Development of digital droplet PCR assay to quantify *Aspergillus species* - Is it useful?

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Introduction/Objectives

Invasive aspergillosis (IA) remains a major complication in patients with haematological malignancies (HM) and post allogeneic haematopoietic stem cell transplantation (HSCT). Early detection of *Aspergillus* infections has the potential to facilitate a more effective management of IA. Digital droplet PCR (ddPCR) has the potential to be more sensitive and more precise than real-time PCR (qPCR) for the detection of low DNA concentrations. By dividing the reaction volume in up to 20,000 separated entities/PCR reactions (droplets) the limit of detection (LOD) and analytical sensitivity can be enhanced and rates of inhibition reduced.

In this study, we transferred a qPCR assay protocol to detect *Aspergillus spp*. into a digital format, compared LODs, and tested clinical, external quality control (QC) and DNA-spiked samples to evaluate the new assay formats.

Methods

A qPCR assay specific for *Aspergillus spp.* (1, 2, 3) and compliant to FPCRI recommendations was adapted to digital PCR using the Bio-Rad (QX200). Different cycling protocols and a gradient of the annealing/elongation temperature were tested to determined best performance. Furthermore, additional digests using different restrictions enzymes (RE) were evaluated to increase sensitivity in serum samples. REs were selected to cut rDNA replicons appropriately to generate amplifiable DNA fragments which can be evenly distributed to single droplets. Dilutions of different *Aspergillus spp.* DNA (5-fold dilutions) were directly compared in both PCR systems and LODs were determined. A second assay (mold-independent target (3)) used as DNA extraction and PCR inhibition control assay (IC) in qPCR, but performed separately to the *Aspergillus* PCR, was performed as a duplex ddPCR reaction. QC samples consisted of blinded DNA spiked samples and specimens obtained from haematological and cystis fibrosis patients with proven and probable IA.

Results

The existing 3-step qPCR protocol (60 cycles of 95°C for 5 s, 54°C for 15 s (detection step), and 72°C for 1 s) (3) performed well in ddPCR and was optimized to detect different *Aspergillus spp*. Using RE digests did not improve ddPCR performance on clinical samples but for spiked samples increased positive droplet number up to 44%. LODs were comparable in both systems for all *Aspergillus spp.* tested. A second mold-independent assay (3) run as duplex in ddPCR facilitated monitoring of PCR inhibition and extraction efficacy in the same sample aliquot without losing sensitivity. DNAs from patient sera being positive in qPCR assays (n=46) were retested by qPCR and ddPCR after one freezing/thawing cycle and showed reduced positivity rates in both assays (48% negative samples).

Conclusion

ddPCR assays can be less prone to PCR inhibition, more reproducible and direct quantification of fungal load in serum is possible. Duplexing using ddPCR reduces costs and enables quantification of two targets in the same reaction. Testing RE digests did not increase sensitivity in clinical samples. Further comparative validation in large multi-centre studies is warranted.

Af-CAR-NK92 cells secreting IL-15 as potential off-the-shelf therapy for invasive pulmonary aspergillosis

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Introduction: Chimeric antigen receptors (CARs) are artificial fusion proteins consisting of an extracellular targeting domain, a spacer, a transmembrane domain, and an intracellular signaling domain, strengthened by a costimulatory domain. Originating from cancer treatment, CAR-engineered immune cells are a promising therapeutic option for treating infectious diseases. Immunocompromised patients often suffer from invasive aspergillosis, which leads to significant mortality, necessitating the exploration of alternative therapeutic options. Previously, we engineered T cells with an *Aspergillus fumigatus* (Af)-specific CAR and demonstrated their potent antifungal activity. CAR-NK92 cells offer certain advantages, such as intrinsic killing and minimal side effects after transplantation. Moreover, Af-CAR-NK92 cells could serve as off-the-shelf allogeneic therapeutics. Given the advantageous influence of IL-15 secretion on the viability, expansion, and cytotoxic potential of CAR-NK cells, we opted to integrate a gene cassette for continuous IL-15 expression into our CAR construct

Objectives: Engineering CAR-NK92 cells targeting *Aspergillus fumigatus* and enhancing their anti-fungal activity by incorporating an IL-15 gene cassette along with the CAR gene, resulting in constitutive IL-15 secretion.

