnosed. This patient was shown unequivocally only at autopsy to have IA. The presence of fungal hyphal invasion in the lung tissue was confirmed by PAS and H&E staining and the episode was subsequently classified “proven”

One patient (patient ID 24) was originally classified “probable” based on positive thoracic CT and GM-EIA but definite negative PCRs. As post-mortem histology could not demonstrate fungal involvement, episode was reclassified “possible”.

DISCUSSION

The fact that prokaryotic *facC* orthologous genes have been passed from different prokaryotic *Streptomyces* to eukaryotic, human pathogen, filamentous fungal strains served the basic concept of this PhD thesis.

It was proved experimentally that out of fungal strains colonizing immunosuppressed patients and provoking systemic fungal infections (so their biological markers can be attainable in the human blood) only the *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus lentulus* possess *facC* orthologous genes. Based on the level of the sequence divergence attainable among these genes we were able to design specific assays hybridizing to certain regions of these genes that are neither cross reacting with each other nor with other strains allowing us to detect them specifically and with high sensitivity in different biological samples (whole blood, serum and BAL).

We developed eight different Taqman® real-time, and one HRM assay. Based on our data the LoD values of the *Aspergillus fumigatus* specific Taqman®-LNA3, and the *Aspergillus terreus* specific Taqman®-LNA4 real-time assays proved to be low (LoD = 1 CFU/biological sample) on different gDNA panels. The validation of our HRM assay identifying *Aspergillus fumigatus* and *Aspergillus lentulus* based on their melting curves of their amplicons is still in progress. The analytical sensitivity of the assay on different *Aspergillus fumigatus,* and *Aspergillus lentulus* gDNA panels; LoD = 1 GE.

During the past few years we worked with serum, whole blood and BAL samples. We optimized numerous protocols depending on the different biological samples. For extracting whole blood and BAL samples the mechanical lysis protocol (performed with the Roche, MagNA Lyser Instrument) carried out by ceramic green beads proved to be the most effective. (method-1). Despite the fact that whole blood and BAL samples contain the highest amount of fungal nucleic acid molecules nowadays serum samples have gained more attention.

We also tested the analytical sensitivity of our real-time assays on the 2013 ISHAM-EAPCRI panel. This panel contained different pathogen, invasive fungal infection (e.g., aspergillosis or candidiasis) causing gDNA isolates in different concentrations. Based on the results, the LoD value of *Aspergillus terreus* specific Taqman®-LNA4 assay proved to be very low. It was able to detect 18 fg (1,2 GE) gDNA form certain clinical isolates of *Aspergillus terreus*. 45% of the the other centres participating in the cross-control study were able to achieve this score (15 out of the other 33 participating centre). The specificity of the Taqman®-LNA4 assay was 100%. However, the *Aspergillus fumigatus* specific Taqman®-LNA3 assay was not able to detect 1,2 GE gDNA of a certain *Aspergillus fumigatus* clinical isolates in 20 µl PCR reaction.

As a member of the ISHAM-EAPCRI laboratory working party we participated in the examination of the 2014 BAL panels containing either *Aspergillus fumigatus* nucleic acids, or *Aspergillus fumigatus* conidia in different concentrations. After analysing the *Aspergillus fumigatus* BAL panels we estimated, that out of our four methods tested the highest score was achieved by the method-1 that is based on a mechanical lysis carried out by ceramic green beads and real-time detection in LC 2.0 capillary system (25 points out of 32). Furthermore, we estimated that our mechanical lysis protocol is able to disrupt the fungal cell wall without fracturing fungal DNA with ceramic green beads and extracting more efficiently *Aspergillus fumigatus* nucleic acids from BAL samples than the Septifast Lysis protocol.

Prompt and accurate diagnosis is of high importance since hospital charges are increasing in a drastic manner in the course of disease. The hospital infection control and patient follow-up would be more effective in case of institutes had cost efficient routine diagnostic procedures allowing biological marker monitoring of patients. Based on the fact that patients suffering from different serious haematological malignancies (mainly acute leukaemia) with long lasting neutropenia are under highest risk developing invasive pulmonary aspergillosis hospital infection control gained importance.

