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# Evaluation of the new *Asp*ID polymerase chain reaction assay for detection of *Aspergillus* species: A pilot study

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## Summary

The newly developed *Asp*ID PCR assay for detection of *Aspergillus* spp. was evaluated with an interlaboratory quality control programme panel and human bronchoalveolar lavage fluid (BALF) samples. With the quality control programme, 8 out of 9 panel members were correctly identified. With the clinical study, 36 BALF samples that had been obtained from 18 patients with invasive pulmonary aspergillosis (IPA) and 18 without IPA were investigated. Sensitivity, specificity, positive and negative likelihood ratio for the *Asp*ID assay were 94.1% (95% CI 73.3-99.9), 76.5% (95% CI 50.1-93.2), 4 (95% CI 1.7-9.5) and 0.1 (95% CI 0.01-0.5) respectively.

**KEYWORDS**
*Asp*ID, BALF, galactomannan, invasive aspergillosis, PCR

Juergen Prattes and Martin Hoenigl contributed equally to this work.

# 1 | INTRODUCTION

Invasive pulmonary aspergillosis (IPA) remains a major cause of morbidity and mortality among severely ill patients. The critical step for successful management of IPA is rapid initiation of antifungal therapy requiring early and reliable diagnosis.[1,2] Several diagnostic approaches including antigen testing, new imaging methods and molecular approaches have been investigated to overcome current limitations, such as limited performance of available diagnostic methods, long turnaround times, and/or limited standardisation.[3-5] Over recent years, *Aspergillus* polymerase chain reaction (PCR) has been shown to be a very promising tool for detection of fungal infections when testing bronchoalveolar lavage fluid (BALF) from immunocompromised patients.[6-11] The newly developed *Asp*ID (OLM Diagnostics, Newcastle, UK) assay is a multiplex real-time PCR assay designed to detect clinically relevant *Aspergillus* spp. This assay targets a pan *Aspergillus* target and simultaneously an *Aspergillus terreus* target for differentiation of *Aspergillus terreus* vs non-terreus *Aspergillus* spp. This is of particular interest as changing epidemiology with an increase in non-fumigatus *Aspergillus* infections has been observed over the past decades.[12]

In this study, the performance of the new *Asp*ID PCR assay was investigated for the first time using both the Quality Control for Molecular Diagnostics (QCMD) 2016 *Aspergillus* spp. DNA EQA Programme panel and human BALF samples.

# 2 | MATERIAL AND METHODS

The accuracy of the *Asp*ID was determined utilising the QCMD 2016 *Aspergillus* spp. DNA EQA Programme panel. The panel consisted of 9 members including *Aspergillus fumigatus* DNA (n = 3), *Aspergillus fumigatus* conidia (n = 3), and negatives (n = 3). Members were either spiked in TE puffer, synthetic sputum, or in plasma matrix. In addition, 36 BALF samples obtained from 18 patients with IPA and 18 patients without IPA were investigated. Samples were obtained between 2013 and 2016 at the Medical University of Graz, Austria. BALF samples were stored at −70°C immediately after collection and have—in part—been used in studies published recently.[4,6,13-15] Patients were classified as having IPA if BALF galactomannan (GM) levels showed an optical density index of >3.0 and patients had clinical and radiological findings compatible with presence of IPA, as suggested by D'Haese and colleagues.[16] Patients without IPA had BALF-GM levels of <0.5 and no clinical or radiological findings suggestive for presence of IPA. Patients were matched 1:1 according to IPA status, primary underlying disease, and intensive care unit admission. For this study, diagnosis of IPA was based on high BALF-GM levels, clinical and radiological findings. We did not utilise the revised EORTC/MSG criteria, as these criteria are designed for diagnosis of invasive fungal infections in patients with active malignancies or severely immunocompromised patients only,[17] and are therefore not applicable to a significant proportion of our study population with mixed underlying diseases.