Material and Methods: We utilized the non-viral Sleeping Beauty transposon system to engineer NK92 cells and introduce the Af-CAR or the Af-CAR along with constitutive expression of IL-15. As a control, we generated cells expressing CARs that specifically target CD19. We selected and expanded the NK92 cells expressing the CAR and conducted *in vitro* functional assays to characterize their antifungal activity. To achieve this, we performed co-culture assays with Af hyphae and measured the secretion of cytokines, the degranulation of the cells, as well as assessed direct hyphal damage.

Results: Using the NK92 cell line, we achieved the generation of cultures containing more than 90% CAR-positive NK92 cells. The secretion of IL-15 proved to be sufficient for the expansion of NK92 cells, resulting in independence from IL-2. When exposed to *Aspergillus fumigatus*, Af-CAR-NK92 cells demonstrated specific activation, as evidenced by cytokine secretion and degranulation, whereas CD19-CAR-NK92 cells did not exhibit activation. We observed a substantial increase in cytokines such as IFN-γ, IL-10, and chemokines like CCL-3 and CCL-4. The constitutive secretion of IL-15 led to higher concentrations of these cytokines and an augmented percentage of CD107a-positive cells. Furthermore, Af-CAR-NK92 cells exhibited inhibition of fungal growth.

Conclusion: Af-CAR-NK92 cells have demonstrated functionality in *in vitro* assays, making them a promising therapeutic option. Their potential is further enhanced by the constitutive expression of IL-15, which augments their activation. Currently, the antifungal activity of Af-CAR-NK92 cells is being investigated *in vivo*.

CD56-mediated activation of human natural killer cells is triggered by *Aspergillus fumigatus* galactosaminogalactan

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Introduction: Invasive *Aspergillus fumigatus* infections are a major cause of severe complications in immunocompromised patients. Delayed natural killer (NK) cell reconstitution in allogenic hematopoietic cell transplant recipients is associated with higher susceptibility to invasive pulmonary aspergillosis, indicating that NK cells are indispensable for fungal clearance. Previously, our group identified CD56 as the only known *pathogen recognition receptor* on NK cells to date that recognizes *A. fumigatus* and is required for their antifungal activity. However, the underlying cellular mechanisms and the fungal ligand of CD56 are still unknown.

The objective of this project are to identify the fungal ligand interacting with CD56 on NK cells and to gain deeper insights into the cellular mechanisms of this interplay.

Materials & Methods: We used a combination of purified cell wall components, biochemical treatments, and *A. fumigatus* mutants with altered cell wall composition and investigated their relevance for the interaction of *A. fumigatus* with NK cells by flow cytometry, microscopy and ELISA. Furthermore, PI3K and Pyk2 inhibitors were used to evaluate their involvement in the signalling pathway of *A. fumigatus*-induced NK cell activation. Moreover, GAG-pulsed NK-cell supernatants engage PMNs into the antifungal immune response.

Results: *A. fumigatus* cell wall galactosaminogalactan (GAG) showed binding to CD56, and especially deacetylated residues of GAG play a role in interaction with CD56 and triggered strong NK-cell activation, along with potent release of cytotoxic effectors and immune-enhancing chemokines. Inhibition of PI3K and Pyk2 decreased *A. fumigatus*/ GAG-mediated activation of NK cells. Supernatants of GAG-stimulated NK cells engage PMNs and enhance their anti-*Aspergillus* activity

Conclusion: Our data suggest that *A. fumigatus* GAG is a ligand of CD56 on human primary NK cells and stimulates potent antifungal effector responses under the involvement of PI3K and Pyk2.