There was an aspergillosis surveillance group established at the University Hospitals of Debrecen comprising geneticists, molecular biologists, microbiologists and physicians, to assess the discriminatory power of combined biomarker testing by considering *facC*-PCR results together with those of the GM-EIA in our local patient population including patients with different hematological malignancies.

Patients were retrospectively categorized according to revised European Organization for the Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) criteria in order to define a case-group of “proven” or “probable” IPA and a control-group “possible” IPA.

As part of our diagnostic strategy combined biomarker testing (CBT) was commenced on the onset of neutropenic fever. Serum samples were prospectively screened for the presence of genus and species specific biomarkers galactomannan (GM) and *facC* genes (mean sample number: 3.11 ±2.36) on three consecutive days.

We established that both CT and BAL screening have high negative predictive values (CT-PV^-=0.85 and BAL-PV^-=0.93), so according to our results they are rather suitable to exclude invasive pulmonary aspergillosis than to confirm it. After completing combined biomarker monitoring we established the diagnostic odds ratios of the biomarker screening tests. In the case of GM-EIA, DOR was 15.33, while in the case of *facC*-PCR it was 28,67.

Finally, we think that our diagnostic method could be suitable for commercial use in a form of a diagnostic kit. The most optimal alternative would be a robotic, modular system.

SUMMARY

The primary aim of this study was to develop PCR methods targeting *facC* orthologous genes for the sensitive detection of invasive aspergillosis in different biological samples. Accordingly, we developed eight Taqman® real-time assays (4 Taqman®-MGB and 4 Taqman®-LNA) to specifically detect *Aspergillus fumigatus* and *Aspergillus terreus* species and one HRM assay to differentiate among *Aspergillus fumigatus* and *Aspergillus lentulus* based on the melting points of their amplicons. We evaluated the PCR amplification efficiency, sensitivity and the specificity of our assays on different genomic DNA panels made in-house and received from the ISHAM-EAPCRI core laboratory working party. We measured the diagnostic utility of our Taqman® real-time assays on biological samples; whole blood and serum spiked with different amounts of *Aspergillus fumigatus* and *Aspergillus terreus* conidia. Taqman®-LNA3 and Taqman®-LNA4 showed the lowest LoD values on whole blood samples therefore we measured their dynamic range on numerous serum samples. As a member of the ISHAM-EAPCRI laboratory working party we also participated with our in-house PCR platforms in international cross-control studies to identify the most important variables influencing the diagnostic performance of *Aspergillus*-PCR and collaborate with other members of the laboratory working party to validate a standard for *Aspergillus*-PCR methodology. Based on the results we can conclude that we were able to develop a very efficient mechanical lysis protocol to disrupt *Aspergillus* conidia without losing the freely floating nucleic acids from the biological samples and we can extract fungal nucleic acids from biological samples in both automated and manual platform with nearly 100% efficiency. Our secondary aim was to validate the diagnostic power of our *facC*-PCR method and the gold-standard *Aspergillus*-GM serology diagnosing invasive pulmonary aspergillosis on large volume serum samples in patients with hematological malignancies and fevered neutropenia. Stratification correction followed by postmortem histology was done and according to this the diagnostic accuracy indexes (AUC for facC-PCR was 0.80 and for GM-EIA was 0.73) and the diagnostic odds ratio of *facC*-PCR proved to be higher (DOR was 28.67) compared to GM-EIA (DOR was 15.33). We also assessed the diagnostic value of standard clinical methods (CT and BAL) and compared to that of combined biomarker testing (*Aspergillus* GM-serology and *facC*-PCR) in our prospective case-control study. The observed agreement was 70.13% generating a kappa statistics of 0.258. With our results we demonstrated evidence that the consideration of standard clinical methods with combined biomarker testing can improve early and more accurate diagnostic decisions.

LECTURES

Paholcsek M., Biró S.: *Aspergillus* diagnostics in Debrecen. 5th Molecular Cell and Immune Biology Winter Symposium, 2012. January 4-7, Galyatető.

Paholcsek M., Biró S.: Hol tart jelenleg az aszpergillusz diagnosztika Debrecenben és világviszonylatban. A Magyar Mikrobiológiai Társaság 2012. évi Nagygyűlése. 2012. October 24-26, Keszthely, Hungary.