BALF samples were thawed and immediately tested for presence of *Aspergillus* DNA in an International Standard Organization (ISO 9001:2008)-certified laboratory, the Molecular Diagnostics Laboratory, IHME, Medical University of Graz. Investigators performing PCR were blinded to IPA status. BALF and QCMD panel samples were processed in the same way. *Aspergillus* DNA was extracted on the NucliSens® easyMAG™ platform (bioMérieux, Marcy-l'Etoile, France) with the NucliSens® easyMAG™ accessory products (bioMerieux) using the specific B protocol. The input volume was 400 μL. After the lysis step (10 min in NucliSens® lysis buffer at room temperature), 4 μL of internal extraction control (IEC) included in the *Asp*ID assay was added. The extracted DNA was eluted automatically with 40 μL of elution buffer. For qPCR and detection with the *Asp*ID, 7 μL of the master mixture and 3 μL of the extracted sample were pipetted into a well of a PCR plate followed by amplification and detection on the LC 480 II instrument (Roche Diagnostics, Penzberg, Germany). According to the manufacturer, *Asp*ID is sensitive to 10 copies of *Aspergillus* target template and may give a positive result if the infecting agent is *Penicillium* species (personal communication with the manufacturer).

Statistical analysis was performed using SPSS, version 24 (SPSS, Inc., Chicago, IL, USA). Receiver operating characteristic (ROC) curves analyses were performed utilising number of *Asp*ID cycles in case of positivity and area under the curve (AUC) values are presented including 95% CI. Optimal cut-offs were calculated using Youdens index. A two-sided *P*-value <.05 was considered statistically significant.

# 3 | RESULTS

When the accuracy of the *Asp*ID was determined with the QCMD panel, 5 out of 6 *Aspergillus* positive samples tested positive for *Aspergillus* and negative for *Aspergillus terreus*. The remaining sample turned out to be false negative. The false negative sample contained *Aspergillus fumigatus* DNA in a TE buffer solution and was classified as "educational" sample (ie containing low amount of fungal DNA). All *Aspergillus* negative samples were correctly identified as negative with the *Asp*ID PCR assay.

QCMD panel characteristics and results are displayed in Table 1, characteristics of patients with and without IPA are displayed in Table 2. When 36 BALF samples were investigated with the *Asp*ID assay, 2 samples, including one from a patient with IPA and one from a patient without IPA, showed inhibition and were excluded from analysis. Twenty BALF samples were found to be positive for *Aspergillus* and negative for *Aspergillus terreus* and 14 negative for both targets. When *Asp*ID results were compared to those obtained from BALF GM determination, 29 (85.3%) were found to be concordant and 5 (14.7%) discordant. Overall, 16/17 BALF samples from patients with IPA turned out as true positive and one as false negative. Thirteen out of 17 BALF samples from patients without IPA

**TABLE 1** QCMD panel characteristics and results obtained. The QCMD sample status is designated as "Core" or "Educational." Participating laboratories are expected to report core proficiency samples correctly within the EQA challenge/distribution

| | QCMD sample content | Matrix | QCMD sample status | *Asp*ID result (cp) |
|---|---|---|---|---|
| Sample 1 | *Aspergillus* negative | TE buffer | Core | Negative |
| Sample 2 | *Aspergillus fumigatus* DNA | TE buffer | Core | Positive (31.86) |
| Sample 3 | *Aspergillus fumigatus* DNA | TE buffer | Core | Positive (30.82) |
| Sample 4 | *Aspergillus fumigatus* DNA | TE buffer | Educational | Negative |
| Sample 5 | *Aspergillus fumigatus* conidia | Synthetic sputum | Core | Positive (33.75) |
| Sample 6 | *Aspergillus* negative | Synthetic sputum | Core | Negative |
| Sample 7 | *Aspergillus fumigatus* conidia | Synthetic sputum | Educational | Positive (35.94) |
| Sample 8 | *Aspergillus* negative | Plasma | Core | Negative |
| Sample 9 | *Aspergillus fumigatus* conidia | Plasma | Core | Positive (28.75) |