Diagnostic performance of three lateral flow assays for measurement of (1–3)-β-D-glucan, mannan antigen and anti-mannan IgG antibodies in patients with candidemia: a retrospective case-control study

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Introduction: Candidemia is the fourth most common blood stream infection on intensive care units and is associated with a high mortality. Rapid diagnosis is essential for timely initiation of antifungal therapy. We aimed to analyse the performance of three lateral flow assays (LFA) for the measurement of (1–3)-β-D-glucan, mannan antigen and anti-mannan antibodies in sera of patients with and without candidemia.
Methods: Serum samples of 248 patients with blood culture-proven candidemia and 202 controls were tested with the QuicG™ Fungus (1-3)-β-D-Glucan LFA, the TECO® Fast *Candida* mannan antigen LFA and the TECO® Fast *Candida* IgG antibody LFA, respectively. The assays have a turn-around-time of approximately 40 min. The control sera were taken from hospitalized patients with negative blood cultures (n=140) and bacteremia (n=60), respectively.
Results: The mean age of the study patients was 64.6 years and 63.5% were male. Candidemia was caused by *Candida* (C.) *albicans* (46.6%), *C. glabrata* (27.7%), *C. parapsilosis* complex (9.9%), *C. tropicalis* (5.1%), *C. krusei* (4.7%), *C. lusitaniae* (1.6%) and other *Candida* spp (1.2%). A mixed candidemia occurred in 3.2% of patients. The sensitivity, specificity and area under the ROC-curve (AUROC) was 54,0% (95%-CI: 47,6 - 60,4), 77,2% (95%-CI: 70,8 - 82,8) and 0,632 (95%-CI: 0,586 to 0,677) for the QuicG™ Fungus (1-3)-β-D-Glucan; 46.2% (95%-CI: 40.0-52.6), 87.1% (95%-CI: 81.6-91.4) and 0.667 (95%-CI: 0.621-0.710) for TECO mannan antigen; 45.1% (95%-CI: 38.9-51.4), 75.6% (95%-CI: 69.1-81.4) and 0.604 (95%-CI:0.557-0.649) for TECO anti-mannan-antibodies.
Conclusion: The diagnostic performance of the QuicG™ Fungus (1-3)-β-D-Glucan, TECO® Fast Candida Ag & IgG LFAs is comparable to the widely used Fungitell™, Platelia *Candida* Ag and Ab Plus enzyme immunoassays (EIA). However, the LFA format is much more practical, especially for small sample numbers, and the time-to-result compared to the EIA is significantly reduced.

Phenotypic and genotypic characterization of azole-resistant *Aspergillus fumigatus* over 11 years

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Objectives

Invasive aspergillosis (IA) mostly occurs in immunocompromised patients, especially in patients with haematological malignancies or after allogeneic and solid organ transplantation. As first-line antifungal therapy, azole antifungal drugs are recommended which have been shown to be effective in the past. In recent years, the emergence of azole-resistant *Aspergillus fumigatus* strains (ARAf) has become a significant challenge in the treatment of IA. This study assessed the epidemiology of ARAf strains in the last eleven years within the University Hospital Essen, Germany.

Methods & Materials

The epidemiology of ARAf was investigated during 2012–2022. All respiratory samples were plated on malt extract agar and incubated for 7 days at 30°C. Identification of isolates was performed using classical macro- and micromorphological characteristics. During the years of collection, all isolates underwent susceptibility testing for at least itraconazole or for both, itraconazole and voriconazole by gradient test. ARAf was defined as non-wild-type minimal inhibitory concentration (MIC). Analysis of mutations mediating resistance was performed using PCR. Patient records were analysed retrospectively regarding sex, age, underlying diseases and 30-day in-hospital outcome.

Results

Over the 11 years, 196 ARAfs (6.1%) and 3002 wild type (WT) isolates of *Aspergillus fumigatus* were found. The number of ARAf cases remained consistent in the years from 2015 to 2019 until an increase in the years 2020 and 2021. 2021 was the year with the highest ARAf rate of 10.8%. Regarding seasonal distribution, WTs occurred mostly in summer and fall whereas most of the ARAfs were isolated in spring and summer. In total, ARAfs were mostly detected in male patients (n=108, 55%) but the gender distribution was variable over time. Median age was 44 years in patients with ARAf and 50 years in patients with WT. L98H/TR34 was the most prevalent mutation (33%) followed by T289/Y121 (5%). Results on 30-day in-hospital outcome and underlying disease will follow.

Conclusion

The findings of this study provide valuable insights into the epidemiological development of infections with ARAf within the last decade. It highlights the emergence of azole-resistant IA in North Rhine-Westphalia and underlines the importance for systematic antifungal susceptibility testing of *A. fumigatus*.