Paholcsek M., Biró S.: Statistical aspects of *Aspergillus* diagnosis; Multicenter *Aspergillus* DNA detection in Whole Blood and Serum. 6th Molecular Cell and Immune Biology Winter Symposium, 2013. January 8-11, Galyatető.

Paholcsek M.: Genetic linkage between bacteria and aspergillosis? A novel invention using highly sensitive and specific TaqMan assays for the identification of *Aspergillus fumigatus* and *Aspergillus terreus* species. Invasive Mycoses in Hematological Malignancies VII. 2013. March 22, Universitätsklinikum Würzburg, Zentrum für Innere Medizin, Germany.

Paholcsek M., Biró S.: An alternative way in the diagnosis of aspergillosis; a possible example of translational medicine. 7th Molecular Cell and Immune Biology Winter Symposium, 2014. January 7-10, Galyatető.

POSTERS

Paholcsek M., Biró S.: Novel methods in *Aspergillus* diagnosis. 4th Molecular Cell and Immune Biology Winter Symposium, 2011. January 11-14, Galyatető.

Springer J., **Paholcsek** M., Alzheimer M., Heinz WH., Schloßnagel H., Einsele H, Loeffler J.: Optimized Molecular Diagnosis if Invasive Aspergillosis in Patients after Allogeneic Stem Cell Transplantation – a Second Confirmatory Assay is Crucial. 37th Meeting of the European Group for Blood and Marrow Transplantation. Le Palais De Congres, 3-6 April 2011, Paris, France

Markovics A., **Paholcsek** M.: Laboratory background of pathogen detection. 7th Molecular Cell and Immune Biology Winter Symposium, 2013. January 8-11, Galyatető.

Fidler G., **Paholcsek** M.: Diagnostic accuracy of PCR compared to galactomannan in serum samples of invasive systematic aspergillosis. 7th Molecular Cell and Immune Biology Winter Symposium, 2014. január 7-10, Galyatető.

Paholcsek M., Fidler G., Biró S.: DNA based aspergillosis diagnostics in Debrecen, Hungary; state of the art and further challenges. 6th Advances Against Aspergillosis Conference, 2014 February 27-March 1, Madrid, Spain.

Registry number: DEENK/142/2015.PL
Subject: Ph.D. List of Publications

Candidate: Melinda Paholcsek
Neptun ID: RHYSRR
Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

1. **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:2.613 (2014)

2. **Paholcsek, M.**, Leiter, É., Markovics, A., Biró, S.: Novel and sensitive qPCR assays for the detection and identification of aspergillosis causing species.
Acta Microbiol. Immunol. Hung. 61 (3), 273-284, 2014.
DOI: http://dx.doi.org/10.1556/AMicr.61.2014.3.3
IF:0.778

List of other publications

3. Karimi Aghcheh, R., Németh, Z., Atanasova, L., Fekete, E., **Paholcsek, M.**, Sándor, E., Aquino, B., Druzhinina, I.S., Karaffa, L., Kubicek, C.P.: The VELVET A orthologue VEL1 of Trichoderma reesei regulates fungal development and is essential for cellulase gene expression.
PLoS One. 9 (11), e112799, 2014.
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4. Fekete, E., Karaffa, L., Karimi Aghcheh, R., Németh, Z., Fekete, É., Orosz, A., **Paholcsek, M.**, Stágel, A., Kubicek, C.P.: The transcriptome of lae1 mutants of Trichoderma reesei cultivated at constant growth rates reveals new targets of LAE1 function.
BMC Genomics. 15, Art. No. 447, 2014.
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5. Springer, J., Morton, C.O., Perry, M., Heinz, W.J., **Paholcsek, M.**, Alzheimer, M., Rogers, T.R., Barnes, R.A., Einsele, H., Loeffler, J., White, P.L.: Multicenter comparison of serum and whole-blood specimens for detection of Aspergillus DNA in high-risk hematological patients.
J. Clin. Microbiol. 51 (5), 1445-1450, 2013.
DOI: http://dx.doi.org/10.1128/JCM.03322-12
IF:4.232

Total IF of journals (all publications): 14,843
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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