cp, crossing point.
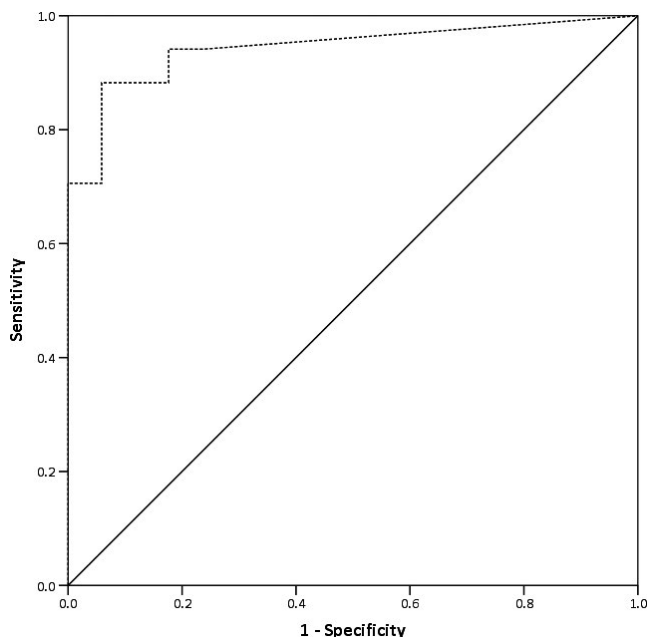


**FIGURE 1** ROC curve analysis for *Asp*ID for differentiating IPA vs no IPA

turned out as true negative and 4 as false positive. Sensitivity, specificity, positive (LR+) and negative likelihood ratio (LR-) for the *Asp*ID assay including 95% confidence interval (95% CI) were 94.1% (95% CI 73.3-99.9), 76.5% (95% CI 50.1-93.2), 4 (95% CI 1.7-9.5) and 0.08 (95% CI 0.01-0.5) respectively. Positive *Asp*ID results from patients with IPA (true positive) showed a median crossing point (cp) value

of 25.5, whereas positive *Asp*ID results from patients without IPA (false positives) showed a median cp value of 36.2 ($P = .011$ calculated by Mann-Whitney-Test). Utilising number of cycles to positivity, ROC curve analysis showed an AUC 0.943 (95% CI 0.860-1.000) for differentiating IPA vs no IPA (Figure 1). With Youdens index, we determined an cp value optimal cut-off of ≤35.65 for differentiating between IPA and no IPA [88.2% sensitivity (95% CI 0.66-0.97), 94.1% specificity (95% CI 0.73-0.99), LR+ 15.0 (95% CI 2.2-101.2) and LR- 0.13 (95% CI 0.03-0.46)], while a slightly higher cp value cut-off of ≤37.23 was associated with 94.1% sensitivity (highest sensitivity in this study) and 83.4% specificity. The latter cut-off resulted in a LR+ of 5.3 (95% CI 1.9-15.0) and a LR- of 0.07 (95% CI 0.01-0.48). Using a cp value cut-off of ≤31.25 for positivity, a specificity of 100% could be reached with an according 70.6% sensitivity. Corresponding LR+ was ∞ and LR− was 0.29 (95% CI 0.14-0.61).

## 4 | DISCUSSION

In this pilot study, the newly developed *Asp*ID PCR assay was evaluated with the 2016 *Aspergillus* QCMD proficiency panel and with BALF samples from patients at risk for IPA for the first time.

With both the QCMD programme panel and the BALF samples the *Asp*ID PCR showed an excellent sensitivity. In this study, the clinical sensitivity of *Asp*ID was 94%, which is higher to that of PCR assays for IPA diagnosis reported in other studies.[8,9,18] This may be due to the classification approach used in this study as a BALF-GM >3 ODI was required for IPA diagnosis. A higher BALF-GM level may

|  | Patients with IPA (n = 18) | Patients without IPA (n = 18) |
|---|---|---|
| Mean age, years (range) | 64.5 (48-84) | 66.5 (25-81) |
| Male | 8 | 9 |
| Female | 10 | 9 |
| Underlying risk factors for IPA[a] | | |
|    Haematological malignancy | 3 | 3 |
|    ICU admission | 12 | 13 |
|    Solid tumour | 4 | 4 |
|    Autoimmune disease | 2 | 2 |
| Mold active treatment at BALF sampling | 5 | 1 |
| Mean BALF GM levels (range) | 6.9 ODI (3.2-25.0) | 0.1 ODI (0.1-0.3) |
| *Aspergillus* positive BALF culture | 7[b] | 0 |

IPA, invasive pulmonary aspergillosis; ICU, intensive care unit; BALF, bronchoalveolar lavage fluid; GM, galactomannan; ODI, optical density index.

[a]A patient may have more than one underlying risk factor.

[b]7/7 *Aspergillus fumigatus.*

be associated with a higher fungal load and may thus lead to an overestimation of the clinical *Asp*ID sensitivity in this study. On the other hand, proven IPA resulted in 100% sensitivity in a previous study.[19] The high clinical sensitivity observed in this study may only be the manifestation of true infection in those patients classified as having IPA. For routine clinical use, sensitivity has to be investigated in a larger cohort of patients presenting intermediate BALF-GM levels.

Clinical specificity was slightly above 76% with 4 out of 17 patients without IPA having a positive *Asp*ID result. However, false positive samples showed significantly higher cp values indicating low fungal DNA concentrations. False positive results may have been observed due to contamination during bronchoscopy and bronchial lavage, during the laboratory workflow, or may be a manifestation of *Aspergillus* colonisation of the respiratory tract of the patient. All of these factors are associated with a potentially lower fungal burden compared to true uncontrolled infection and may resulting in higher cp values. In contrast, all samples that showed cp values of ≤31.25 were associated with IPA. Introduction of a quantitative assay including a quantitation standard would facilitate the introduction of a cut-off value for discrimination of clinically true positives vs negatives. This would be of interest, as severely immunocompromised patients usually receive mold active antifungal prophylaxis potentially leading to lower amount of circulating fungal antigens and DNA.[20] However, it remains in discussion whether a different cut-off for positivity should be used in patients with ongoing mold active antifungal prophylaxis as supposed for BALF-GM.

Sensitivity and specificity can only be estimated for non-terreus *Aspergillus* spp. with *Asp*ID. Both, the QCMD proficiency panel and all clinical samples contained only *Aspergillus fumigatus* or were culture negative. None of the samples tested contained *Aspergillus terreus*. The clinical performance of *Asp*ID regarding detection of *Aspergillus terreus* can thus not be calculated from this study.

Molecular diagnostic approaches for detection of IPA have been constantly improved within the last years and yielded promising results in clinical studies,[21,22] especially when combined with antigen testing.[6,13,18] In addition, performance of PCR testing of BALF samples was shown to be superior to PCR testing of blood samples,[13] which is of particular interest as bronchoscopy is an invasive procedure that may not be repeated as easy as blood drawing. This may be due to a higher fungal load in the airways, the primary site of infection. Also, systemic antifungal therapy may significantly alter PCR performance. While antifungal therapy seems to decrease PCR performance in blood significantly, influence on performance of BALF PCR testing is of less extent.[19,23] One of the major drawbacks for PCR assays used for detection of *Aspergillus* DNA testing is standardisation. Today, the majority of those assays are still based on in-house protocols showing a significant interlaboratory variation. This may be overcome efficiently when employing the *Asp*ID PCR which may be a promising additional assay in the armamentarium of diagnostic tools for detection of IPA. In addition to standardisation of PCR assays, performance of bronchoscopy and BALF sampling may also influence assay performance, for example due to different volumes, concentrations, contamination or use of mucolytic agents. The latter have shown to significantly alter the performance of BALF-GM testing.[24,25] To optimise reproducibility of study results both, standardisation of bronchoscopy and BALF sampling together with improved standardisation of PCR assays employing automated nucleic acid extraction platforms are warranted.

## DISCLOSURES AND CONFLICT OF INTEREST

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